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Haemodynamic effect of nickel chloride in pregnant rats*

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Non-pregnant and pregnant CFY rats were given 3 mg/kg nickel chloride or physiological saline by gavage daily for eight days or during days 7–14 of organogenesis. The haemodynamic investigations were carried out using ^{113}Sn labelled microspheres. Nickel concentrations in maternal and fetal blood, as well as in amniotic fluid were determined by atomic absorption spectrophotometry.

It was found, that nickel crossed the placenta, appeared in the fetal blood and amniotic fluid, where its concentration depended on the dose given to the pregnant animal and the nickel concentration of the maternal blood.

Nickel chloride influenced neither the systemic haemodynamic parameters (arterial blood pressure, total peripheral resistance – TPR, cardiac index) nor the values of the organ (including the placenta) circulation indices, neither in the pregnant nor in the non-pregnant animals.

It is concluded that in the pathomechanism of embryotoxicity (causing weight gain retardation) and teratogenicity (causing major anomalies of the uropoietic apparatus) of nickel, demonstrated earlier, the assumed effects of nickel on maternal and placental circulation probably do not play role (as such effects could not be detected). The direct embryo-damaging effect of nickel crossing the placenta (direct cytotoxic effect) may be held responsible for the embryotoxicity and teratogenicity of nickel.

Keywords: nickel chloride, circulation, pregnancy, rat

Nickel is a widely used industrial metal. On the one hand, it is sensitizing [11] and carcinogenic [23], on the other hand, it is an essential element for a number of living organisms [13]. In relation to solubility, the various nickel compounds differ in toxicity.

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The embryoxic and teratogenic effect of nickel may be taken as proven. In the experiments of Schroeder and Mitchener [19] nickel increased the number of rudimented newborns in the third generation of rats. Following exposure of rat fetuses to nickel Sunderman et al. [24] found microphthalmy and anophthalmy. The exposure was made through a 15-minute inhalation of 0.8–0.3 mg/l nickel carbonyl in air on the 7th or the 8th day of gestation. Lu et al. [8] found teratogenic effect in mice caused by an exposure to 1–6.9 mg/kg $NiCl_2$ on days 7–11 of gestation. According to Sunderman et al. [25] nickel is embryotoxic and teratogenic in several species (chicken, mouse, hamster), but not in rats. According to the investigation of our team, nickel sulphate is embryotoxic and teratogenic in mice and rats, it is embryotoxic in rabbits [28]. Perinatal toxicity of nickel chloride has been recently reported by Smith et al. [21].

A number of literature data prove the cardiovascular effects of nickel. A single dose of nickel decreases the contractile force of the myocardium [6], chronic nickel exposure causes the degeneration of the myocardium and changes of the ECG [7, 9, 26].

In vivo and *in vitro* animal experimental results show that nickel decreases cardiac contractility and causes vasoconstriction in the coronaries [16, 17, 18, 27] and myocardial lesions [14, 15].

Data of human examinations show that the concentration of nickel in serum increases in myocardial infarction and unstable angina [7, 22]. According to Alvarez et al. [1] acute nickel exposure causes catecholamine release and some of the nickel effects, such as hyperglycaemia, might be exerted through this.

In our subacute experiments in non-pregnant rats (daily administration of nickel sulphate for 3 weeks) nickel caused haemodynamic changes and changes in the fine structure of the myocardium (mitochondria, sarcoplasmatic reticulum) [12].

Investigating the mechanism of the teratogenic and embryotoxic effects of nickel on the basis of all the above-mentioned data we assumed that it might exert its teratogenic effect by influencing the maternal cardiovascular system. A redistribution of the maternal circulation may occur which alters the blood flow of the organs playing an important role in the "detoxication" of the organism, such as the liver and the kidneys. This may result in a nickel concentration reaching the embryonic and/or teratogenic levels [5, 19]. The blood flow of the ovaries may also change and the subsequent alteration of the sexual-steroid ratio may exert teratogenic effect [20, 29] or the blood flow of the uterus and/or placenta may decrease and the following hypoxia damages the embryo [2, 3, 20].

Materials and methods

Experimental protocol

Groups of non-pregnant and pregnant CFY rats were given by gavage 8 ml/kg physiological saline or 3 mg/kg nickel chloride dissolved in 10 ml/kg physiological saline daily for eight days and during 7–14 days of organogenesis, respectively. The circulation of the animals was studied in the 24th hour following the last treatment. Further 2–2 pregnant animals received 0, 12, 25 or 50 mg/kg nickel chloride on the 19th day of gestation, and 24 hours later the nickel concentrations in the maternal and fetal blood, as well as in the amniotic fluid were determined by atomic absorption spectrophotometry.

Haemodynamic studies

The rats were anaesthetized with Inactin (Inactin – Byk Gulden Konstanz – 100 mg/kg b.m. i.p.)

Blood pressure in the femoral artery was measured by a Statham P23 Db transducer and recorded by a Rikandeki B–381 three-chanel polygraph (Kogyo Co. Ltd., Tokyo, Japan).

Blood sample for the reference-flow was also taken from the femoral artery.

The cardiac output and its organ fractions were determined by ^{113}Sn labelled microspheres [10].

The ^{113}Sn labelled microspheres (15 ± 1 μm, New England Nuclear, Boston, Mass., USA) were diluted with 1% solution of sterile Tween 80, further dilutions were made with Ficoll–400 (sg: 1077). Homogeneity of the suspension was maintained by vigorous mixing and ultrasound treatment. A volume of 0.4 ml of the suspension was injected into the left ventricle through a polyethylene cannula introduced into heart via the right carotid artery. The number of particles received by one animal was 100 000–200 000, radioactivity of the microspheres was 7–16 μCi (1Ci = 376 GBq)/1×10^6. Blood samples for the determination of the cardiac output were collected (into a preweighed tube) from the right femoral artery during 60 seconds following the administration of microspheres.

$$\text{(Cardiac output, ml/min} = \frac{\text{administered activity} \times \text{blood sample} - \text{speed of sample taking, ml/min}}{\text{activity of blood sample}}\text{)}$$

The animals were killed with i.v. KCl. Their organs were separated, and after measuring the wet weights they were homogenized by boiling in 20% KOH. From the activities measured in the samples after volume correction the activities of whole organs were calculated.

^{113}Sn content of the organs was measured on Nuclear Chicago gamma spectrometer. Organ fractions of the cardiac output were calculated on the basis of the total ^{113}Sn activity administered. Knowing the cardiac output, blood pressure, body mass, organ fractions and organ masses we determined the cardiac index, the TPR/100 g body mass, the nutritive blood flow and vascular resistance of the organs.

Statistical evaluation

The results of the animal experiments were evaluated with analysis of variance and Dunnett's test [4].

Results

Nickel given per os crosses the placenta, appears in the fetal blood and amniotic fluid; its concentration depends on the dose administered to the pregnant animal, as well as on the nickel concentration of the maternal blood (Table I).

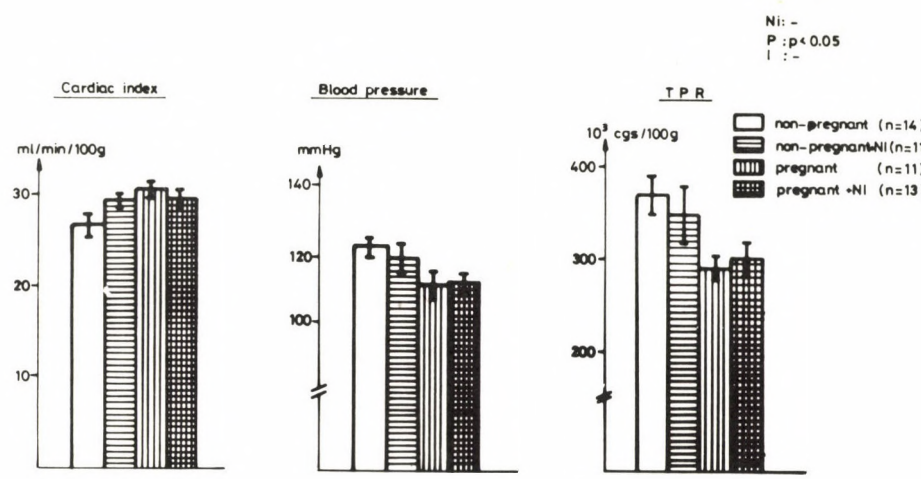
Table I

Concentration of nickel in maternal and fetal blood and in amniotic fluid

Exposure mg/bwkg $NiCl_2$	Maternal blood μg/l	Fetal blood μg/l	Fetal amniotic fluid μg/l
0	3.8	10.6	2.5
12.0	18.5	14.5	16.5
25.0	90.0	65.5	20.0
50.0	91.5	70.5	17.0

Exposure: day 19 of gestation by gavage
Values are means.

Values for maternal and fetal blood and amniotic fluid represent means of values measured in the blood of 2 mothers, and in samples collected from 3 members of 3 litters each, respectively



Figs 1–3. Haemodynamic parameters of non-pregnant and pregnant rats exposed to nickel chloride. Pregnant and non-pregnant rats were given 10 ml/kg physiological saline or 3 mg/kg $NiCl_2$ in 10 ml/kg physiological saline by gavage. Values of significance: Ni: $NiCl_2$, P: pregnancy, I: interaction

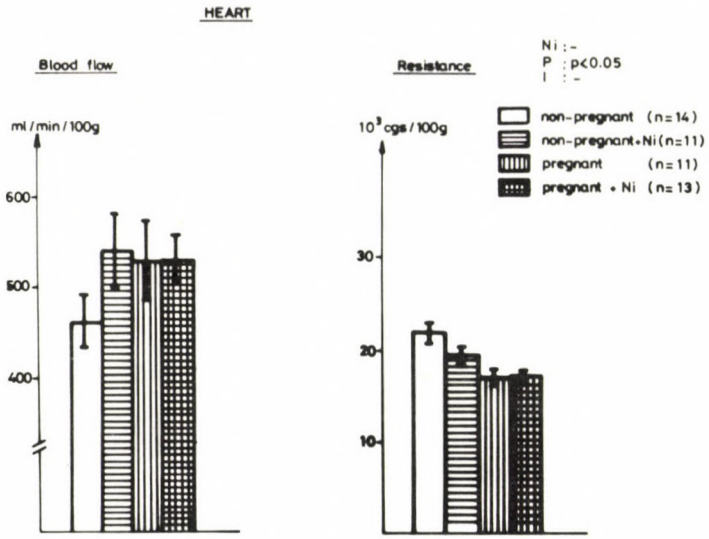

HEART
Blood flow
ml / min / 100g
600
500
400
Resistance
Ni : -
P : p<0.05
I : -
10³ cgs / 100g
30
20
10
non-pregnant (n=14)
non-pregnant+Ni (n=11)
pregnant (n=11)
pregnant + Ni (n=13)

Fig. 2.

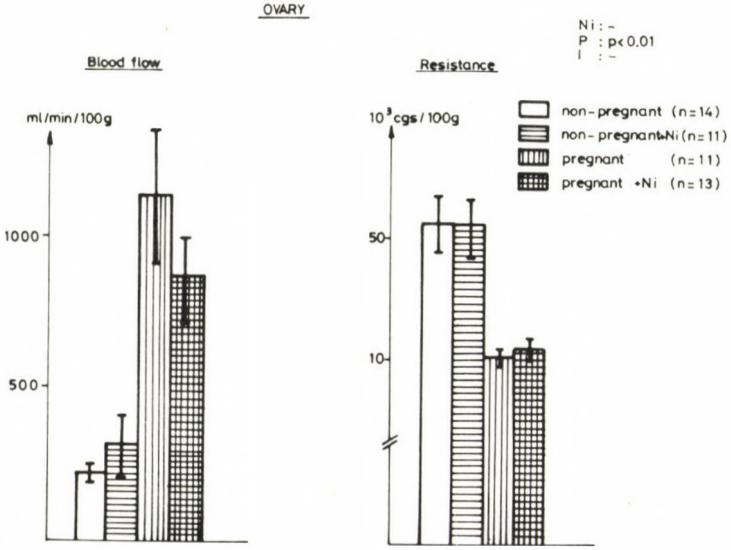

OVARY
Blood flow
ml/min/100g
1000
500
Resistance
Ni : -
P : p<0.01
I : -
10³ cgs / 100g
50
10
non-pregnant (n=14)
non-pregnant+Ni (n=11)
pregnant (n=11)
pregnant +Ni (n=13)

Fig. 3.

Table II

Haemodynamic parameters of non-pregnant and pregnant rats exposed to nickel chloride

	Non-pregnant control n=14	Non-pregnant $NiCl_2$ n=11	Pregnant control n=11	Pregnant $NiCl_2$ n=13	Values of significance p (less than) $NiCl_2$	Pregnancy	Interaction
Total body							
Body wt (BW), g	228.6 ± 13.7	220.4 ± 8.4	258.2 ± 5.4	263.0 ± 9.9	–	–	–
Cardiac output, ml/min	60.1 ± 2.9	64.3 ± 5.5	79.0 ± 2.7	77.6 ± 1.9	–	–	–
Brain							
Blood flow	81.6 ± 3.5	86.5 ± 5.0	71.1 ± 5.2	76.2 ± 4.4	–	–	–
Resistance	124.2 ± 7.1	114.6 ± 8.4	125.1 ± 9.1	122.2 ± 7.0	–	–	–
Kidney							
Blood flow	422.2 ± 20.1	378.1 ± 47.4	298.7 ± 10.9	328.1 ± 6.6	–	–	–
Resistance	24.1 ± 1.4	33.7 ± 8.1	30.3 ± 1.7	28.3 ± 1.8	–	–	–
Lung							
Blood flow	46.1 ± 5.2	44.3 ± 5.6	76.4 ± 11.5	90.0 ± 3.6	–	0.05	–
Resistance	237.6 ± 17.9	240.1 ± 19.6	141.2 ± 18.6	128.7 ± 7.9	–	0.05	–
Liver							
Blood flow	14.8 ± 1.3	12.2 ± 1.0	15.2 ± 1.8	13.6 ± 2.3	–	–	–
Resistance	775.5 ± 107.3	838.8 ± 68.59	647.6 ± 57.9	834.4 ± 99.8	–	–	–

Uterus							
Blood flow	60.3 ± 13.6	114.3 ± 53.6	38.5 ± 3.8	52.5 ± 5.8	–	–	–
Resistance	354.4 ± 128.3	202.5 ± 54.4	256.0 ± 27.8	197.5 ± 21.1	–	–	–
Placenta							
Blood flow	–	–	73.3 ± 7.1	87.8 ± 10.2	–	–	–
Resistance	–	–	134.7 ± 15.1	121.2 ± 15.1	–	–	–
Gut							
Blood flow	115.4 ± 11.4	117.5 ± 15.8	107.8 ± 8.9	94.3 ± 8.1	–	–	–
Resistance	94.7 ± 7.9	98.8 ± 14.6	87.0 ± 6.6	108.8 ± 14.5	–	–	–
Skin							
Blood flow	13.9 ± 1.1	13.3 ± 1.4	12.5 ± 0.9	12.9 ± 1.2	–	–	–
Resistance	794.6 ± 89.1	846.1 ± 134.2	746.3 ± 54.4	754.9 ± 61.2	–	–	–
Carcass							
Blood flow	16.2 ± 0.9	19.9 ± 2.2	19.8 ± 1.8	18.8 ± 1.6	–	–	–
Resistance	636.8 ± 43.7	533.9 ± 51.4	489.3 ± 46.6	529.4 ± 56.1	–	–	–

Values are mean ± SEM. For the organs: blood flow, ml/min per 100 g organ weight, resistance, 10^3 cgs/100 g organ weight.
Degree of difference: not significant (–), Significant, $p < 0.05$

Nickel chloride influenced the values of neither the systemic (blood pressure, TPR, cardiac index), nor the organs' (including the placenta) circulation parameters, neither in the non-pregnant, nor in the pregnant animals (Figs 1, 2, 3, Table II).

Pregnancy increased the cardiac index, decreased the arterial blood pressure and the TPR (Fig. 1); increased the blood flow and decreased the vascular resistance of the heart (Fig 2), the lungs (Table II) and the ovaries (Fig. 3).

Discussion

Our present study indicate, that the haemodynamic changes [12] observed in pregnant animals following a three-week nickel sulphate administration develop neither in pregnant nor in non-pregnant animals after a shorter treatment. This could be explained by the different nickel salts administration. In the previous experiment nickel sulphate, in the present study nickel chloride was used. It is improbable, however, that this can be held responsible for the difference, as the toxicity of the two salts is similar, and can be attributed to the nickel content itself. For this we assume, that the difference in the exposure time is responsible for the difference in the effects. In our present experiment we studied the effects of nickel during organogenesis (7–14 days of gestation). An 8-day administration of the 3 mg/kg dose not seem to be long enough to develop the cardiovascular effects of nickel. Nickel affects neither the circulation of organs responsible for detoxication (liver, kidneys), nor that of the endocrine organs, which play basic role in maintaining the pregnancy, (ovaries) nor that of the uterus and placenta. So we concluded, that the cardiovascular effects of nickel do not play a role in the mechanism of its teratogenic effect. The teratogenic and other embryotoxic effects are rather the result of the direct cytotoxic effect of nickel [28]. It is all the more probable, because the placenta of the rat does not make a significant barrier to nickel – as it is proved by our measurements. Our methods made it not possible to investigate the cardiovascular effects of nickel, appearing on the foetal side, in the foetus. The question remains, whether the effects of nickel, found in adult animals – such as the decrease of the myocardial contractility, coronary vasoconstriction [16, 17, 18], myocardial lesions [14, 15], – develop also in foetuses. Their possible development might be responsible for the embryotoxic effect of nickel.

REFERENCES

1. Alvarez, C., Blade, C., Cartana, J.: Alpha 2-adrenergic blockade prevents hyperglycaemia and hepatic glutathione depletion in nickel-injected rats. Toxicol. Appl. Pharmacol. **121**, 112–117 (1993).
2. Beck, F., Lloyd, J. B.: Comparative placental transfer. In: Handbook of Teratology. Wilson, J. G., Fraser, F. C. (eds) Plenum Press, New York – London 1977, Vol. 3, pp. 155–188.
3. Benirischke, K.: Effects of placental pathology on the embryo and fetus. In: Handbook of Teratology. Wilson, J. G., Fraser, F. C. (eds) Plenum Press, New York – London 1977, Vol. 3, pp. 79–118.
4. Dunnett, C. W.: A multiple comparison procedure for comparing several treatments with control. J. Amer. Statist. Assoc. **50**, 1096–1121 (1955).
5. Gilette, J. R.: Hemodynamic aspects of excretion and metabolism of drugs. In: Handbook of Teratology. Wilson, J. G., Fraser, F. C. (eds) Plenum Press, New York – London 1977 , Vol. 3, pp. 54–55.
6. Kaufmann, R., Fleckenstein, A.: Ca-kompetitive elektro-mechanische Entkoppelung durch Ni- und Ca-ionen am Warmblütermyokard. Arch. ges. Physiol. **282**, 290–297 (1965).
7. Leach, C. N. Jr., Linden, J. V., Hopfer, S. M., Crisostomo, M. C., Sunderman, F. W. Jr.: Nickel concentrations in serum of patients with acute myocardial infarction or unstable angina pectoris. Clin. Chem. **31**, 556–560 (1985).
8. Lu, C., Matsumoto, N., Lijime, S.: Teratogenic effects of nickel chloride on embryonic mice and its transfer to embryonic mice. Teratology **19**, 137–142 (1979).
9. Mather, A. K.: Arch. Toxicol. **37**, 59 (1977). cit: Patty's Industrial Hygiene and Toxicology. Clayton, G. D., Clayton, F. E. (eds) Wiley-Interscience Publ., New York – Chichester Brisbane 1981, p. 1827.
10. McDevitt, D. G., Nies, A. S.: Simultaneous measurement of cardiac output and its distribution with microspheres in the rat. Cardiovasc. Res. **10**, 494–498 (1976).
11. Menné, T., Borgan, O., Green, A.: Nickel allergy and hand dermatitis in a stratified sample of the Danish female population: an epidemiological study including a statistic appendix. Acta Dermatovenerol. **62**, 35–41 (1982).
12. Morvai, V., Szakmáry, É., Ungváry, Gy., Szénási, G.: The effects of simultaneous alcohol and nickel sulphate poisoning on the cardiovascular system of rats. Acta Physiol. Hung. **82**, 239–251 (1993).
13. Norseth, T.: Nickel. In: Handbook on the Toxicology of Metals, Friberg, L., Nordberg, G. F. and Vouk, V. (eds) Elsevier Science Publishers B. V. 1986, Chapter 18, pp. 462–481.
14. Patai, K., Balogh, I.: Nickel- and calcium-induced fetal myocardial changes in the mouse: the hazards of cigarette smoke in pregnancy. Acta Chir. Hung. **29**, 315–321 (1988).
15. Reichrtova, E., Sulicova, L., Vesela, A., Bencko, V.: Bioaccumulation of metals from nickel smelter waste in P and F1 generation of exposed animals. Part III. Morphological responses. J. Hyg. Epidemiol. Microbiol. Immunol. **34**, 337–341 (1990).
16. Rubányi, G., Hajdu, K., Pataki, T., Bakos, M.: The role of adrenergic receptors in Ni^{2+}-induced coronary vasoconstriction. Acta Physiol. Acad. Sci. Hung. **59**, 161–167 (1982).
17. Rubányi, G., Kovách, A. G. B.: Cardiovascular actions of nickel ions. Acta Physiol. Acad. Sci. Hung. **55**, 345–353 (1980).
18. Rubányi, G., Ligeti, L., Koller, A., Kovach, A. G.: Possible role of nickel ions in the pathogenesis of ischemic coronary vasoconstriction in the dog heart. J. Mol. Cell. Cardiol. **16**, 533–546 (1984).
19. Schroeder, H. A., Mitchener, M.: Toxic effects of trace elements on the reproduction of mice and rats. Arch. Environ. Health **23**, 102–106 (1971).

20. Shepard, T. H.: Maternal metabolic and endocrine imbalances. In: Handbook of Teratology. Wilson J. G., Fraser, F. C. (eds) Plenum Press, New York – London 1977, Vol. 3, pp. 54–55.
21. Smith, M. K., George, E. L., Stober, J. A., Feng, H. A., Kimmel, G. L.: Perinatal toxicity associated with nickel chloride exposure. Environ. Res. **61**, 200–211 (1993).
22. Solovjev, A. V., Ermolin, G. A., Dikov, M. M., Soroka, V. R., Riabkova, N. V.: Myoglobin and nickel in the blood of patients in the acute period of myocardial infarct. Vrach. Delo. **10**. 62–64 (1989).
23. Sunderman, F. W., Jr.: Mechanisms of nickel carcinogenesis. Scand J. Work Environ. Health **15**, 1–12 (1989).
24. Sunderman, F. W., Allpass, P. R., Mitchell, J. M., Basett, R. C., Albert, D. M.: Eye malformations in rats: Induction by prenatal exposure to nickel carbonyl. Science **203**, 550–553 (1979).
25. Sunderman, F., Reid, M. C., Sken, S. K., Kevorkian, C. B.: Embryotoxicity and teratogenicity of nickel compounds. In: Reproductive and developmental toxicity of metals. Clarkson, ThW., Norberg, G. F., Sager, P. R. (eds) Plenum Press, New York 1983, p. 399–416.
26. Szabó, K., Balogh, I., Gergely, A.: Endogenous nickel release in injured patients: A possible cause of myocardial damage. Injury **16**, 613–620 (1985).
27. Szabó, K., Faragó, M., Balogh, I., Gergely, A., Rubányi, G., Kovach, A. G.: The effect of alpha and beta adrenergic blockade on Ni induced coronary spasm in scalded rats. Acta Physiol. Hung. **68**, 129–145 (1986).
28. Szakmáry, É., Ungváry, Gy., Náray, M., Szeberényi, Sz., Tátrai, E., Morvai, V.: The offspring damaging effect of nickel on rats, mice and rabbits. Egészségtudomány **37**, 83–91 (1993). (in Hungarian).
29. Ungváry, Gy.: Correlation of maternal hepatotoxicity and embryotoxicity in poisonings coast by benzene and its alkyl derivatives. D. Sc. Thesis. Budapest 1985 (in Hungarian).

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Electrophysiological characterization of a novel Class III antiarrhythmic agent, GLG–V–13 in the mammalian heart

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GLG–V–13, a novel 3,7-diheterabicyclo[3.3.1]nonane, was examined both *in vivo* and *in vitro* in order to characterize its electrophysiological, haemodynamic and inotropic properties. Left ventricular epicardial monophasic action potential (MAP), surface ECG and mean arterial blood pressure (MBP) were recorded in six pentobarbital-anaesthetized, artificially ventilated and thoracotomized guinea-pigs. When studied in an intravenous dose interval ranging between 0.5 μg/kg and 500 μg/kg, GLG–V–13 dose-dependently lengthened the MAP duration ($p < 0.05$ at doses above 5 μg/kg), the atrioventricular conduction time ($p < 0.05$ at doses above 1 μg/kg) and the RR interval ($p < 0.05$ at doses above 25 μg/kg). At the highest dose (500 μg/kg) these variables were increased by 30%, 13% and 23%, respectively. Only minor effects were noted on intraventricular conduction time (QRS interval) and MBP. In rabbit atrial and papillary muscle preparations, GLG–V–13 (0.32 to 3.2 mg/l) did not exert negative inotropic action. In 10 intact anaesthetized mongrel dogs, left ventricular endocardial MAP at 90% repolarization (MAP_{90}) was measured during atrial pacing before and after administration of GLG–V–13, 3 and 6 mg/kg i.v., respectively.

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Compared to the drug-free state, the agent induced a significant prolongation of the MAP_{90} at all pacing frequencies (2.0 to 4.5 Hz). In 15 anaesthetized Harris-dogs, studied 24–96 h after anterior myocardial infarction, the antiarrhythmic/proarrhythmic potential of GLG–V–13 was compared to that of lidocaine. ECG, His bundle, left ventricular infarct (IZ_{epi}) composite and normal zone composite electrograms were recorded. Programmed electrical stimulation and burst pacing (4.0–7.0 Hz) were delivered to the right ventricular outflow tract. In the drug-free state, sustained monomorphic ventricular tachycardia (SMVT) was inducible in 6 dogs (6/15). After lidocaine, SMVT was induced in 7 additional dogs (13/15). GLG–V–13 prevented induction of SMVT in 5/6 dogs, a proarrhythmic action was seen in 1 dog only. GLG–V–13 slowed the heart rate, increased the AH and the HV intervals, prolonged the paced (2.5 Hz) QT interval and increased the ventricular effective refractory period (VERP). These effects were associated with 2:1 conduction block of late potentials in the IZ_{epi} electrograms, a phenomenon also observed during rapid atrial pacing (2.5–3.5 Hz), suggestive of a marked and partially rate-independent prolongation of refractoriness in the ischemically damaged myocardium. The high antitachyarrhythmic efficacy, together with the low proarrhythmic potential and lack of cardiodepressant and reverse use dependent properties of GLG–V–13, may merit further investigation of this novel Class III antiarrhythmic agent.

Keywords: GLG–V–13, Class III, monophasic action potential, guinea-pig, rabbit, dog, myocardial infarction, proarrhythmia

Undoubtedly, antiarrhythmic drug research and the clinical use of antitachyarrhythmic agents have undergone striking changes after the Cardiac Arrhythmia Suppression Trial (CAST) [21, 49]. Apparently, Na^+-channel blocking antiarrhythmics, especially the types subclassified as Ic, suppressed premature ventricular depolarizations effectively, but increased the rate of sudden arrhythmic cardiac death [21, 47]. The proarrhythmic action of Class Ic agents is attributed to a marked depression of impulse conduction, which becomes increasingly apparent at rapid heart rates (use-dependence), resulting in functional block and reentrant ventricular tachycardia [4, 12, 18]. Furthermore, Ic antiarrhythmics either do not, or only slightly, prolong ventricular refractoriness [4, 49]. Significant prolongation of the ventricular effective refractory period (VERP) can be a means to prevent or terminate ventricular tachycardia leading to ventricular fibrillation [23, 31, 38, 44]. Therefore, attention has recently been focused on compounds selectively prolonging cardiac action potential duration (APD), i.e., an action classified as Class III [45]. Unfortunately, the ability of most of these agents to prolong the APD and the repolarization time is diminished or disappearing at fast heart rates, an undesirable phenomenon referred to as reverse use-dependence [23]. On the other hand, the APD lengthening is exacerbated by bradycardia [23]. Hypothetically, the ideal Class III antiarrhythmic agent should prolong atrial or ventricular refractoriness exclusively at rapid heart rates (positive use-dependence) without any depressant effect on conduction of the cardiac impulse [23]. At present, available Class III antiarrhythmics do not meet both these requirements [5, 23, 39]. Unfortunately, Class III action also

incorporates a potential risk of proarrhythmia, the bradycardia-dependent prolongation of the APD predisposes Purkinje or subepicardial M cells to early afterdepolarization-induced triggered firing, clinically manifested as torsades de pointes [5, 11, 26, 51, 53].

The synthesis and development of some novel bispidine-analogues (3,7-diheterobicyclo[3.3.1]nonanes = DHBNs) with heart rate lowering, refractory period prolonging and antiarrhythmic properties (GLG – V – 13, GLG – IV – 57, GLG – III – 93) were established [13 – 16, 46, 48]. The goal of the present work was to characterize the electrophysiological and inotropic profile of one of these new compounds, GLG – V – 13, in the guinea-pig, rabbit and canine heart. GLG – V – 13's effect on the duration of ventricular repolarization was measured by registering left ventricular MAPs from hearts of anaesthetized guinea-pigs and dogs *in vivo*. The effects of the novel agent on the mechanical activity of the cardiac muscle was analyzed in rabbit atrial and ventricular muscle preparations *in vitro*. Furthermore, the electrophysiological effects and the antiarrhythmic/proarrhythmic potential of GLG – V – 13 were compared to that of lidocaine in anaesthetized dogs with a 1 – 4-day-old myocardial infarction [42]. Based upon its moderate cardiodepressant effects and its reported low incidence of clinical proarrhythmia, lidocaine was selected as a prototypical Class Ib agent [17, 26, 47]. Preliminary results of these experiments have been published in abstract form [13 – 16].

Materials and methods

Animal studies were performed in accordance with guidelines established by the National Institute of Health and state regulations from the US Department of Agriculture. In addition, all protocols were approved by the Animal Studies Subcommittee and Research & Development Committee at the Department of Veterans Affairs Medical Center, Oklahoma City, Oklahoma, USA and by the Ethics Committee on animal experiments in Gothenburg, Sweden.

Anaesthetized guinea-pigs

Guinea-pigs (760 – 910 g) were housed in the animal department for at least one week before the experiment. During that period the animals had free access of food (Ewos K1 pellets) and tap water. Anaesthesia was induced by an intraperitoneal injection of pentobarbital (40 – 50 mg/kg) and catheters were introduced into one carotid artery for blood pressure recording and blood sampling and into one jugular vein for drug infusion. Needle electrodes were placed on the limbs for recording of ECG (lead II). A thermistor was placed in the rectum and the animal was placed on a heating pad, set to maintain a rectal temperature between 37.5 and 38.5 °C. A tracheotomy was performed and the animal was artificially ventilated with room air by use of a small animal ventilator to keep blood gases within the normal range for the species. In order to reduce autonomic influences both vagi were cut in the neck, and 0.5 mg/kg propranolol was given intravenously 15 minutes before the start of the experiment. The left ventricular epicardium was exposed by a left-sided thoracotomy, and a custom-designed suction electrode for recording of the MAP was applied and left in position as long as an acceptable signal could be

recorded, and otherwise moved to a new position [10]. A bipolar electrode was clipped to the left atrial appendage for pacing (2 ms duration, twice the diastolic threshold) by means of a custom-made constant current stimulator. The pacing rate was set just above the spontaneous sinus rate (interstimulus interval: 258 ± 10 ms) during 1 minute every fifth minute throughout the study. The mean arterial blood pressure (MBP), the MAP signal and the lead II ECG were recorded on a Mingograph ink-jet recorder (Siemens-Elema, Solna, Sweden). All signals were collected (the sampling frequency was 1000 Hz and each sampling period 10 seconds) on a Compaq personal computer (Descpro 386s) during the last 10 seconds of each pacing sequence and the last 10 seconds of the following minute of sinus rhythm. Finally, the signals were processed using a custom-designed computer program [1]. The test procedure consisted of two basal control recordings 5 minutes apart during both pacing and sinus rhythm. After the second control recording the first dose of GLG – V – 13 was infused into the jugular vein catheter for 30 s (infusion volume 0.2 ml). Three minutes later pacing was started and a new recording was made. Five minutes after the previous dose the next dose of test substance was administered. Ten consecutive doses were given during each experiment.

Of the numerous variables measured in the computer analysis some were selected as more important for the electrophysiological and haemodynamic characterization of GLG – V – 13. Hence, the MAP duration at 75% repolarization during pacing, the atrioventricular (AV) conduction time (defined as the interval between the atrial pacing pulse and the start of the ventricular MAP) during pacing, the QRS interval of the surface ECG and the rise time of the MAP (indices of intraventricular conduction) at pacing and the heart rate defined as the RR interval during sinus rhythm were measured. The mean of the two control recordings was set to zero and the effects recorded after consecutive doses of GLG – V – 13 were expressed as percentage changes from this value. By plotting these percentage values against the cumulative dose administered before each recording, dose-response curves were constructed by linear connection of the data points obtained.

Intact anaesthetized dogs

The mongrel dogs, weighing 10 – 16 kg, were anaesthetized with sodium pentobarbital (30 mg/kg i.v.). A custom-made contact MAP electrode-catheter was positioned in the left ventricle for measuring the duration of the MAP at 90% repolarization (MAP_{90}, 30,50). An electrode catheter was introduced into the femoral vein and was advanced into the right atrium for atrial pacing. Following basal recordings of the MAP_{90} at pacing rates varying between 2.0 and 4.5 Hz, GLG – V – 13 was intravenously administered (3 and 6 mg/kg, respectively) and the measurements repeated.

Anaesthetized Harris-dogs

Fifteen mongrel dogs, weighing 15 – 25 kg, were anaesthetized with sodium pentobarbital (30 mg/kg i.v.). Using aseptic technique, the heart was exposed by a left thoracotomy in the 4th intercostal space during positive pressure ventilation (Harvard 607 dual phase control respirator; South Natick, MA, USA). The left anterior descending coronary artery (LAD) was dissected free and a two-stage ligation was performed just distal to the takeoff of the first diagonal branch [22]. The chest was closed and the dog received antibiotic and analgesic therapy post-operatively.

Electrophysiologic studies were performed in the post-absorptive state, 1 – 4 days (2.4 ± 0.36 days; mean ± SEM) after the occlusion of the LAD. On the day of the study, anaesthesia was reinduced and a 12-lead ECG was recorded to confirm the presence of an anterior myocardial infarction which showed QS complexes in V_{2-5}. During the experiment, limb lead II (L – II) and aVR were registered continuously. The "paced QT interval" was measured in the last stimulated complex of 2.5 Hz atrial pacing trains ($QT_{p,\,2.5\,Hz}$) [28]. To record His bundle activation, a 5 or 6 F electrode catheter was

introduced into the proximal aortic root through one of the common carotid arteries (Hb_{eg}) [40]. Another electrode catheter was inserted into the femoral vein and was advanced to the right atrium to deliver atrial pacing stimuli. The MBP was measured by means of a Statham P – 24D transducer (Viggo-Spectramed Inc., Critical Care Div., Oxnard, CA., USA) through a cannula introduced into the femoral artery. In order to record the activation of the subepicardium overlying the infarct, composite electrodes [3, 41] were placed onto the ischemically injured anteroapical epicardium (IZ_{epi}). The non-infarcted posterior wall (NZ_{epi}) of the left ventricle was used as a comparison. The ECG and the electrograms were recorded continuously on a multichannel oscilloscope (Electronics for Medicine VR – 12; Pleasantville, NY, USA), and recordings were made at paper speeds of 50 – 250 mm/sec on a Gould ES 1000 paper drive (Gould Electronics, Valley View, OH, USA). The filter frequencies used were 0.1 to 200 Hz for ECGs and 30 to 250 Hz for electrogram recordings, respectively. Measurements were accurate up to 3 msec at a paper speed of 250 mm/sec. For induction of sustained monomorphic ventricular tachycardia (SMVT = ventricular tachycardia of uniform QRS morphology > 30 sec at a rate of > 250/min; 43), the heart was electrically stimulated from the right ventricular outflow tract. Two stimulation protocols were applied: a conventional programmed extrastimulus technique and a fast (4.0 – 7.0 Hz) intermittent pacing protocol consisting of 3-beat-bursts [17, 43]. The former protocol utilized a Medtronic 5328 programmable stimulator, the latter was performed with an S88 Grass stimulator (Grass Medical Instruments; Quincy, MA, USA). During the programmed electrical stimulation (PES) using a baseline cycle length of 300 msec, 1 (S_1S_2), 2 ($S_1S_2S_3$) or 3 ($S_1S_2S_3S_4$) premature stimuli were applied. The strength of the stimuli, with a duration of 2 ms, was twice the diastolic threshold. The VERP, defined as the longest S_1S_2 interval at which S_2 failed to result in V_2, was measured by the S_1S_2 technique. Lidocaine and GLG – V – 13 were administered over a period of 1 min in cumulative i.v. doses of 3 and 6 mg/kg, respectively. The latter drug was injected 1 hour after lidocaine, after which the electrophysiological effects of the former had completely dissipated [34, 43].

Inotropic studies on rabbit atrial and papillary muscle preparations

New Zealand white rabbits (body weight, 2.4 – 4.0 kg) were anaesthetized with ether and killed using blunt head trauma. The heart was excised and then rinsed in modified Tyrode's solution equilibrated with 100% oxygen. The Tyrode's solution contained (in *mM*) 140 NaCl; 5 KCl; 1.0 $MgCl_2$; 10 glucose, 5 HEPES; 2.0 $CaCl_2$; 1.0 Na_2HPO_4; 0.2 aspartic acid. The pH was adjusted to 7.4 using 5 M NaOH. The atrial appendages and right ventricular papillary muscles were removed and placed in glass tissue bath (50 ml) containing Tyrode's solution at 37 °C. These baths were continuously bubbled with O_2. The preparations were attached to Grass FT – 03 isometric force transducers via 4 – 0 suture and were stimulated to contract isometrically at 0.5, 1.0, 2.0 and 3.0 Hz, respectively; paired pulses stimulation was delivered at 1.0 Hz [35, 52]. The resting tension was adjusted to produce maximal force development. The pacing pulse was a unidirectional square-wave of 4 ms duration from a Grass S48 stimulator and SIU – 5 or SIU – 7 stimulus isolation unit. Force development was recorded continuously on a Grass 7 polygraph. The preparations were paced at 1 Hz and allowed to equilibrate for one hour before control measurements were obtained. GLG – V – 13 was applied in cumulative half-log units (0.32, 1.0, 3.2, 10 and 32 mg/l). These concentrations were comparable with the canine-effective plasma levels (1.3 – 2.0 mg/l) measured *in vivo* [8]. A period of 20 min was allowed between individual drug concentrations. Return to control values was determined 20 min after replacement of the bath solution with modified Tyrode's solution void of drug (washout).

Statistical analysis

Quantitative data are presented as the means ± SEM and n indicates the number of observations. Student's t-test for unpaired or paired observations or one-way analysis of variance (ANOVA) for repeated measures were applied when appropriate. A p value less than 0.05 was considered as statistically significant.

Drugs used

In the guinea-pig experiments a stock solution (2.5 mM) of GLG – V – 13 (Fig. 1; 3-[4-(1H-imidazol-1-yl)benzoyl]-7]isopropyl-3,7-diazabicyclo[3.3.1]-nonane dihydroperchlorate) was prepared by dissolving the compound in polyethylene glycol, ethanol and distilled water (1:2:3). The stock solution was then further diluted with physiological saline to the final concentrations used. For dogs, GLG – V – 13 was dissolved in 50:50 95% ethanol/H_2O, 3 mg/ml. Lidocaine hydrochloride (Vedco Inc., St. Joseph, Mo., USA) was dissolved in saline.

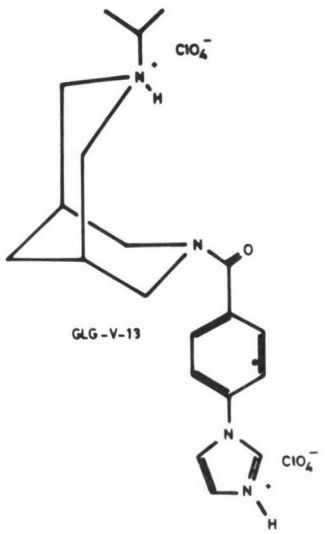

Fig. 1. Chemical structure of GLG – V – 13. Molecular weight: 539.37 g/mol

Results

Effects of GLG – V – 13 in the anaesthetized guinea-pig

When studied in an intravenous dose interval ranging between 0.5 μg/kg and 500 μg/kg, GLG – V – 13 induced a dose-dependent lengthening of the MAP duration ($p < 0.05$ at doses above 5 μg/kg), AV-conduction time ($p < 0.05$ at doses above 1 μg/kg) and RR interval ($p < 0.05$ at doses above 25 μg/kg) (Fig. 2). At the highest dose, i.e., 500 μg/kg, these variables were increased by 30%, 13% and 23%,

respectively. No significant changes were noted on the QRS interval or on the rise time of the MAP. A minor, transient drop in MBP was observed (data not shown).

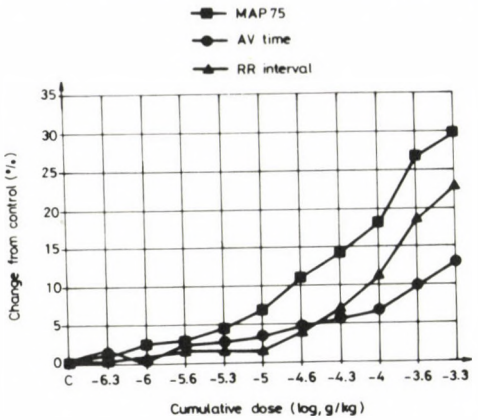

Fig. 2. Dose-dependent effects of GLG – V – 13 on the duration of the monophasic action potential (at 75% repolarization, MAP_{75}), atrioventricular conduction time (AV time) and RR interval in the anaesthetized guinea-pig. MAP_{75} and AV time were measured during a constant atrial pacing frequency set just above the spontaneous sinus rate. The RR interval was measured during sinus rhythm. Values shown are means SEM, n = 6

Effects of GLG – V – 13 on the duration of MAP in anaesthetized dog

Both doses of GLG – V – 13 induced a statistically significant prolongation of the MAP_{90} at pacing rates varying between 2.0 and 4.5 Hz with a slight tendency of reverse frequency-dependence at the higher pacing rates (Fig. 3).

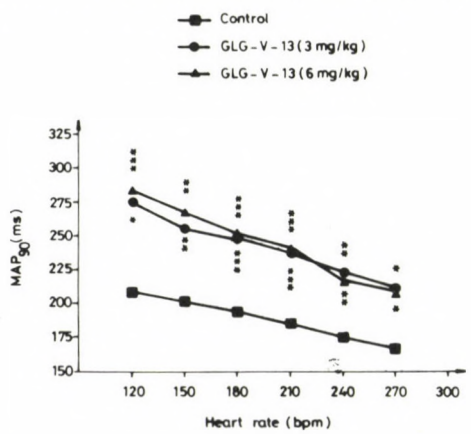

Fig. 3. Effects of GLG – V – 13 (3 or 6 mg/kg i.v.) on the duration of the monophasic action potential (at 90% repolarization, MAP_{90}) at atrial pacing rates varying between 2.0 and 4.5 Hz (120 to 270 beats/min) in the intact anaesthetized dog. Values shown are means SEM, n = 10. *;$p < 0.05$, **;$p < 0.001$ *vs.* control

Comparison of the electrophysiological effects and antiarrhythmic/proarrhythmic potential of GLG – V – 13 and lidocaine in the infarcted canine heart

Compared to baseline (152 ± 25/min), lidocaine did not influence sinus heart rate (151 ± 26/min). GLG – V – 13, however, caused an immediate lowering of the heart rate (110 ± 20/min, $p < 0.01$). After bolus administration of lidocaine, a short-lasting (1.0 – 1.5 min), but consistent, decrease of the MBP (105 ± 17 to 84 ± 18 mm Hg; $p < 0.001$) was observed. GLG – V – 13 induced a shorter (30 – 40 sec) and non-significant reduction of MBP (94 ± 17 to 84 ± 24 mm Hg). Lidocaine did not induce either AH (baseline 57 ± 8, post-lidocaine 57 ± 7 msec), or HV (baseline 30 ± 3, post-lidocaine 33 ± 2 msec) interval prolongation. GLG – V – 13, however, increased both the AH and the HV intervals (66 ± 7 msec, $p < 0.05$ and 37 ± 7 msec, $p < 0.01$, respectively). VERP (baseline 142 ± 14 msec) was prolonged both by lidocaine (167 ± 24 msec, $p < 0.01$) and GLG – V – 13 (187 ± 18 msec, $p < 0.001$). Lidocaine did not prolong the QT interval (baseline 199 ± 28, post-lidocaine 200 ± 30 msec, NS), whereas GLG – V – 13 did ($QT_{p,\ 2.5\ Hz}$ = 288 ± 37 msec, $p < 0.005$; Fig. 4).

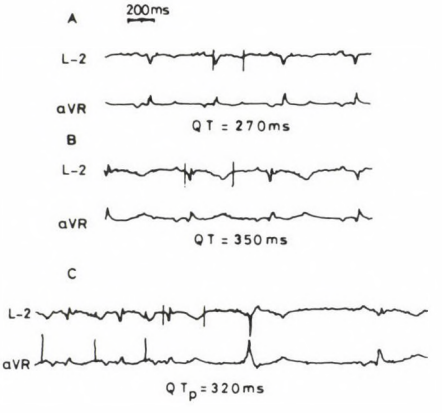

Fig. 4. Effect of GLG – V – 13 on the QT interval of an anaesthetized dog with a 4-day-old myocardial infarction. Panel A: baseline drug-free state with a heart rate of 110 beats/min and QT = 270 msec. Panel B: after GLG – V – 13, 6 mg/kg, heart rate decreased to 86 beat/min with a QT = 350 ms. Panel C: $QT_{p,\ 2.5\ Hz}$ measured in the last paced complex of a 150 beats/min atrial stimulation train was 320 ms

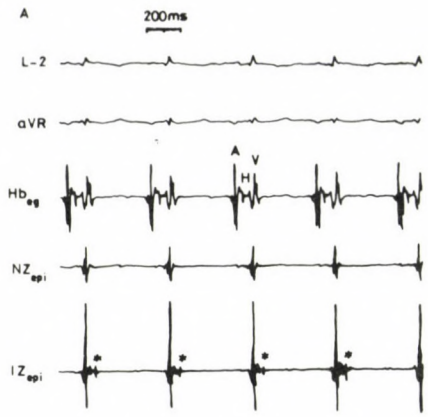

Fig. 5a. Electrogram recorded from the peri-infarction subepicardium (IZ_{epi}) of an anaesthetized Harris-dog during sinus rhythm (120 beat/min) demonstrating late potentials (asterisks)

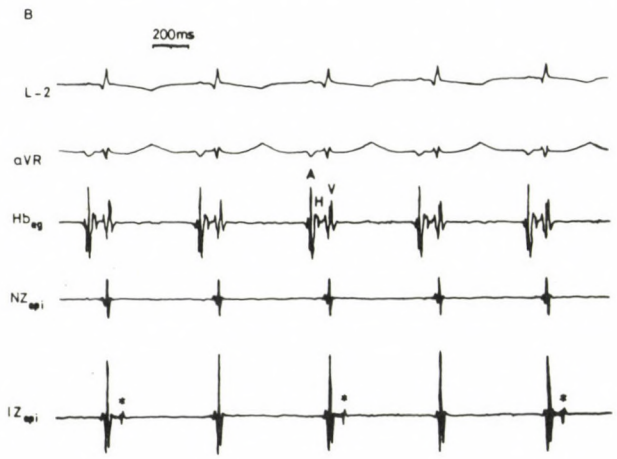

Fig. 5b. Administration of GLG – V – 13 (3 mg/kg) in the same experiment (see also Fig. 5a) induced a 2:1 local conduction block of late potentials (asterisks) in the IZ_{epi} electrogram

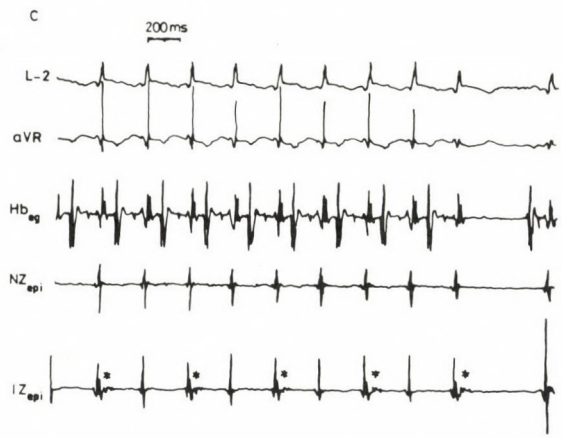

Fig. 5c. Persistence of 2:1 conduction block of IZ_{epi} late potentials (asterisks; see also Fig. 5b) during atrial pacing at a rate of 3.5 Hz suggesting that the prolongation of refractoriness in the ischemically-injured epicardium persisted at the higher heart rate

Adapting one or both of the pacing protocols, 6 out of the 15 dogs were SMVT inducible in the drug-free state. As published previously [17, 34, 43], lidocaine pretreatment highly facilitates the induction of SMVT in this particular canine model of myocardial infarction. Hence, in the present study injection of lidocaine resulted in SMVT induction in 7 additional dogs. Sustained reentry was induced after 3 mg/kg lidocaine in 4 animals and after 6 mg/kg in an additional 3 dogs. After the administration of GLG – V – 13, 5 of the 6 animals which were inducible in the control state, did not manifest SMVT. A proarrhythmic response was seen in only one, i.e., one dog, non-inducible in the baseline, showed SMVT after the administration of GLG – V – 13. GLG – V – 13-induced torsades de pointes VT was not observed. The duration of heart rate lowering, ventricular refractoriness-prolonging and SMVT-preventing effects of GLG – V – 13 were 1.5 – 2.0 hours. GLG – V – 13's antitachyarrhythmic action was associated with 2:1 conduction block of late potentials in the IZ_{epi} electrograms (Figs 5a and 5b). During atrial pacing at rapid heart rates (2.5 – 3.5 Hz), prolonged refractoriness in the IZ_{epi} electrograms with the 2:1 block pattern persisted suggesting a marked and partially rate-independent prolongation of refractoriness in the ischemically injured subepicardium (Fig. 5c).

Inotropic effects of GLG – V – 13 in vitro

No differences in maximal force development and time to peak tension were observed in the atrial or the papillary muscle preparations during superfusion with

GLG – V – 13 (0.32, 1.0 and 3.2 mg/l) compared to the control. Only at the higher (therapeutically irrelevant) concentrations (10 and 32 mg/l), was the force generation, not the time to reach peak tension, of both the atrial and the ventricular muscles depressed (Tables I and II).

Table I

Effects of GLG – V – 13 on the maximal force development in rabbit atrial muscle

	Stimulation frequency (Hz)				
Concentration (mg/l)	0.5	1.0	2.0	3.0	Paired Pacing
Pre-Drug	3.2 ± 1.4	4.1 ± 1.5	4.6 ± 1.5	4.5 ± 1.5	4.9 ± 1.6
0.32	3.3 ± 1.5	4.2 ± 1.6	4.5 ± 1.7	4.6 ± 1.6	5.0 ± 1.7
1.0	3.0 ± 1.2	3.9 ± 1.4	4.3 ± 1.6	4.1 ± 1.4	4.5 ± 1.4
3.2	3.5 ± 1.3	4.2 ± 1.5	4.7 ± 1.6	4.0 ± 1.6	5.3 ± 1.6
10.0	2.9 ± 1.2	3.6 ± 1.5	4.0 ± 1.6	4.0 ± 1.6	4.5 ± 1.5
32.0	1.7 ± 0.5*	2.7 ± 1.0*	3.1 ± 1.3*	3.4 ± 1.5*	3.3 ± 1.0*
Washout	2.9 ± 1.3	3.6 ± 1.5	4.2 ± 1.9	4.5 ± 1.9	4.9 ± 1.4

Maximal force development expressed as g. n = 5, *; $p < 0.05$ *vs.* control

Table II

Effects of GLG – V – 13 on the maximal force development in rabbit papillary muscle

	Stimulation frequency (Hz)				
Concentration (mg/l)	0.5	1.0	2.0	3.0	Paired Pacing
Pre-Drug	0.65 ± 0.09	1.12 ± 0.11	1.76 ± 0.14	1.97 ± 0.19	2.21 ± 0.21
0.32	0.60 ± 0.09	1.12 ± 0.13	1.81 ± 0.14	2.08 ± 0.20	2.18 ± 0.20
1.0	0.55 ± 0.06	1.20 ± 0.14	2.00 ± 0.23	2.04 ± 0.23	2.24 ± 0.20
3.2	0.47 ± 0.04	1.09 ± 0.11	1.93 ± 0.15	2.06 ± 0.19	2.27 ± 0.21
10.0	0.41 ± 0.05*	0.90 ± 0.09	1.72 ± 0.13	1.88 ± 0.24	2.08 ± 0.21
32.0	0.36 ± 0.03*	0.72 ± 0.11*	1.19 ± 0.17*	1.36 ± 0.23*	1.31 ± 0.23*
Washout	0.61 ± 0.10	1.09 ± 0.11	1.66 ± 0.12	1.73 ± 0.10	2.18 ± 0.18

Maximal force development expressed as g/mm^2. n = 10, *; $p < 0.05$ *vs.* control

Discussion

There is no doubt that the autonomic nervous system via enhanced sympathetic impulse traffic plays an important role in the development of life-threatening ventricular tachyarrhythmias (VT/VF) and sudden arrhythmic cardiac death [31, 47].

Therefore, β-adrenergic blocking drugs (Class II action in the Vaughan Williams classification scheme) are able to decrease cardiac mortality and the incidence of sudden arrhythmic cardiac death despite the fact that they diminish the number of ventricular premature depolarizations only slightly [47]. On the other hand, it was elucidated in 1970 that the non-selective β-blocker d,l-sotalol can induce a lengthening of the cardiac action potential duration (APD) and of the refractory period without affecting sodium channels designated as Class III action [45]. Therefore, d,l-sotalol should be considered as the first combined Class II/III antiarrhythmic drug [25, 31]. Recently, it came to light that post-infarction cardiac mortality and possibly the incidence of sudden cardiac death, is reduced solely by β-blocking agents, d,l-sotalol and amiodarone, but not by Na^+- and Ca^{2+}-channel blocking agents [47]. Based on these clinical observations, efforts to develop additional Class III agents with associated/ancillary antiadrenergic properties as is the case with d,l-sotalol or amiodarone have been undertaken [27, 36]. On the other hand, the recognition that Class III agents are very effective in the prevention or termination of reentrant atrial and ventricular tachyarrhythmias resulted in another trend, namely to design new Class III molecules without β-blocking properties [2, 5, 9, 38, 44, 53]. Such compounds have been designated "pure" Class III agents in which the primary electrophysiological effect is to prolong the cardiac APD [7, 38, 44]. For most compounds this effect is mediated via blocking one or more K^+-channels without influencing the function of the fast inward Na^+-channel and the propagation of the cardiac impulse [7].

This study demonstrates that the novel compound, GLG-V-13, which chemically belongs to DHBNs, prolongs the MAP-duration by approximately 20% in both the anaesthetized guinea-pig and dog, respectively. Moreover, the same degree of lengthening of the VERP was demonstrated in the normal *right* ventricular myocardium of the infarcted canine heart. In the mammalian heart the atrial, ventricular and His-Purkinje tissues respond with a shortening of their refractory period when the heart rate increases [23]. Theoretically, an antiarrhythmic drug with no or minimal effects on conduction velocity, and which induces a rate-dependent lengthening of refractoriness (positive use-dependence) in the reentry circuit would approach the ideal antiarrhythmic agent against reentrant tachycardias [23]. Therefore, in the development and evaluation of new Class III antiarrhythmic compounds, the relationship between heart rate and refractoriness must be evaluated. Most Class III antiarrhythmics have the least effect during tachycardias, i.e., at rapid cycle lengths, because of the reverse use dependent shortening of APD/MAP and QT interval [23]. In normal dogs treated with GLG-V-13 we observed the maintenance of a significant MAP_{90}-prolongation also at higher atrial pacing rates (210-270 beats/min) with a slight tendency of reverse use-dependence. Moreover, the observation of 2:1 conduction block of late potentials in the IZ_{epi}

electrogram after the administration of GLG – V – 13 indicated a marked prolongation of refractoriness also in the ischemically injured subepicardium, an effect previously reported for another DHBN, BRB – I – 28, both in vitro [33] and in vivo [15]. The differential response of normal and ischemic Purkinje fibres and the higher sensitivity of functionally abnormal cardiac tissue to Class III agents was demonstrated both *in vitro* [20] and *in vivo* [19]. The prolongation of IZ_{epi}-refractoriness observed by us in the infarcted canine heart should be considered also as a Janus-faced action, which could play a role in the prevention of pacing-induced reentrant SMVT on the one hand, and may predispose ischemic Purkinje tissue to EAD-based triggered firing on the other [20, 39]. It seems to be worthy to mention in this regard that we did not observe GLG – V – 13-induced torsades de pointes VT in the infarcted canine experiments. One has to consider, however, that some species (e.g. the rabbit) appear more sensitive to the proarrhythmic effect of K^+-channel blockers than other species [e.g. the dog; 6, 39]. On the other hand, a recent preliminary study confirms the differential effects of d-sotalol on normal and infarcted myocardium in a canine infarct model and suggests that as opposed to normal tissue d-sotalol does not demonstrate reverse use-dependence in the functionally abnormal tissue [19]. Further studies to elucidate the differential effects of GLG – V – 13 on normal and ischemically injured myocardium are in progress. The *in vivo* electrophysiological profile and the demonstration that GLG – V – 13 induces a significant block of the rapid component of the delayed rectifier K^+-current (I_{kr}; Mattias et al., unpublished observations) provide strong evidence that this compound is a new I_{kr}-blocking Class III agent like almokalant, dofetilide or E – 4031 [5, 7].

GLG – V – 13's negative chronotropic effect could be explained by the prolongation of the APD of sino-atrial (SA) pacemaker cells, which has been reported for the potassium channel-blocking agents tedisamil [2] and melperone [37]. Furthermore, the observation that GLG – V – 13, administered to pentobarbital-anaesthetized dogs pretreated with propranolol, evokes an *additional* significant decrease of the heart rate supports the idea that the negative chronotropic effect is mainly due to a direct Class III action on SA-nodal automaticity (Scherlag et al., unpublished observations). On the other hand, the presence of the imidazole-ring has been shown in cases to convey Class II/III activity on other structural moieties [27] and DHBNs could respond similarly, i.e., the possibility exists that GLG – V – 13 has associated antiadrenergic property. The cellular mechanism of the heart rate lowering action of GLG – V – 13 remains to be clarified and awaits more appropriate protocols.

The incidence of proarrhythmic response is greater in patients suffering from congestive heart failure, and the negative inotropic effect of antiarrhythmics may contribute to greater susceptibility to proarrhythmia [18, 26]. Hence, it is an important observation that dosages of GLG – V – 13 that can be assumed to be

clinically relevant, in the present study do not influence the mechanical activity of rabbit atrial and papillary muscle preparations. Class III antiarrhythmic agents increase the amount of Ca^{2+} entering the cardiac cells due to the prolongation of the plateau of the action potential and consequently delay the inactivation of the Ca^{2+}-channel [24]. Such effects could result in a positive inotropic response or counteract a decrease of the contractility due to any inherent depressive feature of the molecule.

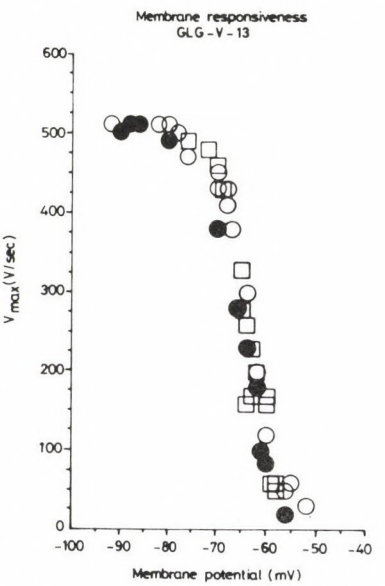

Fig. 6. Membrane responsiveness curve for GLG-V-13 in canine cardiac Purkinje fibers. Membrane responsiveness curves are shown for control (●), 1.0 mg/l GLG-V-13 (o) and 3.2 mg/l GLG-V-13 (□) demonstrating the lack of Na^+-channel blocking activity of the novel antiarrhythmic compound

The prototypical agent of the new antiarrhythmic DHBN-family, BRB-I-28, inhibits fast, Na^+-dependent depolarization (Class I action) [32, 33]. The fast Na^+-channel onset/offset kinetics in the presence of BRB-I-28 (the V_{max} recovery half-time is 160±26 msec), the lack of effect on APD and the use-dependent depression of V_{max} puts BRB-I-28 in the Ib subclass of Na^+-channel blockers [32]. Based on observations in anaesthetized rabbits *in vivo* [6], one may assume, although speculative, that additional Na^+-channel blocking activity of GLG-V-13 may attenuate its proclivity to induce proarrhythmia related to delayed repolarization, i.e., torsades de pointes. Microelectrode recordings obtained in isolated canine Purkinje fibers exposed to GLG-V-13 (0.32, 1.0, 3.2 or 10.0 mg/l), however, did not show any change of V_{max}, membrane responsiveness (Fig. 6), action potential amplitude and resting or overshoot potentials (Patterson et al., unpublished observations). Furthermore, the agent did not induce widening of QRS complex in the guinea-pig or

in the dog. These findings are consonant with the lack of Na^+-channel blocking activity of GLG – V – 13 and the classification of GLG – V – 13 as a pure Class III agent thus seems appropriate. The minor prolongation of the AH and HV intervals observed in the Harris-dog preparation are not, however, a characteristic feature of the novel investigational Class III agents [38] and the underlying cause of these observations are still unclear.

To summarize, GLG – V – 13 is a novel and effective Class III antitachyarrhythmic agent lacking negative inotropic and reverse use dependent properties. In addition, the new agent seems to have a better antiarrhythmic/proarrhythmic profile than lidocaine in the infarcted canine heart. The design and pharmacological characterization of new antiarrhythmics should be done with the simultaneous consideration of both antiarrhythmic and proarrhythmic effects in the aftermath of CAST. The high proarrhythmic potential of lidocaine in the Harris-dog preparation observed by us [17, 29, 43] provides a model which enables testing of the antiarrhythmic/proarrhythmic profile of new compounds quantitatively.

REFERENCES

1. Axenborg, J. E., Hirsch, I.: A PC-based on-line system for physiological in vivo and in vitro experiments. Comput. Methods Programs Biomed. **41**, 55 – 67 (1993).
2. Beatch, G. N., Abraham, S., MacLeod, B. A., Yoshida, N. R., Walker, M. J. A.: Antiarrhythmic properties of tedisamil (KC8857), a putative transient outward K^+ current blocker. Br. J. Pharmacol. **102**, 13 – 18 (1991).
3. Brachmann, J., Kabell, G., Scherlag, B. J., Harrison, L., Lazzara, R.: Analysis of interectopic activation patterns during sustained ventricular tachycardia. Circulation **67**, 449 – 456 (1983).
4. Brugada, J., Boersma, L., Kirchoff, C., Allessie, M.: Proarrhythmic effects of flecainide. Experimental evidence for increased susceptibility to reentrant arrhythmias. Circulation **84**, 1808 – 1818 (1991).
5. Carlsson, L., Abrahamsson, C., Drews, L., Duker, G., Schiller-Linhardt, G.: Proarrhythmic effects of the Class III agent almokalant: importance of infusion rate, QT dispersion, and early afterdepolarizations. Cardiovasc. Res. **27**, 2186 – 2193 (1993).
6. Carlsson, L., Drews, L., Duker, G., Schiller-Linhardt, G.: Attenuation of proarrhythmias related to delayed repolarization by low-dose lidocaine in the anesthetized rabbit. J. Pharmacol. Exp. Therap. **267**, 1076 – 1080 (1993).
7. Carmeliet, E.: Mechanisms and control of repolarization. Eur. Heart J. **14**, Suppl. H, 3 – 13 (1993).
8. Chen, C. L., Sangiah, S., Roder, J. D., Chen, H., Berlin, K. D., Garrison, G. L., Scherlag, B. J., Lazzara, R.: Determination of GLG – V – 13, a novel antiarrhythmic agent, in plasma and urine by high-performance liquid chromatography. J. Liquid Chromatogr. **17**, 913 – 927 (1994).
9. Cross, P. E., Arrowsmith, J. E., Thomas, G. N., Gwilt, M., Burges, R. A., Higgins, A. J.: Selective Class III antiarrhythmic agent. 1. Bis(arylalkyl)amines. J. Med. Chem. **33**, 1151 – 1155 (1990).
10. Duker, G., Almgren, O., Axenborg, J. E.: Computerized evaluation of drug-induced changes in guinea-pig epicardial monophasic action potentials. Pharmacol. Toxicol. **63**, 85 – 90 (1988).

11. Fazekas, T.: Az amiodaron. (In Hungarian) Orvosképzés **61**, 112–123 (1986).
12. Fazekas, T., Brugada, J., Smeets, J. L. R. M., Wellens, H. J. J.: Az Ic antiarrhythmicumok (propafenon) proaritmiás hatásmechanizmusa. (In Hungarian) Cardiol. Hung. **20**, 303–311 (1991).
13. Fazekas, T., Mabo, P., Berlin, K. D., Scherlag, B. J., Lazzara, R.: Comparative electrophysiological effects of a new chemical class of anti-arrhythmic agents, 3,7-diheterobicyclo[3.3.1]nonanes, in the 1-4-day-old infarcted dog heart. The Physiologist **35**, 221 (1992).
14. Fazekas, T., Scherlag, B. J., Mabo, P., Berlin, K. D., Garrison, G. L., Chen, C. L., Sangiah, S., Patterson, E., Lazzara, R.: The comparative antiarrhythmic and proarrhythmic activity of a 3,7-diheterobicyclo[3.3.1]nonane, BRB–I–28, and lidocaine in the 1-4-day-old infarcted dog heart. Acta Physiol. Hung. **81**, 289–299 (1993).
15. Fazekas, T., Scherlag, B. J., Mabo, P., Berlin, K. D., Garrison, G. L., Patterson, E., Lazzara, R.: The antiarrhythmic-proarrhythmic and inotropic profile of two new agents, BRB–I–28 and GLG–V–13: actions in the 1-4-day-old infarcted canine heart. PACE **16**, 925 (1993).
16. Fazekas, T., Scherlag, B. J., Mabo, P., Patterson, E., Berlin, K. D., Garrison, G. L., Lazzara, R.: Egy új refrakter periódust megnyújtó antiaritmiás vegyület, a GLG–V–13 szívelektrofiziológiai jellemzése. (In Hungarian) Cardiol. Hung. **22**, 9–13 (1993).
17. Fazekas, T., Scherlag, B. J., Mabo, P., Patterson, E., Lazzara, R.: Facilitation of reentry by lidocaine in canine myocardial infarction. Am. Heart. J. **127**, 345–352 (1994).
18. Fazekas, T., Smeets, J. L. R. M., Wellens, H. J. J.: Az antiaritmiás gyógyszerek aritmogén hatása. A proaritmiák korszerű klinikai szemlélete. (In Hungarian), Orv. Hetil. **132**, 2243–2248 (1991).
19. Freigang, K. D., Schoels, W., Sterns, L. D., Offner, B., Bauer, A., Kuebler, W., Brachmann, J.: Differential effect of d-sotalol on normal and infarcted tissue in the canine infarct model. J. Am. Coll. Cardiol. 800–4, 198A (1994).
20. Gough, W. B., El-Sherif, N.: The differential response of normal and ischaemic Purkinje fibres to clofilium, d-sotalol and bretylium. Cardiovasc. Res. **23**, 554–559 (1989).
21. Greene, H. L., Roden, D. M., Katz, R. J., Woosley, R. L., Salerno, D. M., Henthorn, R. W. and the CAST Investigators: The Cardiac Arrhythmia Suppression Trial: First CAST... Then CAST–II. J. Am. Coll. Cardiol. **19**, 894–898 (1992).
22. Harris, A. S.: Delayed development of ventricular ectopic rhythms following experimental coronary occlusion. Circulation **1**, 1318–1328 (1950).
23. Hondeghem, L. M., Snyders, D. J.: Class III antiarrhythmic agents have a lot of potential but a long way to go. Reduced effectiveness and dangers of reverse use dependence. Circulation **81**, 686–690 (1990).
24. Josephson, M. A., Singh, B. N.: Haemodynamic effects of Class III antiarrhythmic agents. In: Control of Cardiac Arrhythmias by Lengthening Repolarization. Singh, B. N. (Ed.), Futura Publishing Co., Mount Kisco, New York, 153–173 (1988).
25. Julian, D. G., Prescott, R. J., Jackson, F. S., Szekely, P.: Controlled trial of sotalol for one year after myocardial infarction. Lancet **1**, 1142–1147 (1982).
26. Lazzara, R., Szabó, B., Patterson, E., Scherlag, B. J.: Mechanisms for proarrhythmia with antiarrhythmic drugs. In: Cardiac Electrophysiology. From Cell to Bedside. Zipes, D. P., Jalife, J. (Eds.), W. B. Saunders Co., 402–407 (1990).
27. Lis, R., Morgan, T. K., Marisca, Jr., A. J., Gomez, R. P., Lind, J. M., Davey, D. D., Phillips, G. B., Sullivan, M. E.: Synthesis of novel (aryloxy) propanolamines and related compounds possessing both class II and class III antiarrhythmic activity. J. Med. Chem. **33**, 2883–2891 (1990).

28. Lynch, J. J., Heaney, L. A., Wallace, A. A., Gehret, J. G., Stein, B. S.: Failure of lidocaine to suppress lethal ischemic ventricular arrhythmias in a canine model of previous myocardial infarction. J. Cardiovasc. Pharmacol. **16**, 41 – 49 (1990).
29. Patterson, E., Gibson, J. K., Lucchesi, B. R.: Electrophysiologic actions of lidocaine in a canine model of chronic myocardial ischemic damage – arrhythmogenic actions of lidocaine. J. Cardiovasc. Pharmacol. **4**, 925 – 934 (1982).
30. Patterson, E., Jackman, W. M., Scherlag, B. J., Lazzara, R.: The monophasic action potential in clinical cardiology. Clin. Cardiol. **14**, 505 – 510 (1991).
31. Patterson, E., Lynch, J. J., Luchessi, B. R.: Antiarrhythmic and antifibrillatory actions of the beta adrenergic receptor antagonist, dl-sotalol. J. Pharmacol. Exp. Therap. **230**, 519 – 526 (1984).
32. Patterson, E., Scherlag, B. J., Berlin, K. D., Lazzara, R.: Electrophysiological actions of BRB – I – 28 in canine myocardial tissues. J. Pharmacol. Exp. Therap. **259**, 558 – 565 (1991).
33. Patterson, E., Scherlag, B. J., Berlin, K. D., Lazzara, R.: Electrophysiologic actions of BRB – I – 28 in ischemically injured canine myocardium. J. Cardiovasc. Pharmacol. **21**, 637 – 646 (1993).
34. Patterson, E., Scherlag, B. J., Lazzara, R.: Electrophysiologic actions of clofilium and lidocaine in ischemically injured canine epicardium. J. Pharmacol. Exp. Therap. **262**, 375 – 382 (1992).
35. Patterson, E., Shlafer, M., Walden, K. M., Khazaeli, M. B., Montgomery, D. G., Lucchesi, B. R.: Changes in cardiac muscle function and biochemistry produced by long-term amiodarone and amiodarone + triiodthyronine administration in the rabbit. Pharmacology **35**, 130 – 140 (1987).
36. Phillips, G. B., Morgan, Jr., T. K., Lumma, W. C., Gomez, R. P., Lind, J. M., Lis, R., Argentieri, T., Sullivan, M. E.: Synthesis, cardiac electrophysiology, and β-blocking activity of novel arylpiperazines with potential as Class II/III antiarrhythmic agents. J. Med. Chem. **35**, 743 – 750 (1992).
37. Refsum, H., Platou, E. S.: Antiarrhythmic properties of melperone, a neuroleptic agent that lengthens cardiac repolarization. In: Control of Cardiac Arrhythmias by Lengthening Repolarization. Singh, B. N. (Ed.), Futura Publishing Co., Mount Kisco, New York, 353 – 365 (1988).
38. Roden, D. M.: Current status of Class III antiarrhythmic drug therapy. Am J. Cardiol. **72**, 44B – 49B (1993).
39. Roden, D. M.: Early after-depolarizations and torsades de pointes: implications for the control of cardiac arrhythmias by prolonging repolarization. Eur. Heart J. **14**, Suppl. H, 56 – 61 (1993).
40. Scherlag, B. J., Abelleira, J. L., Samet, P.: Electrode catheter recordings from the His bundle and left bundle in the intact dog. In: Research in Physiology. Kao, F. F., Koizumi, K., Vassale, M. (Eds.), Aulo Gaggi Publisher, Bologna 223 – 238 (1971).
41. Scherlag, B. J., Patterson, E., Berbari, E., Lazzara, R.: Slow conduction in experimental arrhythmias: mechanisms and role in reentry. In: Cardiac Electrophysiology and Arrhythmias. Fisch, C., Surawicz, B. (Eds.), Elsevier Science Publishing Co., New York, 96 – 107 (1991).
42. Scherlag, B. J., Patterson, E., Lazzara, R.: Advantages of an acute experimental preparation that mimics clinical arrhythmias. J. Electrocardiol. S61 – S63 (1988).
43. Scherlag, B. J., Patterson, E., Lazzara, R., Bailey, B. R., Thomson, M. D., Berlin, K. D.: Comparative electrophysiological and hemodynamic actions of BRB – I – 28 and lidocaine in the normal and infarcted dog heart. J. Electrophysiol. **2**, 461 – 477 (1988).
44. Singh, B. N.: Arrhythmia control by prolonging repolarization: the concept and its potential therapeutic impact. Eur. Heart J. **14**, Suppl. H, 14 – 23 (1993).
45. Singh, B. N., Vaughan Williams, E. M.: A third class of anti-arrhythmic action: effects on atrial and ventricular intracellular potentials, and other pharmacological actions on cardiac muscle of MJ1999 and AH3474. Br. J. Pharmacol. **39**, 675 – 689 (1970).

46. Smith, G. S., Thomson, M. D., Berlin, K. D., Holt, E. M., Scherlag, B. J., Patterson, E., Lazzara, R.: A study of the synthesis and antiarrhythmic properties of selected 3,7-diheterobicyclo[3.3.1]nonanes with substituents at the 2,4-positions and at the 9-position. Eur. J. Med. Chem. **25**, 1–8 (1990).
47. Teo, K. K., Yusuf, S., Furberg, C. D.: Effects of prophylactic antiarrhythmic drug therapy in acute myocardial infarction. An overview of results from randomized controlled trials. J.A.M.A. **270**, 1589–1595 (1993).
48. Thompson, M. D., Smith, G. S., Berlin, K. D., Holt, E. M., Scherlag, B. J., van der Helm, D., Muchmore, S. W., Fidelis, K. A.: Synthesis and antiarrhythmic properties of novel 3-selena-7-azabicyclo[3.3.1]nonanes and derivatives. Single-crystal X-ray diffraction analysis of 7-benzyl-3-selena-7-azabicyclo[3.3.1]nonan-9-one and 7-benzyl-3-selena-7-azabicyclo[3.3.1]nonane hydroperchlorate. J. Med. Chem. **30**, 780–788 (1987).
49. Vaughan Williams, E. M.: Significance of classifying antiarrhythmic actions since the Cardiac Arrhythmia Suppression Trial. J. Clin. Pharmacol. **31**, 123–135 (1991).
50. Vos, M. A., Fazekas, T., Gorgels, A. P. M., Leunissen, J. D. M., Wellens, H. J. J.: Action of $MgSO_4$ differs from moricizine and verapamil on ouabain-induced ventricular tachycardia in normomagnesemic conscious dogs. J. Cardiovasc. Pharmacol. **23**, 252–258 (1994).
51. Wiesfeld, A. C. P., Crijns, H. J. G. M., Tobé, T. J. M., Almgren, O., Bergstrand, R. H., Aberg, J., Haaksma, J., Lie, K. I.: Electropharmacologic effects and pharmacokinetics of almokalant, a new Class III antiarrhythmic, in patients with healed or healing myocardial infarcts and complex ventricular arrhythmias. Am. J. Cardiol. **70**, 990–996 (1992).
52. Wohlfart, B.: Analysis of the mechanical alternans in rabbit papillary muscle. Acta Physiol. Scand. **115**, 405–414 (1982).
53. Wong, W., Pavlou, H. N., Birgersdotter, U. M., Hilleman, D. E., Mohiuddin, S. M., Roden, D. M.: Pharmacology of the Class III antiarrhythmic agent sematilide in patients with arrhythmias. Am. J. Cardiol. **69**, 206–212 (1992).

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In vitro degradation of Thymopoietin$_{32-35}$ in human, dog and rat plasma

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TP4 (Arg–Lys–Asp–Val) is a synthetic immunomodulatory tetrapeptide of Chemical Works of Gedeon Richter Ltd.

The aim of this study was to give comparative data on the in vitro degradation of ^{14}C–TP4 in plasma of different species at two TP4 concentrations to allow suitable extrapolation of preclinical data to human system.

The results show that the degradation was very fast in each plasma and varied by the species and concentrations studied. In rat, dog and human plasma for initial concentration of 80 μg/ml and 800 μg/ml the apparent half-life values proved to be 1.4, 1.8, 2.7 and 3.9, 6.3, 13.6 minutes, respectively. The most susceptible breakdown point was the peptide bond between Arg and Lys. The correlation between TP4 degradation and arginine formation was demonstrated by a straightline with a correlation coefficient of 0.99565 and a slope close to −1.

Keywords: immunomodulator, TP4, peptide, plasma degradation, rat, dog, human

Thymopoietin$_{32-35}$ – termed as TP4 – is a synthetic tetrapeptide Arg–Lys–Asp–Val [3] possessing some immunological effects similar to those of thymopentin (Arg–Lys–Asp–Val–Tyr) that represents the "active site" of the immunomodulatory thymic hormon thymopoietin [1]. The stability of thymopentin in human plasma has been studied and the degradation proved to be very rapid, showed

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an apparent $t_{1/2}$ of about 30 seconds [4]. The peptide bond linking Arg and Lys was most susceptible to the enzymatic hydrolysis. The results led to a very important conclusion drawn by the authors: "sustained circulating levels may not be required for activity of pentapeptide."

This paper presents a comparative study for the TP4 in vitro degradation in human, dog and rat plasma. The TP4 used for the investigations contained ^{14}C-labelled arginine. The degradation of the compound was followed in time by quantitating the radioactivity related to TP4 and its arginine containing fragments.

Materials and methods

The TP4, Arg–Lys–Asp, Arg–Lys peptides were synthesized in Chemical Works of Gedeon Richter Ltd. [2, 3]. ^{14}C–TP4 of 626 MBq/mmol specific activity was synthesized in the Central Research Institute for Chemistry of Hungarian Academy of Sciences. The nuclide was introduced into delta position of arginine. The radiochemical purity was >95% as determined by paper electrophoresis. The ^{14}C–TP4 was stored in sterile distilled water solution at 10 mg/ml concentration and −20 °C.

All other chemicals were of analytical grade. Pooled plasma samples were used in the study.

Degradation study

The ^{14}C–TP4 degradation was studied in 0.5 ml of plasma at 37 °C and TP4 concentration of 80 and 800 μg/ml. ^{14}C–TP4 solution of 10 mg/ml was added to the plasma preincubated at 37 °C for 5 minutes. From the reaction mixtures samples of 50 μl were taken at appropriate times and mixed with equal volume of 0.5 M perchloric acid solution at 4 °C to stop the reaction and deproteinize the samples. After rigorous stirring the samples were centrifuged at 4 °C and 4150 g for 30 minutes. The pH of the supernatants were neutralized by the addition of 2.5 M KOH solution (5–6 μl). Because of formation of insoluble salts, the samples were once more centrifuged. The final supernatants were stored at −20 °C till the electrophoretic analysis.

Electrophoresis

For electrophoretic separation of TP4 and its degradation products sodium acetate–acetic acid buffer of 0.05 M and pH 4.2 was used. The electrophoresis was performed on Whatmann 3 MM paper. A marker (reference) solution was applied at the two edges and the middle of Whatmann paper to allow the identification the radioactive TP4 and its degradation products in trial samples. The marker solution contained 1 mg/ml of TP4 and its possible arginine containing degradation products as Arg–Lys–Asp, Arg–Lys peptides and arginine. Between the markers two plasma supernatants were applied. The sample volumes were 5 μl. The electrophoresis was carried out at 500 V and 25 mA using Shandon Vokam 500 power supply Gelman Deluxe electrophoretic chamber. After 30 minutes the paper was removed and dried quickly, the different sample strips were cut out. The strips of trial samples were sliced into pieces. These pieces were placed in scintillation vials containing 0.5 ml of distilled water. After 30-min shaking 10 ml of scintillation cocktail was added. The cocktail composed of 1000 ml benzyl alcohol, 200 ml absolute ethanol and 6 g 2,5-diphenyl oxasol. The radioactivity was measured by LKB Rackbeta scintillation counter. The marker strips were developed by ninhydrine. The TP4 content of samples was expressed as percentage of the total recovered radioactivity in samples.

Results

The precision of the electrophoretic analysis was demonstrated in Table I which shows the mean TP4 and arginine contents with respective coefficient of variation for the analysis of the same samples within one run and between different runs of electrophoretic separations.

Table I

Precision of TP4 and arginine determination in trial plasma supernatants by paper electrophoresis

		TP4 content of samples (% of total activity)		Arginine content of samples (% of total activity)	
	n	mean	c.v.	mean	c.v.
Intra-run assay	4	83.7	1.0	5.64	5.32
	4	10.2	10.4	78.1	2.23
Inter-run assay	2	36.1	7.5	55.4	8.1
	2	19.6	10.5	72.0	6.5
	3	9.7	7.8	79.9	2.0

The precision of the overall procedure of the TP4 degradation study was presented in Table II by the mean TP4 and arginine values with respective coefficients of variation obtained by parallel incubations.

In the studied systems arginine was the only detected degradation fragment from the possible arginine containing fragments except for the human samples at the first 30 seconds. In these human samples approximately 5% of the total radioactivity appeared as arg–lys dipeptide. Apart from this exception, TP4 and arginine content of samples correlated, as it could be assessed from a high correlation coefficient and a slope close to −1. This correlation indicated that the rate of decay of TP4 matched the rate of formation of arginine (Fig. 1).

Table II

Precision of the overall procedure of the TP4 degradation studies

Incubation time (minutes)		TP4 content (% of total activity)		Arginine content (% of total activity)	
	n	mean	c.v.	mean	c.v.
30	2	77.0	3.0	14.0	14.1
60	3	62.7	1.7	24.5	10.9
120	3	46.7	3.8	46.7	4.5

Mean values obtained by parallel incubations in dog plasma at TP4 concentration of 80 μg/ml

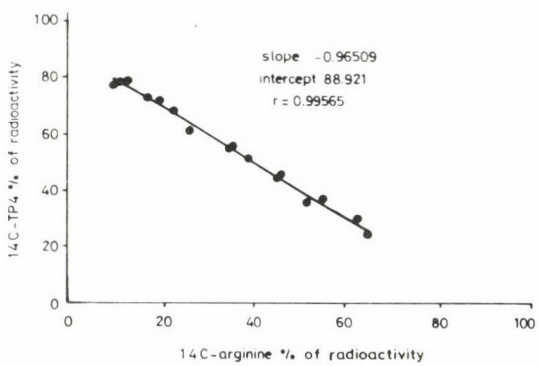

Fig. 1. Correlation between ^{14}C–TP4 degradation and ^{14}C-arginine formation in plasma

Figure 2 shows the TP4 degradation time curves for human, dog and rat plasma at starting TP4 concentration of 80 and 800 μg/ml. The rate of degradation in the three species was demonstrated in Fig. 3 by using semilogarithmic plot. Table III presents the estimated apparent plasma half-life values for the three species.

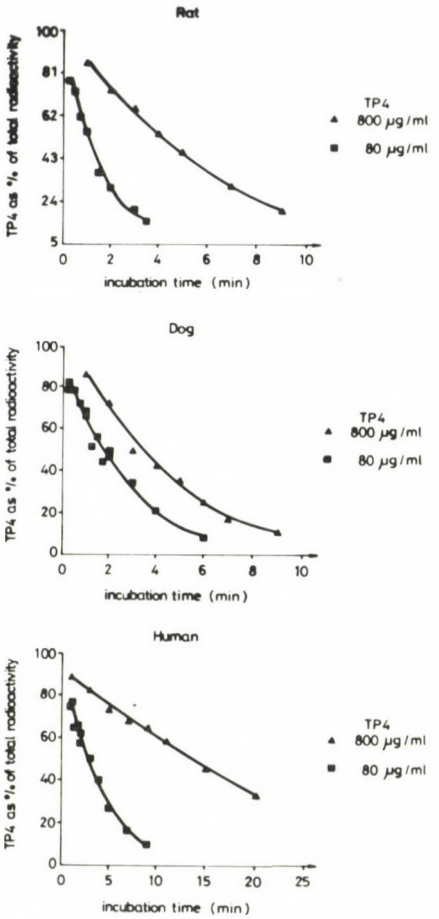

Fig. 2. ^{14}C–TP4 degradation in rat, dog and human plasma at concentrations of 80 and 800 µg/ml

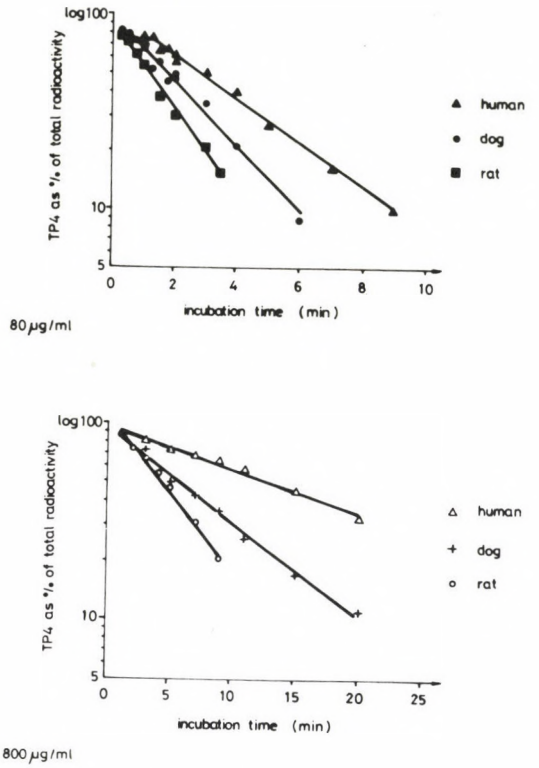

Fig. 3. Comparison on ^{14}C–TP4 in vitro degradation in plasma of rat, dog and human at TP4 concentrations of 80 and 800 µg/ml

Table III

Apparent half-life of TP4 in plasma for different species

TP4 concentration (µg/ml)	Half-life (minutes)		
	Rat	Dog	Human
80	1.4	1.8	2.7
800	3.9	6.3	13.6

Discussion

It can be concluded from the results that the degradation of TP4 in plasma was rapid and showed differences between the species. The rate of the degradation was different for the two concentrations investigated (Fig. 3, Table III). The rat plasma had the most intensive TP4-hydrolysing activity and the human plasma had the lowest one. As Fig. 2 shows the decay of TP4 was curvilinear in all species especially for the 80 μg/ml starting concentration. Although the semilogarithmic plots of the data gave straight lines (Fig. 3) it is possible that the true first order phase of TP4 plasma degradation falls even below 80 μg/ml and the half-life values may be even shorter than the ones estimated for the 80 μg/ml TP4 concentration. Since the possible in vivo concentration values are expected to be lower than 80 μg/ml at the therapeutic dose, the TP4 disappearance from the plasma might be very fast, even not considering other factors.

In summary, the results of the present study are consistent with the data described earlier for TP5 degradation [1]. Our data show that the degradation of TP4 is equally fast and the most susceptible breakdown point of the tetrapeptide is the peptide bond linking arginine to lysine as it is the case for TP5. On the basis of the rapid in vitro degradation long lasting in vivo plasma levels can not be expected.

REFERENCES

1. Goldstein, G., Scheid, M. P., Boyse, E. A., Schlesinger, D. H., Van Wauve: A synthetic pentapeptide with biological activity characteristic of the thymic hormone thymopoietin. Science **204**, 1309–1310 (1979).
2. Hungarian Patent Appl. No. 1755/1981.
3. Kisfaludy, L., Nyeki, O., Schon, I., Denes, L., Ember, J., Szpornyi, L., Hajos, Gy., Szende, B.: Immuno-regulating peptides, i. synthesis and structure-activity relationships of thymopentin analogs. Hoppe-Seyler's Z. Physiol. Chem. **364**, 933–940 (1983).
4. Tischio, J. P., Patrick, J. E., Weintraub, H. S., Chasin, M. C., Goldstein, G.: Short in vitro half-life of thymopoietin pentapeptide in human plasma. Int. J. Peptide Protein Res. **14**, 479–484 (1979).

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Pre-column fluorescence derivatization using leucine-coumarnylamide for HPLC determination of mono- and dicarboxylic acids in plasma

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A sensitive HPLC assay utilising a simple fluorescence pre-column labelling technique was developed for plasma level monitoring of different types of mono- and dicarboxylic acids.

Carboxylic acids form mixed anhydrides with ethyl chloroformate in the presence of triethylamine; the mixed anhydrides further react with L-leucine-4-methyl-7-coumarinylamide, forming highly fluorescent and stable amides. Drugs with no chromophore (azelaic acid, and its longer carbon chain analogues) or week UV absorption (artelinic acid, enalaprilat) were used as model compounds. The plasma samples were extracted using ion exchange solid phase cartridges. The separation was performed on an Axxiom C_{18} (5 μm, 4.6 × 250 mm) column. The detector wavelengths were set at 330 nm for excitation and 390 nm for emission.

Keywords: pre-column fluorescent derivatization, mono- and dicarboxylic acids, solid-phase extraction, reversed-phase HPLC

The concentration measurement of mono- and dicarboxylic acids with low wavelength UV absorption (enalaprilat) or with no chromophore (azelaic acid) usually requires high-cost immunoassay [1] or instrumentation, like GC–MS [2], plus

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in the latter case the synthesis of deuterated standards. For assaying great number of samples during therapy not a more sensitive, but a less expensive and simpler HPLC method would be required.

In the mid eighties Björkman reported a pre-column derivatization method for the stereo specific assaying of non-steroid anti-inflammatory drugs (NSAID) [3]. That paper describes a derivatization reaction where the carboxylic acid functional groups of the molecules react with chiral reagent forming acidamides. The reaction takes less than 3 minutes to perform.

The above-mentioned reaction is the basis of the new fluorescence derivatization described in this paper. The paper also describes the application of the method in the analysis of plasma samples.

Materials and methods

Reagents and chemicals

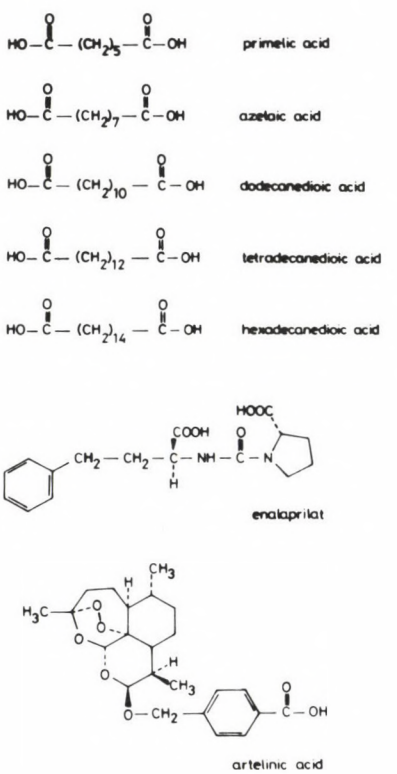

Fig. 1. Structure of the test compounds

Azelaic acid (nonanedioic acid), pimelic acid, dodecanedioic acid, tetradecanedioic acid and hexadecanedioic acid were supplied by Sigma (St. Louis, USA), artelinic acid by Walter Reed Army Institute for Drug Research (Washington, DC., USA) and USP standard enalaprilat was used (Fig. 1). The compounds were dissolved in methanol:water 1:1 v/v and further diluted also with methanol:water 1:1 v/v. Ethyl chloroformate (ETC), triethylamine (TEA) from Sigma (St. Louis, USA) and the fluorescent reagent L-leucine-4-methyl-7-coumarinylamid-hydrochloride (L-4) [4] were purchased from Fluka (Buchs, Switzerland), acetonitrile (HPLC grade), methanol (HPLC grade), phosphoric acid (Fisher Reagent ACS), hydrochloric acid (Fisher Reagent ACS) and NaH_2PO_4 (Fisher Certified ACS) from Fisher Scientific (Fair Lawn, USA), Bondelut NH_2 and SCX 500 mg cartridges were obtained from Varian (Harbor City, USA). For controls, standard curve samples blank rat plasma was purchased from Pel Free Biologicals (Rogers, USA), blank dog plasma from Rockland Company (Gilbertsville, USA) and blank human plasma from Irwin Memorial Blood Bank (USA).

ETC and TEA were diluted with acetonitrile to prepare 60 mM and 50 mM solution, respectively, L-4 was dissolved in methanol to prepare 3 mM solution.

Extraction

Azelaic acid, pimelic acid, etc. and artelinic acid determination 1 ml of plasma:

1. Load plasma sample onto NH_2 solid-phase cartridges (previously washed with 3 ml of methanol and 12 ml of 0.1 M NaH_2PO_4 pH 7 buffer; artelinic acid plasma samples were diluted with the same buffer prior to the load).
2. Wash cartridges with 3 ml of water and 3 ml of acetonitrile, then dry with vacuum.
3. Elute with 1 ml of 0.5 M formic acid in acetonitrile, and evaporate to dryness under nitrogen stream.
4. Derivatize.

Enalaprilat in 1 ml of plasma:

1. Load plasma sample onto SCX solid phase cartridges (previously washed with 3 ml of methanol and 12 ml of 1% acetic acid).
2. Wash cartridges with 3 ml of water followed by 3 ml of acetonitrile, and dry with vacuum.
3. Elute with 2 ml of 0.5 M HCl in acetonitrile, and evaporate to dryness under nitrogen stream.
4. Derivatize.

Derivatization (see reaction mechanism in Fig. 2. Scheme adapted from [5])

1. Add 100 µl of 50 mM TEA solution to the extracted residue, and vortex for 1 minute.
2. Add 50 µl of 60 mM ETC solution, and vortex for 1 minute.
3. Add 200 µl of 3 mM L-4 solution, vortex for 1 minute and wait for 4 minutes.
4. Evaporate to dryness under nitrogen stream.
5. Reconstitute in 200 µl of acetonitrile:water 1:1 v/v.
6. Inject 5-15 µl onto column.

The derivatization is performed at room temperature.

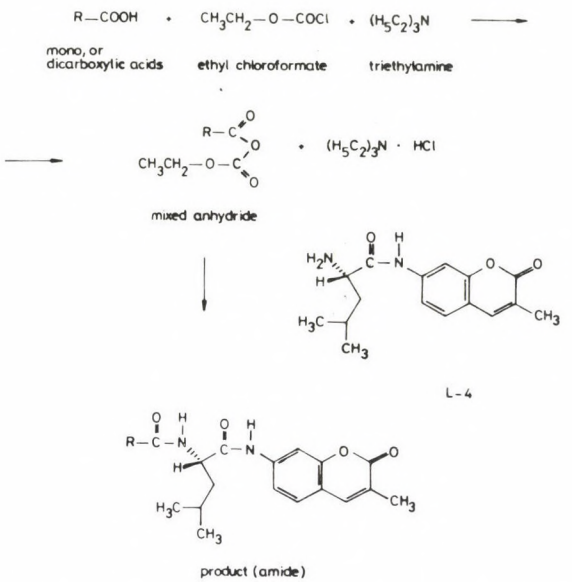

Fig. 2. Reaction mechanism of the new fluorescence derivatization method

Chromatography

The chromatographic system used consisted of Milton Roy gradient pump (CM 4000), a WISP 710B autosampler (Waters Associates, Milford, USA), an Axxiom octyl reversed phase column (5 μm particle size; 250×4.6 mm; Richard Scientific, Novato, USA), a Shimadzu 535 fluorescence detector, operating at 330 nm as excitation wavelength and 390 nm as emission wavelength, sensitivity was in high position, and range was set to 64 (Shimadzu Scientific Instruments, Inc., Columbia, USA) and a Hewlett-Packard 3390A integrator (Hewlett-Packard Co., Santa Clara, USA).

Table I

Gradient elution program for the HPLC separation of the test compounds

Time (minutes)	Fatty acids		Artelinic acid		Enalaprilat	
	A%	B%	A%	B%	A%	B%
0	55	45	60	40	52	48
15	55	45	60	40	52	48
30	10	90	10	90	10	90

The compounds were separated by gradient elution. The mobile phase A was 1000 ml water plus 2 ml 85% phosphoric acid, the mobile phase B 1000 ml acetonitrile plus 2 ml 85% phosphoric acid. The gradient program is shown in Table I.

Animal experiment

Different doses of azelaic acid were administered to dogs during a toxicological study. The plasma concentrations of azelaic acid and pimelic acid (metabolite of azelaic acid) were determined with a negative ionisation GC–MS method using selective ion monitoring of the pentafluorobenzyl derivatives of the compounds. Latter method was validated and used for hundreds of samples. Detailed description is not given here, since this is not topic of this paper. After GC–MS analysis few samples were reassayed with HPLC to determine the reliability of the new method by comparing GC–MS and HPLC results.

Results and discussion

Fatty acids and the other test compounds as well are highly polar molecules. Their extraction with organic solvents from biological fluids would be difficult because of the interfering endogenous compounds. In addition, fatty acids have no chromophore and the others have low-wavelength UV absorption maxima, where the high level endogenous components have. An always returning demand is to develop simple, reliable and suitably sensitive analytical methods, which do not require expensive and sophisticated instrumentation. We made attempts to fulfil the above-mentioned requirements for the measurements of these kinds of compounds in human and dog plasma.

Speaking about the chemical reaction, one can see that a relatively wide range of mono- and dicarboxylic acid was successfully derivatized. Figure 3 shows HPLC chromatograms of the products.

The peaks represent equal amounts of the compounds, thus the yields are comparable. As it is shown in the first chromatogram the peak height of dodecanedioic acid (a dicarboxylic acid) is found to be more than twice than that of artelinic acid (a monocarboxylic acid), while the yield is similar for symmetrical (azelaic acid and others in the second) and non-symmetrical (enalaprilat in the third chromatogram) dicarboxylic acids. At room temperature even after 3 days standing in test tube dry, or dissolved, the fluorescent products were stable. The stability was not tested for a longer period.

The labelling method is also applicable in the case of plasma extracts of these carboxylic acids. In Fig. 4 chromatograms of 1 μg/ml plasma samples are presented. Calibration curves obtained in human and dog plasma are linear from the low ng/ml to the low μg/ml range. Even though the theoretical detection limit is 1 ng/ml for dicarboxylic acids, the actual limit is depending on the fatty acid levels in the plasma used for the preparation of calibrators and QC samples, thus having a control plasma containing very low levels of fatty acids is essential in this assay. The fatty acid concentration in the plasma is depending on the food intake; low fatty acid concentration plasma can be obtained from animals starved for a longer period. In

case plasma samples with low fatty acid concentration are not available, water may be used as control matrix. In latter case though the accuracy of the measurement will be questionable.

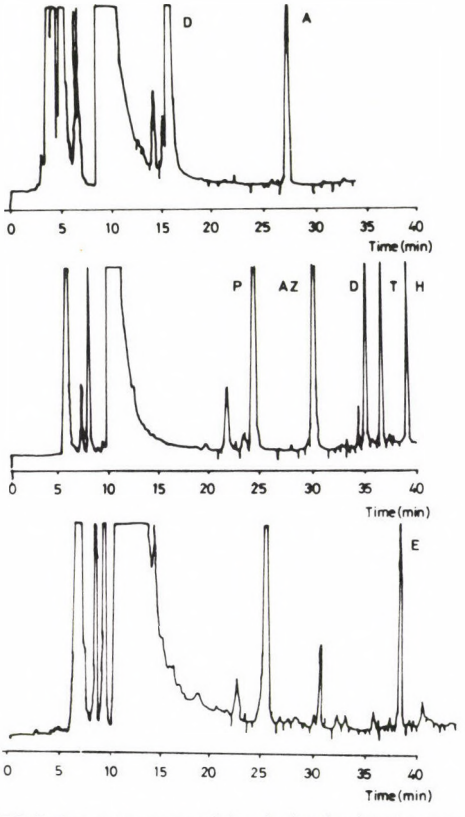

Fig. 3. HPLC chromatograms of the derivatized test compounds

The plasma extracts of artelinic acid and enalaprilat give 10 ng/ml and 20 ng/ml sensitivity limits, respectively.

Data in Table II show that the plasma concentrations determined by HPLC and GC–MS methods are very similar for both azelaic and pimelic acid, confirming the reliability of the derivatization method.

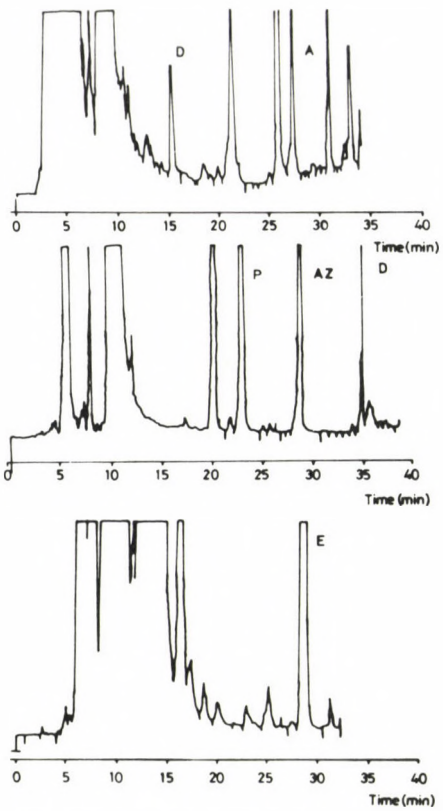

Fig. 4. HPLC chromatograms of the derivatized plasma extracts

Table II

The comparison of azelaic and pimelic acid concentrations (ng/ml) in dog plasma determined with GC–MS and HPLC

Treatment #	Dog ID	Azelaic acid		Pimelic acid	
		GC–MS	HPLC	GC–MS	HPLC
1	1356	31300	25800	1330	*
2	1356	75500	75600	5850	5300
3	1356	14700	15000	1390	1580
4	1356	52500	40600	3218	2970

* technical problem

REFERENCES

1. Jackson, B., Cubela, R. B., Johnston, C. I.: Measurement of angiotensin converting enzyme inhibitors by Radio-Inhibitor Binding displacement: Application to lisinopril in chronic renal failure. J. Hypertens. 5, Suppl. **5**, S587–S589 (1987).
2. Niwa, T., Ohki, T., Maeda, K., Saito, A., Kobayashi, K.: Pattern of aliphatic dicarboxylic acids in uremic serum including a new organic acid, 2,4-dimethyladipic acid. Clin. Chim. Acta **99**(1), 71–83 (1979).
3. Björkman, S.: Determination of the enantiomers of indoprofen in blood plasma by high-performance liquid chromatography after rapid derivatization by means of ethyl chloroformate. J. Chromatogr., **339**, 339–346 (1985).
4. Kanaoka, Y., Takahashi, T., Nakayama, H.: A new fluorogenic substrate for aminopeptidase. Chem. Pharm. Bull., **25**, 362–363 (1977).
5. Spahn, H.: Formation of diastereomeric derivatives of 2-arylpropionic acids using L-leucinamide. J. Chromatogr., **423**, 334–339 (1987).

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Structure activity relationship in toxicology

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One of the basic principles of the modern pharmacological research is that there is a close correlation between the chemical structure and the pharmacological effect. In contrast to pharmacology, in toxicology high doses of the drugs are used in order to provoke toxic symptoms generally realized through other receptors than the pharmacological effect.

The evaluation of the structure activity relationship (SAR) in toxicology is hindered by the fact that it is not possible to establish in every case that the toxic effect

1. is developed by the original compound or its metabolite(s)
2. is realized through one or more receptors.

Furthermore it is necessary to take into consideration that the studies in toxicology are not carried out with so many compounds as the studies in pharmacology and – horribile dictu – the toxicological data are not always public. Thus the prediction of the toxicity of a compound on the base of its chemical structure is difficult, and the prediction whether a new drug is toxic to such an extent that excludes its development, is impossible. What we can do is trying to predict – in the knowledge of the metabolism of the drug – its possible carcinogenic, hepatotoxic, CNS etc. toxic effect.

Keywords: chemical structure, toxicology, pharmacology, relationship

One of the most important principles of the modern pharmacological research is that there is a close correlation between the chemical structure and the pharmacological effect. This supposition is reasonable if we take into consideration that the action of most pharmacons becomes realized through specific receptors. Thus it is well known that one of the methods of planning new compounds is the

Some results described in this paper were presented in the Symposium on Pharmacokinetics and Metabolism. Mátrafüred, 15–16 April, 1994, Hungary

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computer technique where the interaction of the receptor with new molecules can be simulated.

General aspects. Pharmacology versus toxicology

1. During toxicology studies we must reach the toxic symptoms in animals, and when possible even in humans during the phase I study. This means the use of high doses, in animals sometimes extremely high, which are irrelevant to pharmacology and to the human therapy. It is quite clear that the toxic symptoms are realized – in most of the cases – through other receptors than the pharmacological response.

2. In toxicology we measure the effect of the drug and all of its metabolites. In pharmacology we generally measure the effect of the drug itself. Most of the drugs undergo metabolism in vivo, the structure of the metabolites is not always known, especially that of the so-called minor metabolites which may be very important in long-term toxicity, but do not play any role in the pharmacological action.

3. Systematic investigation of the metabolites of drugs for toxicity does not exist, due to economical consideration. In contrast, pharmacology investigates very carefully the action of the metabolites, due to economical interest of the company.

4. In pharmacology a lot of compounds with similar structure are screened for their action, but it is not the case in toxicology, i.e. the data used to draw conclusion between chemical structure and toxicology action are very poor.

5. Compounds proved to be toxic in animal studies are never administered to human beings.

6. In many cases toxicological results remain unpublished.

Example of Valproic acid (VPA)

VPA (2-n-propyl-pentanoic acid) was introduced in several countries as an anticonvulsant drug. Its mechanism of action is unknown, but the ability to elevate brain GABA level has been suggested. VPA produces some 50 metabolites in rats and humans. The major pathways of metabolism is β-oxidation with glucuronidation, but ω-hydroxylation, ω-1-hydroxylation, β-hydroxylation, δ-dehydrogenation, γ-dehydrogenation and glycine conjugation also occur [for review see Refs 1 and 2].

VPA brings about liver damage and displays teratogenic effect [3, 4, 5]. 2-n-propyl-4-pentenoic acid has been suspected to be the ultimate toxic/teratogenic compound. This compound when incubated in vitro with rat liver microsomes has produced 8 metabolites among them 2-n-propyl-hydroxymethyl-γ-butyrolactone, possibly via epoxide intermediate (Fig. 1). 6 metabolites have been tested for teratogenecity and only 2-n-propyl-4-pentenoic acid was found to be clearly teratogenic. So it can be supposed that the final toxic teratogenic compound is 2-n-propyl-4-hydroxymethyl-γ-butyrolactone. From these results one could conclude that (saturated and unsaturated) fatty acids are potential toxic teratogenic compounds. If we suppose that this effect requires a carboxyl group and a double bond the simplest compound would be acrylic acid for liver injury and teratogenecity. But acrylic acid proved to be inactive in this respect.

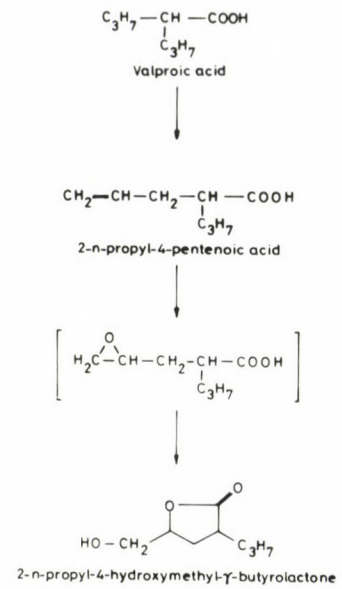

Fig. 1. Valproic acid and its metabolism to possible toxic/teratogenic compounds

Conclusion

From the example of VPA, a relatively simple compound it can be seen how difficult it is to draw conclusion on the possible toxic effect of a compound from its chemical structure. And it is quite clear that without knowing the structure of the metabolites this conclusion is practically impossible.

The first step in the prediction of the toxicity of a compound must be the experimental determination or at least computerized calculation of the structure of the metabolites, and then each metabolite should be investigated for being a potential carcinogenic, mutagenic, teratogenic, liver, cardiovascular, CNS etc. toxic agent. The power of the computer programs is based, however, on earlier data generally obtained in animal experiments, being more or less reliable, therefore their human relevance may be rather poor.

In my opinion the prediction by computer the toxicity of new drug candidates might be a useful recommendation for the companies, but the direct toxicity testing of drugs in animals will be required for a while.

REFERENCES

1. Di Carlo, F. J., Bickart, P., Auer, C. M.: Structure-metabolism relationship (SMR) for the prediction of health hazards by the Environmental Protection Agency. I. Background for the practice of predictive toxicology. Drug Metab. Rev. **17**, 171 – 184 (1986).
2. Di Carlo, F. J., Bickart, P., Auer, C. M.: Structure-metabolism relationships (SMR) for the prediction of health hazards by the Environmental Protection Agency. II. Application to teratogenecity and other toxic effects caused by aliphatic acids. Drug Metab. Rev. **17**, 187 – 220 (1986).
3. Kesterson, J. W., Granneman, G. R., Machinist, J. M.: The hepatotoxicity of valproic acid and its metabolites in rats. I. Toxicologic, biochemical and histopathologic studies. Hepatology, **4**, 1143 – 1152 (1984).
4. Lewis, J. H., Zimmerman, H. J., Garrett, C. T., Rosenberg, E.: Valproate-induced hepatic steatogenesis in rats. Hepatology, **2**, 870 – 873 (1982).
5. Ong, L. L., Schardein, J. L., Petrere, J. A., Sakowski, R., Jordan, H., Humphrey, R. R., Fitzgerald, J. E., de la Iglesia, F. A.: Teratogenesis of calcium valproate in rats. Fund. Appl. Toxicol. **3**, 121 – 126 (1983).

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The effect of combined pre-treatment on the metabolism of antipyrine in the rat

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Determination of the effect of the known enzyme-inducers phenobarbital-Na and spironolactone on the activity of the MFO was carried out. Male Wistar rats were treated either with 40 mg/kg phenobarbital-Na or 40 mg/kg spironolactone, and also in combination with 100 mg/kg of the MFO was inhibitor α-methyldopa. The activity of the MFO was assessed by changes in antipyrine half-life.

Our results show that given alone both phenobarbital-Na and spironolactone are potent inducers of the MFO. Co-treatment with the enzyme-inhibitor α-methyldopa, however, led to different results in two cases: It slightly diminished the enzyme inducing effect of phenobarbital-Na, while the effect of spironolactone has completely ceased.

These findings may suggest that phenobarbital-Na and spironolactone are acting on different cytochrome isoenzymes.

Keywords: antipyrine half-life, enzyme inhibitors, enzyme inducers, rats

It is well known that certain drugs are able to induce or inhibit the activity of the microsomal drug metabolising cytochrome isoenzymes (MFO).

In the present study we have studied the effect of the known enzyme inducers phenobarbital-Na and spironolactone, as well as the enzyme inhibitor α-methyldopa. We have also studied the effect of combined treatment with either phenobarbital-Na and α-methyldopa or spironolactone and α-methyldopa.

Some results described in this paper were presented in the Symposium on Pharmacokinetics and Metabolism. Mátrafüred, 15 – 16 April, 1994, Hungary

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The drug metabolising activity of the MFO was determined by observing the changes in the half-life of antipyrine.

Animals and methods

Male Wistar rats weighing 140 – 160 g were used. The rats were kept in plastic cages and fed with LATI food pellets. The room temperature was constant and there was a fixed rhythm light regulation as well. The animals were kept fasting for 16 hours before decapitation.

There were six treatment groups with 30 animals in each: I. α-methyldopa 100 mg/kg, II. phenobarbital-Na 40 mg/kg, III. spironolactone 40 mg/kg, IV. phenobarbital-Na 40 mg/kg + α-methyldopa 100 mg/kg, V. spironolactone 40 mg/kg + α-methyldopa 100 mg/kg, VI. control.

The drugs were administered through a gastric tube in a watery suspension for a three-day period. Control animals were given physiological saline of the same amount.

24 hours after the last treatment the animals were given 100 mg/kg antipyrine i.v. Ten animals/treatment group were decapitated 30, 90 or 120 min after the administration of antipyrine. Blood was collected in heparinised tubes. Antipyrine half-life, apparent volume of distribution was determined according to Brodie et al. [6]

The measurements were performed on SPECORD – M – 40 spectrophotometer.

Results

The results are summarized in Table I. As the apparent volume of distribution was the same for all treatment groups, it can be safely assumed, that all changes in antipyrine half-life were due to the effects of pre-treatments.

Table I

Effect of pre-treatments on biological half-life and on volume of distribution of antipyrine in rats

	Antipyrin t1/2 (min)		aVd (ml/kg)		Change
	mean	±SE	mean	±SE	(%)
Control	122.5	4.7	994.0	37.4	
Methyldopa	212.5	6.7	1000.0	28.6	73
Phenobarbital-Na	60.0	7.2	985.0	29.5	−50
Phenobarbital-Na + α-Methyldopa	71.0	4.6	997.0	40.3	−41
Spironolactone	60.0	5.8	990.0	32.4	−50
Spironolactone + α-Methyldopa	119.0	7.0	994.0	30.6	− 2

Discussion

In the present study we have assessed the effect of pre-treatment with known enzyme inducers and enzyme inhibitors given alone or in combination. As it can be seen in Table I, our results confirm that given alone α-methyldopa is an inhibitor of MFO, while phenobarbital-Na and spironolactone are potent inducers of the same enzyme system.

The combined pre-treatment led to different results. α-methyldopa has been able to lessen the enzyme inducing effect of phenobarbital-Na only slightly, while it completely countered the effect of spironolactone. This phenomenon can be explained by supposing that the two enzyme inducer drug – spironolactone and phenobarbital-Na – studied in the present study are acting on different cytochrome izoenzymes.

REFERENCES

1. Conney, A. H.: Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev. **19**, 317–366 (1967).
2. Natsuhori, M., Witkamp, R. F., Van't Klooster, G. A. E., Van Miert, A. S. J. P. A. M.: Metabolism of antipyrine and sulphadimidine in dwarf goats: effects of the enzyme inducing agents phenobarbital, troleandomycin and rifampicin. Xenobiotica, **22**, 1243–1250 (1992).
3. Danhof, M., Krom, D. P., Breimer, D. D.: Studies on the different metabolic pathways of antipyrine in rats: influence of phenobarbital and 3-methylcholantrene treatment. Xenobiotica, **9**, 695–702 (1979).
4. Danhof, M., Verbeek, R. M. A., Van Boxtel, C. J. A., Boeijinga, K. J., Breimer, D. D.: Differential effects of enzyme induction on antipyrine metabolite formation. Br. J. Clin. Pharmacol., **13**, 379–386 (1982).
5. Vesell, E. S.: The antipyrine test in clinical pharmacology: Conceptions and misconceptions. Clin. Pharm. and Ther. **26**, 275–286 (1979).
6. Brodie, B. B., Axelrod, J., Soberman, R., Levy, B. B.: The estimation of antipyrine in biological materials. J. Biol. Chem. **179**, 25–29 (1949).

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Effect of ouabain in catecholamine-induced cardiac hypertrophy

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Changes in the sensitivity to ouabain of the hypertrophied myocardium were studied in isolated left ventricular trabeculae of rat heart using conventional microelectrode technique, and in anaesthetized rats using limb-lead electrocardiography (ECG). Hypertrophy was induced by administration of 5 mg/kg isoprenaline once daily for 7 days. The age-matched normal control rats received the same volume of 0.9% NaCl solution. The heart weight to body weight ratio increased from 3.23 ± 0.07 mg/kg to 4.37 ± 0.12 mg/kg, which means a 35% hypertrophy. The action potential duration at 50% repolarization (APD_{50}) was 25.1 ± 2.4 ms in normal case and 63.5 ± 4.6 ms in hypertrophy. The S–T interval of the ECG was 24.9 ± 1.9 ms in normal rats and 64.4 ± 3.4 ms in hypertrophy. The toxic effect of ouabain was studied by determining the concentrations of the drug at which arrhythmogenic transient depolarizations (TDs; in microelectrode experiments) or sustained ventricular tachyarrhythmias (VTAs; in ECG experiments) appeared. The dose of ouabain inducing TDs was 345 ± 25 μM in normal case and 630 ± 40 μM in hypertrophy. In ECG studies, the dose inducing VTAs was 19.1 ± 3.6 mg/kg in normal rats and 34.3 ± 3.1 mg/kg in hypertrophy. The results suggest that catecholamine-induced cardiac hypertrophy damages the membrane-bound Na^+, K^+-ATPase, thus decreasing the number of the ouabain binding sites, which consequently, makes the myocardium membrane more insensitive to ouabain.

Keywords: cardiac hypertrophy, action potential, ECG, transient depolarizations, arrhythmias, Na^+, K^+-ATPase, ouabain

Cardiac hypertrophy is essentially a compensatory mechanism in which the ventricular muscle enlarges to meet the increased circulatory demands of the body under a wide variety of stressful situations [20]. Many authors have raised the possibility that catecholamines play the role of a molecular signal which links the

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increased work load of the heart to myocardial hypertrophy [5, 8, 14, 19, 23]. It has also been demonstrated that chronic administration of catecholamines induces myocardial hypertrophy [6, 9, 10, 16, 21, 22]. In this hypertrophy model, the myocardial membrane was depolarized, the action potential duration was prolonged and the V_{max} was decreased. Arrhythmogenic transient depolarizations could be induced by a train of action potentials, which might be due to the hypertrophy-induced damages in the membrane-bound Na^+, K^+-ATPase [9].

Since, Chevalier et al. [3] have shown a diminished toxicity of ouabain in pressure overload cardiac hypertrophy, the present study was undertaken to determine the changes in the sensitivity of the myocardium to the toxic concentrations of ouabain in catecholamine-induced cardiac hypertrophy.

Materials and methods

Induction of hypertrophy

Cardiac hypertrophy was induced in male Wistar rats weighing 180 – 240 g by intraperitoneal injection of 5 mg/kg isoprenaline once daily for 7 days [16, 21]. Age-matched normal control animals received the same volume of 0.9% NaCl solution only. The animals were used for experiments 24 hours after the last injection. The degree of hypertrophy was estimated by measuring the wet heart weight and the body weight, and calculating the heart weight to body weight ratio (HW/BW).

Electrophysiology

The experiments were carried out on left ventricular trabeculae taken from rats with or without cardiac hypertrophy. The rats were killed by a sharp blow on the head and the heart was quickly removed and transferred into a dissection chamber containing Tyrode solution with the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, NaH_2PO_4 1.0, $MgCl_2$ 1.2, $NaHCO_3$ 24.9 and glucose 11.5. The solution was aerated with a gas mixture of 95% O_2 and 5% CO_2, the pH was 7.4 and the temperature was maintained at 37 °C. The left ventricle was opened and a suitable trabecula was excised and mounted in a 2 ml organ chamber. The preparations were electrically paced by isolated rectangular pulses (1 ms duration, 3 times threshold voltage, 3 Hz frequency), via bipolar platinum electrodes placed on the tissue. Membrane potentials were recorded with conventional microelectrodes filled with 3 M KCl solution (5 – 15 M, resistance), and connected to a high input impedance amplifier (Axoclamp – 2A). The potential signals were monitored on an oscilloscope (Nicolet type 3091) and recorded on a chart-recorder [9].

Electrocardiography

The generally used limb-lead electrocardiography (ECG) was carried out in rats with or without cardiac hypertrophy. The animals were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.). The lead-II ECG was recorded before and 15 min after application of ouabain. The drug was administered in increasing concentrations (5 mg/kg steps) through a cannula inserted into the femoral vein. Stainless-steel needles impaled subcutaneously in the limbs of the rats served as electrodes and were connected to the head-stage of an electrocardiograph (Medicor MR-11-3). The ECG was recorded on a chart recorder.

Drugs and statistics

Drugs used in this study were ouabain (Serva, Feinbiochemica, Heidelberg), dl-isoproterenol (Sigma Chemical Co., St Louis), all the other reagents were purchased from Reanal (Budapest).

All values are given as mean ± S.E.M. The statistical significance of difference from control was estimated using Student's t-test. A P value of less than 0.05 was considered significant.

Results

Transient depolarizations

In accordance with the literature data [16, 21, 22] and our former results [9, 10], chronic administration of dl-isoproterenol (5 mg/kg/day, i.p. for 7 days) induces about 35% increase in the mass of the ventricular muscle. The heart weight to body weight ratio was increased from 3.23 ± 0.07 mg/g (in the age-matched normal rats) to 4.37 ± 0.12 mg/g (in the catecholamine-treated rats).

Figure 1 shows representative action potentials and arrhythmogenic transient depolarizations (TDs) recorded in normal (Fig. 1A) and hypertrophied (Fig. 1B) left ventricular myocardium of the rat. In this example, the cell membrane was depolarized by about 13% and the action potential duration (APD_{50}) markedly prolonged, by about 153%, as compared to those of the normal control (Fig. 1Aa and Fig. 1Ba).

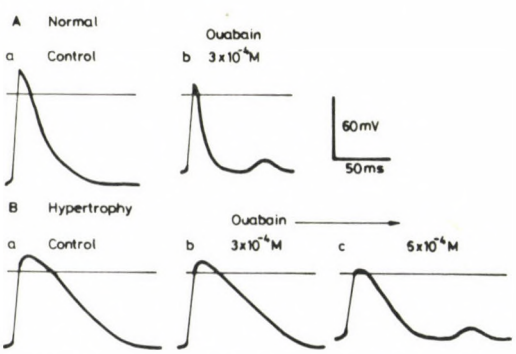

Fig. 1. Induction of arrhythmogenic transient depolarizations (TDs) by different concentrations of ouabain in left ventricular trabeculae isolated from a normal (A) and from a catecholamine-treated rat (B). Note that the control action potential is much longer and induction of TD requires much higher concentration of ouabain in hypertrophy

The concentration of ouabain inducing TDs was 300 μM in a normal (Fig. 1Ab), and 600 μM in a hypertrophied preparation (Fig. 1Bc). The data of 9 (normal) and 12 (hypertrophy) experiments are summarized in Table I. In both normal and hypertrophy, the early effect of ouabain on the APD_{50} was a slight increase which

occurred within the first minute of the application (not shown). Then, the cell membrane started to depolarize and the action potential became smaller and shorter. These changes were progressive and within 10 to 15 min, the action potential was reduced to a spike-like one because the plateau disappeared. The arrhythmogenic TDs appeared during this late, and toxic effect of the drug (Figs 1A and 1B).

Table I

Hypertrophy-induced changes in heart weight to body weight ratio (HW/BW), resting potential (V_m), action potential duration at 50% repolarization, S–T interval of ECG and ouabain sensitivity of the myocardium. Means ± S.E.M. n: number of animals; TD: transient depolarization; VTA: ventricular tachyarrhythmia

Treatment	n	HW/BW	V_m	APD_{50}	S–T	Dose of ouabain inducing	
		(mg/kg)	(mV)	(ms)	(ms)	TD (μM)	VTA (mg/kg)
Normal	9	3.23±0.12	−83.7±3.2	25.1±2.4	24.1±1.9	345±25	19.1±3.6
Hypertrophy	12	4.37±0.16[a]	−72.9±3.4[a]	63.5±4.6[a]	64.4±3.4[a]	630±40[a]	34.3±3.1[a]

[a] $p < 0.05$

Ventricular tachyarrhythmias

The standard body surface ECG (limb-Lead II) was recorded from 9 rats (normal) and 12 rats (hypertrophy). The animals were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.). Typical experiments are shown in Fig. 2 (normal) and Fig. 3 (hypertrophy), and Table I summarizes the changes in some parameters of the ECG. Normal rats demonstrated no arrhythmias during monitoring, while it could be seen in some of the catecholamine-treated animals. Rats showing arrhythmias prior to ouabain treatment were not used for the experiments. The most pronounced changes in the ECG configuration in the rats with cardiac hypertrophy, were as follows: the direction of the T wave became negative and flattened, and the S–T interval was prolonged by about 167% (Fig. 3A) as compared to those of the normal control (Fig. 2A; see also Table I).

The arrhythmogenic (toxic) concentrations of ouabain were determined by adding bolus injections of the drug in 5 mg/kg increments, through a cannula inserted into the femoral vein. The effect of the glycoside appeared within the first min. The monitoring time was 10 min with each concentration. During this period, the changes in the ECG reached a steady-state. As shown in Fig. 2 (normal) and Fig. 3 (hypertrophy), the sensitivity of the hypertrophied myocardium to ouabain was markedly decreased. Animals with hypertrophy required about twice as much

ouabain to develop arrhythmias as the age-matched normal rats. The toxic concentration of the glycoside was 19.1±3.6 mg/kg in normal rats and 34.3±3.1 mg/kg in rats with hypertrophy (Table I).

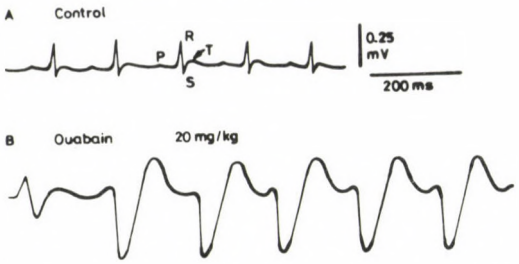

Fig. 2. Induction of ventricular tachyarrhythmias (VTAs) with ouabain in a normal rat. A: Control recordings of the limb-lead-II ECG. P, R, S, T show the corresponding waves of the ECG. B: ECG taken 10 min after a bolus injection of 20 mg/kg ouabain

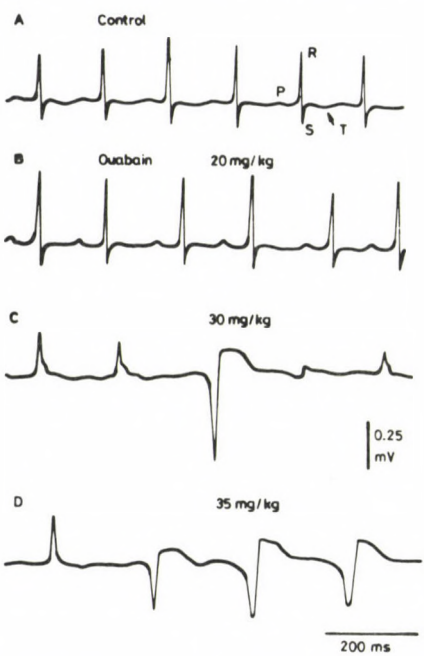

Fig. 3. Induction of ventricular tachyarrhythmias (VTAs) with ouabain in a catecholamine-treated rat. A: Control recordings of the limb-lead-II ECG. P, R, S, T show the corresponding waves of the ECG. B: ECG taken 10 min after a bolus injection of 20 mg/kg ouabain. C: 30 mg/kg ouabain induces only a single premature ventricular beat. D: Sustained VTAs induced by 35 mg/kg ouabain

Discussion

The present study provides further evidence in supporting that repeated administration of low doses of isoproterenol induces marked (about 35%) cardiac hypertrophy in rats, within a relatively short time period (7 days). This phenomenon was first described by Rona et al. [16], and recently attracted particular attention, because catecholamines were thought to play an important role in the development of cardiac hypertrophy [8]. This hypothesis seems to be confirmed by increasing number of evidence. Ostman-Smith [14] has demonstrated that exercise-induced cardiac hypertrophy can be prevented by chemical sympathectomy. Several investigators have raised the possibility that catecholamines may be one of the molecular signals which links the increased circulatory demand to myocardial hypertrophy [5, 19, 23]. All of these reports agree upon that the development of catecholamine-induced cardiac hypertrophy is mediated by stimulation of myocardial alfa-adrenoceptors, and that it is independent of hemodynamic and contractile changes. The catecholamine-induced cardial hypertrophy has been used as a laboratory model in biochemical, morphological and electrophysiological investigations [6, 9, 10, 15, 21, 22].

The present study revealed considerable alterations in the shape of the ECG recorded from rats in which cardiac hypertrophy was induced by chronic administration of isoproterenol. The most prominent changes were the inversion of the T wave and the prolongation of the S–T interval, as compared to the control. T wave inversion could be attributed to a delayed repolarization process in the hypertrophied myocardium. It has been demonstrated that the body surface ECG of many mammals exhibit a concordance in the polarity of the R wave and the T wave. The explanation for this phenomenon is that some regions of the heart (base) with longer action potential must be excited earlier and repolarized later than regions (apex) with shorter action potentials [11]. In hypertrophy, the repolarization is slowed down, as reflected by a prolonged S–T interval and a longer action potential. This causes the action potential to propagate more slowly, thus the repolarization of apex delayes, consequently, the T wave becomes negative and flattened. The marked prolongation of the repolarization seen in hypertrophy can be explained by a decreased outward K^+ current [1], by an increased inward Ca^{2+} current [18], or by a combination of both mechanisms [9, 12].

In our hypertrophy model, the toxicity of ouabain was markedly diminished, as reflected by the concentration of the glycoside required to evoke arrhythmogenic transient depolarizations (TDs) and ventricular tachyarrhythmias (VTAs). The ouabain concentration inducing VTAs was 20 mg/kg in normal case and 35 mg/kg in hypertrophy. Taking into account that a rat has about 70 ml blood/kg body weight,

these doses mean 400 μM and 700 μM, respectively, which are very close to the doses inducing TDs, namely, 300 μM in normal case and 600 μM in hypertrophy.

These findings are in good agreement with the results of Chevalier et al. [3], who demonstrated about a 50% reduction in the ouabain toxicity when the cardiac hypertrophy was induced by aortic stenosis in the rat. As an explanation, the molecular basis for the reduced toxicity of ouabain in hypertrophied myocardium, would be a reduced contribution of the membrane-bound digitalis receptors, the Na^+, K^+-ATPase. In rat heart, two types of ouabain binding sites have been identified on the sarcolemma. One with a high affinity for ouabain appears as the pharmacological receptor related to positive inotropy, whereas the low affinity enzyme form is associated with toxicity [7]. Chevalier et al. [3] showed that the functionally available low affinity sites of the enzyme were twofold less in the hypertrophied than in the normal hearts. The reduced contribution of the low affinity isoform of the Na^+, K^+-ATPase could be due to a membrane damage caused by an intracellular Ca^{2+} overload during the chronic isoproterenol administration [2, 4]. The fact that the action potential is much longer in the hypertrophied myocardium could further reduce the effectiveness of the glycoside, because the action potential must be shortened prior to the appearance of the arrhythmogenic TDs [17].

In summary, it can be concluded that catecholamine-induced cardiac hypertrophy, similarly to the pressure overload hypertrophy, reduces the sensitivity of the myocardium to ouabain. This reduction is explained by a decreased contribution of the low affinity sites of the Na^+, K^+-ATPase. Our results provide further evidence to the view that catecholamines might be the molecular signal linking the increased work load of the heart to myocardial hypertrophy.

REFERENCES

1. Aronson, R. S.: Characteristics of action potentials of hypertrophied myocardium from rats with renal hypertension. Circ. Res. **47**, 443–454 (1980).
2. Bloom, S., Davis, D. L.: Calcium as mediator of isoproterenol-induced myocardial necrosis. Am. J. Pathol. **69**, 459–470 (1972).
3. Chevalier, B., Berrebi-Bertrand, I., Mouas, C., Lelievre, L. G., Swynghedauw, B.: Diminished toxicity of ouabain in the hypertrophied rat heart. Pflugers Arch. **414**, 311–316 (1989).
4. Fleckenstein, A., Janke, J., Doring, H. J., Pachinger, O.: Ca overload as the determinant factor in the production of catecholamine-induced myocardial lesions. In: Recent Advances in Studies on Cardiac Structure and Metabolism, vol. 2, Cardiomyopathies, ed. E. Bajusz, University Park Press, Baltimore 1973, pp. 455–466.
5. Ikeda, U., Tsuruya, Y., Yaginuma, T.: $Alpha_1$-Adrenergic stimulation is coupled to cardiac myocyte hypertrophy. Am. J. Physiol. **260**, H953–H956 (1991).
6. Jones, S. M., Hunt, N. A., Monte, F. D., Harding, S. E.: Contraction of cardiac myocytes from noradrenaline-treated rats in response to isoprenaline, forskolin and dibutyryl cAMP. Eur. J. Pharmacol. **191**, 129–140 (1990).

7. Lelievre, L. G., Charlemagne, D., Mouas, C., Swynghedauw, B.: Respective involvements of high- and low-affinity digitalis receptors in the inotropic response of isolated rat heart to ouabain. Biochem. Pharmacol. **35**, 3449–3455 (1986).
8. Malik, A. B., Geha, A. S.: Role of adrenergic mechanisms in the development of cardiac hypertrophy. Proc. Soc. Exp. Biol. Med. **150**, 796–800 (1975).
9. Mészáros, J.: Sodium-pump injury and arrhythmogenic transient depolarizations in catecholamine-induced cardiac hypertrophy. Eur. J. Pharmacol. **210**, 325–331 (1992).
10. Mészáros, J., Lévai, G.: Catecholamine-induced cardiac hypertrophy uncouples beta-adrenoceptors from slow calcium channels. Eur. J. Pharmacol. **210**, 333–338 (1992).
11. Noble, D.: The initiation of the Heartbeat. Clarendon Press, Oxford 1979.
12. Nordin, C.: Abnormal Ca^{++} handling and the generation of ventricular arrhythmias in congestive heart failure. Heart Failure **5**, 143–151 (1989).
13. Nordin, C., Siri, F., Aronson, R. S.: Electrophysiological characteristics of single myocytes isolated from hypertrophied guinea pig hearts. J. Mol. Cell. Cardiol. **21**, 729–739 (1989).
14. Ostman-Smith, I.: Cardiac sympathetic nerves as the final common pathway in the induction of adaptive cardiac hypertrophy. Clin. Sci. **61**, 265–272 (1981).
15. Reithmann, C., Werdan, K.: Noradrenaline-induced desensitization in cultured heart cells as a model for the defects of the adenylate cyclase system in severe heart failure. Naunyn-Schmiedeberg's Arch. Pharmacol. **339**, 138–144 (1989).
16. Rona, G., Chappel, C. I., Balázs, T., Gaudry, R.: An infarct-like myocardial lesion and other toxic manifestation produced by isoproterenol in the rat. Arch. Pathol. **67**, 443–455 (1959).
17. Ruiz-Ceretti, E., Samson, J. P., Reisin, I., Schanne, O. F.: Ionic and electrical effects of ouabain on isolated rabbit hearts. J. Mol. Cell. Cardiol. **9**, 51–61 (1977).
18. Ryder, K. O., Bryant, S. M., Hart, G.: Membrane current changes in left ventricular myocytes isolated from guinea pigs after abdominal aortic coarctation. Cardiovasc. Res. **27**, 1278–1287 (1993).
19. Simpson, P.: Stimulation of hypertrophy of cultured neonatal rat heart cells through an $alpha_1$-adrenergic receptor and induction of beating through an $alpha_1$- and $beta_1$-adrenergic receptor interaction. Evidence for independent regulation of growth and beating. Circ. Res. **56**, 884–894 (1985).
20. Swynghedauw, B.: Cardiac Hypertrophy and Failure. John Libbey, London 1990.
21. Taylor, P. B., Tang, Q.: Development of isoproterenol-induced cardiac hypertrophy. Can. J. Physiol. Pharmacol. **62**, 384–389 (1984).
22. Tse, J., Powell, J. R., Baste, C. A., Priest, R. E., Kuo, J. F.: Isoproterenol-induced cardiac hypertrophy: Modifications in characteristics of beta-adrenergic receptor, adenylate cyclase, and ventricular contractions. Endocrinol. **105**, 246–255 (1979).
23. Zierhut, W., Zimmer, H. G.: Significance of myocardial alpha- and beta-adrenoceptors in catecholamine-induced cardiac hypertrophy. Circ. Res. **65**, 1417–1425 (1989).

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Conjugate effects of saralasin and indomethacin on kidney function in anesthetized dog

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The intravenous administration of the cyclooxigenase blocking indomethacin results in a very rapid and sharp decrease of renal blood flow (RBF) and in a decrease of water and sodium excretion in anesthetized dogs. The result is very similar to that of the angiotensin II infusion.

The present study was undertaken to examine in anesthetized dogs, how the angiotensin II receptor antagonist salarasin can influence the kidney function and the renal effects of the intravenously administered indomethacin.

0.2 μg/kg/min saralasin infused directly into the left renal artery induced a slight drop in the arterial blood pressure, and a slight but significant decrease in the renal vascular resistance without affecting other renal parameters.

The intrarenal infusion of saralasin, when it preceded the administration of indomethacin, almost completely abolished the renal hemodynamic effects of indomethacin and decreased its effect on the renal water and sodium excretion.

It seems therefore probable that in anesthetized dogs the renin-angiotensin system may play a role in the development of the renal effects of indomethacin.

Keywords: salarasin, indomethacin, RBF, renal function, anesthetized dog

It is already clear, that certain prostaglandins may alter renal blood flow. PGE_2 [54] and PGD_2 [5] given directly into the renal artery of the dog produce dose-related increases in RBF. Arachidonic acid administered in the same way induces a significant increase in the renal blood flow. This increase in RBF could be blocked by prior treatment with an inhibitor of PG synthesis [52].

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The prostaglandin synthesis inhibitors, in particular indomethacin and meclofenamat given to anesthetized dogs, produce a demonstrable decrease in RBF [18, 30, 33, 53].

When awake, chronically instrumented animals were given indomethacin, smaller or no change in RBF was observed [27, 55]. These studies suggest, that during anesthesia, and particularly following surgery [29], the prostaglandins do play a role in dilating the renal vasculature. Rather than suggesting a primary role, prostaglandins might function in modulating manner to attenuate the vasoconstricting effects of both angiotensin and norepinephrine [10, 19]. There is an increased sensitivity to exogenous angiotensin II following the administration of drugs that inhibit PG synthesis [43]. It has been demonstrated by Burger et al. [6] that in dogs barbiturate anesthesia induces an angiotensin dependent constriction of the renal vasculature. In our former experiments we could observe a very rapid and sharp decrease in the RBF following indomethacin administration in anesthetized dogs [29, 30].

Intrarenal infusion of angiotensin II sharply decreases the RBF, urine flow and sodium excretion [28, 46] and its effect is very similar to that of indomethacin in anesthetized dogs.

In hypotension and salt depletion the blockade of angiotensin II receptors substantially reduced the deleterious action of PG-inhibitory agent on renal function [4, 25].

The aim of the present study was to investigate, how the blockade of the angiotensin II receptors could modify the effects of PG synthesis inhibition on the renal function in mildly volume expanded anesthetized dogs.

Methods

The experiments were performed on mongrel dogs of either sex weighing between 12–20 kg. For 24 hours prior to the experiment the animals were kept on water only. Anesthesia was induced with sodium pentobarbital (25 mg/kg i.v.) and maintained with periodic additional doses. Immediately after the anesthesia animals received an infusion of Ringer solution of body temperature equal to 1% of body weight, containing para-aminohippuric acid (PAH) and inulin ensuring a plasma concentration of 2 mg and 30 mg per 100 ml respectively, followed by a sustaining infusion of 0.25 ml/kg/min for the remainder of the experiment.

Cannulation of the femoral arteries and veins on both sides were performed in all animals, for arterial blood collections and infusions.

Mean arterial blood pressure was measured by a Statham strain gauge transducer connected to a polyethylene catheter in the right femoral artery and recorded on a RADELKIS recorder.

From a lower midline incision the bladder was exposed and the ureters were catheterized supravesically using fine polyethylene cannules.

A short segment of the left renal artery was exposed by a flank incision and cleared of adhering tissue to accomodate positioning of an electromagnetic flow probe. Mean renal blood flow (RBF) was

measured by connecting the flow probe to a flow-meter (NYCOTRON 375) and recorded by relaying the signal to a RADELKIS OH 814/1 recorder.

A thin cannula was inserted in the left renal artery and 0.5 ml/min of Ringer solution was infused by means of a pump directly in the blood following to the left kidney during the experiment.

Approximately 40 min were allowed to elapse following surgery for equilibration of the preparation.

About 60 min after starting the intravenous infusion urine was collected separately from both kidneys in 10-min periods.

Blood was taken at the midpoint of each clearance period. After centrifugation the supernatant plasma was collected and the erythrocytes were reinfused in Ringer solution. In the middle of the periods the values of the arterial blood pressure and the renal blood flow were registered.

In the first series of experiments, the two control periods (periods 1 and 2) were followed by the infusion of 0.2 μg/kg/min (2.07 nmol/kg/min) Saralasin (Sar^1 Ile^8 Angiotensin II SIGMA) dissolved in 0.5 ml/min Ringer solution into the left renal artery. Two further 10-min clearance periods were made (periods 3 and 4).

In the other series, 0.2 μg/kg/min Saralasin was infused into the left renal artery, and the renal parameters were investigated in two 10-min clearance periods (periods 1 and 2) afterwards the intrarenal Saralasin infusion being kept constant for further 20 min, 4 mg/kg of indomethacin dissolved in 50 ml of 4-fold diluted 0.1 M phosphate buffer solution was infused i.v. within 10 minutes. From the beginning of the i.v. indomethacin infusion further four (periods 3, 4, 5 and 6) 10-min clearance periods were made.

The results got in these experiments were compared to the results of experiments, where following the first two control periods, only 4 mg/kg indomethacin was administered intravenously.

PAH concentration in the urine and plasma was determined by the method of Smith et al. [48], that of inulin by the method of Little [32]. Urinary and plasma sodium and potassium concentrations were measured by flame photometry. Total osmolarity of urine and plasma was measured by the method of freezing-point depression in a Fishke osmometer. Hematocrit was determined by means of Hawskley microhematocrit centrifuge, plasma protein concentration by biuret method [21].

The clearance of PAH (C_{PAH}) and that of inuline ($C_{inuline}$) were determined by the usual formules. The data for C_{PAH}, $C_{inuline}$, potassium excretion ($U_K \cdot V$) were referred to 100 g kidney tissue weight. The total vascular resistance (R_{kidney}/kg) was calculated for 1000 g kidney weight.

Significance of the data was evaluated by paired and unpaired analysis using Student's t-test. A p value less than 0.05 was considered significant.

Results

In the first series of experiments, following two control clearance periods, 0.2 μg/kg/min Saralasin was infused directly into the left renal artery. As it can be seen in Table I and Figs 1 and 2 Saralasin infused into the left renal artery of anesthetized dogs induced a slight but significant ($p < 0.05$) decrease in the arterial blood pressure, and a significant drop from 1.58 ± 0.09 to 1.42 ± 0.09 in the vascular resistance of the left kidney ($p < 0.01$). There was no other change in the parameters studied either in the left or the right kidney.

Table I

Infusion of 0.2 μg/kg/min Salarasin into the left renal artery ($\bar{x} \pm S.E.M.$, $\mu = 10$)

	Control periods		Infusion of Salarasin	
	1	2	3	4
Blood pressure mm Hg	148 ± 4.36	147 ± 5.55	143 ± 5.93	140 ± 5.68 *
RBF_{dir} (left) ml/min	575 ± 25.3	577 ± 27.3	597 ± 26.8	602 ± 28.1 NS
R_{kidney}/kg	1.58 ± 0.09	1.57 ± 0.11	1.48 ± 0.10	1.42 ± 0.09 **
C_{PAH} ml/min	280 ± 14.9	281 ± 18.4	299 ± 22.5	293 ± 18.1 NS
C_{inulin} ml/min	91.9 ± 3.86	93.5 ± 4.79	97.5 ± 5.62	90.8 ± 4.81
Urine output ml/min	2.67 ± 0.56	3.03 ± 0.80	3.10 ± 0.80	3.07 ± 0.68 NS
$U_{Na}xV$ μmol/min	323 ± 78.8	346 ± 114.5	338 ± 109.1	342 ± 83.9
$U_{K}xV$ μmol/min	101 ± 27.8	119 ± 35.9	114 ± 27.8	105 ± 18.3
U_{osm} mosm/l	463 ± 32.8	440 ± 47.4	450 ± 55.6	443 ± 57.3
FF	0.263 ± 0.017	0.269 ± 0.017	0.272 ± 0.018	0.250 ± 0.015

*$p < 0.05$
**$p < 0.01$
} as compared to the control values

In the second series of experiments the base of comparison was given by the experiments, when following two control periods (periods 1 and 2) 4 mg/kg indomethacin was administered intravenously. The infusion of indomethacin resulted in an increase in the mean arterial blood pressure from 130±5.1 mm Hg to 160±3.1 mmHg ($p < 0.001$). The RBF in the left kidney decreased from 478±14.8 to 136±26.0 ml/min ($p < 0.01$), the left renal vascular resistance increased from 1.64±0.07 to 3.30±0.34 ($p < 0.001$). There was no change in the C_{inulin}, the filtration fraction increased from 0.314±0.01 to 0.541±0.03 ($p < 0.01$). Urine excretion dropped from 3.00±0.53 ml/min to 1.63±0.33 ml/min ($p < 0.01$), sodium excretion from 313±70 μmol/min to 196±47 μmol/min ($p < 0.05$). The urinary osmotic activity increased from 349±26 mosm/l to 534±71 mosm/l ($p < 0.01$). There was no significant change in the hematocrit values and plasma protein concentration. Our data are in good agreement with those given in the literature, namely, that in anesthetized dogs the blockade of PG synthesis increases the blood pressure and renal vascular resistance and decreases urine- and sodium excretion.

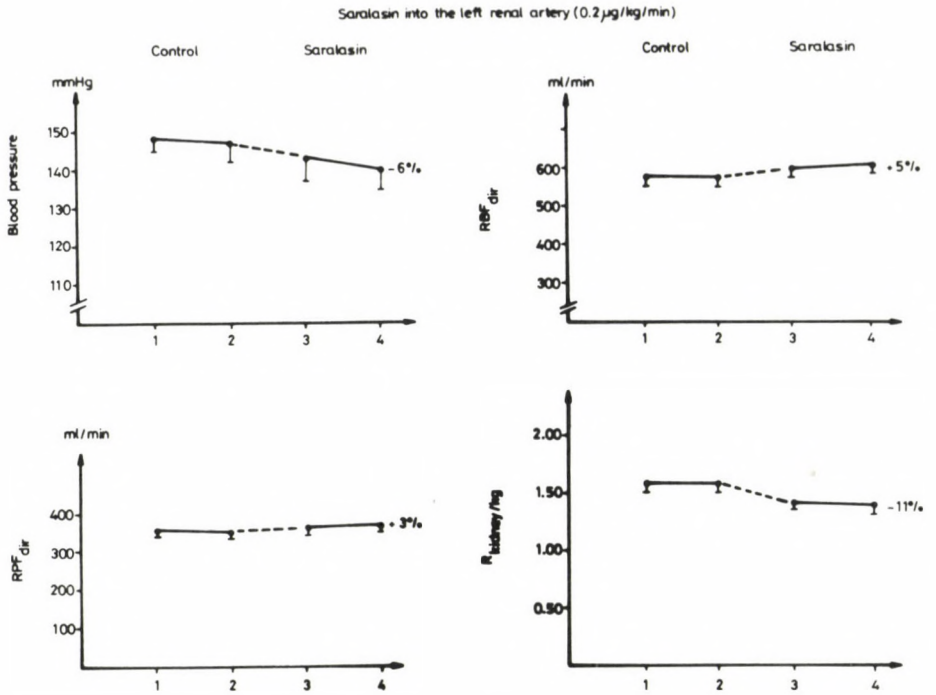

Fig. 1. The effect of 0.2 µg/kg/min Saralasin infused into the left renal artery on the left kidney's parameters. The average values of 10 experiments and S.E.M. are given. The numbers on the abscissa represent the 10-min periods following each other

The results of these experiments were compared with those, where in order to investigate the possible role of angiotensin II in the development of the indomethacin effects in anesthetized animal, indomethacin was administered following Saralasin pretreatment (Table II, Figs 3, 4 and 5).

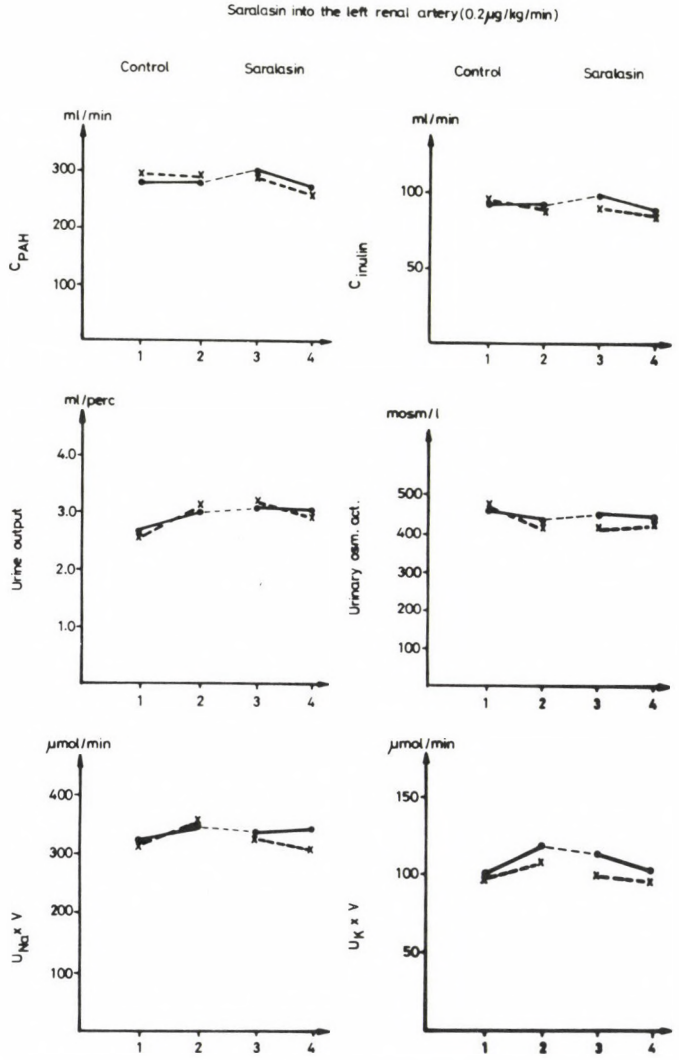

Fig. 2. The effect of 0.2 μg/kg/min Saralasin infused into the left renal artery on the parameters or both kidneys, The solid line represents the left-, the broken line the right kidney's parameters. The numbers on the abscissa give the successive 10-min periods

Table II

Effect of the iv. infusion of indomethacin following the intrarenal infusion of Saralasin (S+I) (n = 8) as compared to the effect of i.v. infusion of indomethacin without any pretreatment (I) (n = 11) left kidney parameters

		Saralasin or Ringer intravenously		Indomethacin iv.	Periods following the iv. indomethacin administration		
		1	2	3	4	5	6
Blood pressure	S+I	143 ± 5.9	140 ± 5.7	146 ± 6.2	152 ± 4.8	156 ± 4.7	158 ± 4.3 *
mm Hg	I	130 ± 5.1	130 ± 5.6	145 ± 5.0	156 ± 4.5	160 ± 2.9	160 ± 3.1 ***
RBF_{dir} ml/min	S+I	597 ± 26.8	602 ± 28.1	587 ± 25.4	587 ± 26.0	576 ± 22.8	569 ± 26.2
	I	478 ± 14.8	478 ± 15.7	331 ± 19.0	318 ± 20.0	305 ± 21.0	316 ± 26.0 **
R_{kidney}/kg	S+I	1.48 ± 0.10	1.42 ± 0.09	1.53 ± 0.09	1.58 ± 0.09	1.64 ± 0.09	1.70 ± 0.11
	I	1.64 ± 0.07	1.65 ± 0.08	2.84 ± 0.31	3.15 ± 0.33	3.38 ± 0.35	3.30 ± 0.34 ***
C_{PAH} ml/min	S+I	299 ± 22.3	273 ± 18.1	261 ± 13.5	236 ± 13.5	239 ± 13.8	228 ± 14.5
	I	271 ± 15.6	260 ± 16.7	212 ± 14.0	176 ± 13.0	196 ± 12.1	189 ± 12.3
C_{inulin} ml/min	S+I	97.5 ± 5.63	90.8 ± 4.82	101.5 ± 5.63	98.2 ± 6.66	105.9 ± 7.20	105.7 ± 9.11
	I	91.8 ± 4.26	92.9 ± 4.62	87.5 ± 4.60	91.4 ± 6.80	106.0 ± 5.71	106.0 ± 7.91
Urine output	S+I	3.09 ± 0.80	3.07 ± 0.68	3.42 ± 0.66	3.08 ± 0.60	2.66 ± 0.52	2.57 ± 0.55
ml/min	I	3.00 ± 0.53	3.11 ± 0.56	2.82 ± 0.49	2.07 ± 0.38	1.77 ± 0.32	1.63 ± 0.33 **
$U_{Na}xV$	S+I	339 ± 109	343 ± 84	426 ± 104	439 ± 91	344 ± 65	291 ± 63
µmol/min	I	313 ± 70	300 ± 61	278 ± 60	258 ± 46	224 ± 67	196 ± 47 *
$U_K xV$	S+I	114 ± 27.8	105 ± 18.3	114 ± 19.8	108 ± 15.3	99 ± 14.9	101 ± 18.7
µmol/min	I	80 ± 10.3	88 ± 8.8	83 ± 9.0	77 ± 8.5	76 ± 7.0	73 ± 5.2
U_{osm} mosm/l	S+I	451 ± 56	443 ± 57	444 ± 58	498 ± 59	540 ± 72	533 ± 78
	I	349 ± 26	307 ± 33	322 ± 28	404 ± 33	486 ± 57	534 ± 71 **
C_{H_2O} ml/min	S+I	−0.66 ± 0.52	−0.67 ± 0.55	−0.90 ± 0.59	−1.31 ± 0.49	−1.30 ± 0.51	−1.09 ± 0.53
	I	−0.24 ± 0.31	+0.34 ± 0.40	+0.01 ± 0.24	−0.54 ± 0.13	−0.82 ± 0.17	−0.87 ± 0.14
Hematocrit %	S+I	39 ± 1.6	39 ± 1.4	39 ± 1.5	39 ± 1.5	38 ± 1.6	39 ± 1.5
	I	39 ± 1.4	39 ± 1.1	39 ± 1.1	38 ± 1.1	38 ± 1.1	38 ± 1.2
Plasma prot. cc.	S+I	4.80 ± 0.21	4.75 ± 0.20	4.70 ± 0.22	4.64 ± 0.21	4.56 ± 0.19	4.53 ± 0.19
g/dl	I	5.13 ± 0.35	5.05 ± 0.33	4.92 ± 0.32	4.84 ± 0.36	4.92 ± 0.39	4.85 ± 0.40
FF	S+I	0.272 ± 0.018	0.250 ± 0.015	0.284 ± 0.013	0.275 ± 0.021	0.300 ± 0.022	0.301 ± 0.018
	I	0.314 ± 0.012	0.318 ± 0.014	0.433 ± 0.023	0.463 ± 0.025	0.560 ± 0.036	0.541 ± 0.032 **

$\bar{x} \pm$ S.E.M.

*p < 0.05
**p < 0.01
***p < 0.001
} as compared to the control values

In this series following the intrarenal Saralasin infusion periods (0.2 µg/kg/min Saralasin into the left renal artery) (periods 1 and 2), the Saralasin infusion being kept constant for further 20 min, 4 mg/kg of indomethacin was administered intravenously. When the i.v. indomethacin infusion followed the Saralasin pretreatment, under the effect of indomethacin, the mean arterial blood pressure increased from 143 mm Hg to 158 mm Hg ($p < 0.05$). The left renal blood flow did not change (597 ± 26.8 ml/min and 569 ± 26.2 ml/min respectively), and there was no significant change in the left renal vascular resistance (from 1.48 ± 0.10 to 1.70 ± 0.11, $p > 0.10$). The C_{inulin} remained unchanged and there was no significant change in the filtration fraction. The drop in the urine excretion from 3.09 ± 0.80 ml/min to 2.57 ± 0.55 ml/min and in sodium excretion from

339 ± 109 μmol/min to 291 ± 63 μmol/min was mathematically not significant ($p > 0.30$ and $p > 0.60$ respectively). There was no significant change in the urinary osmotic activity ($p > 0.50$). The hematocrit value and plasma protein concentration did not show significant changes throughout the experiment.

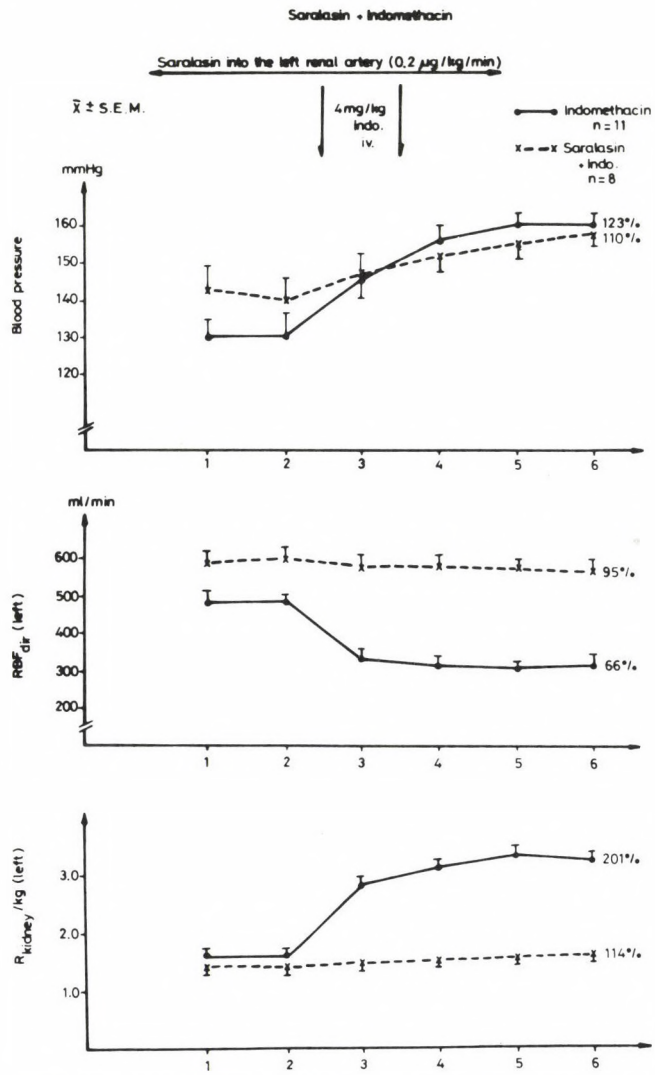

Fig. 3. The conjugated effect of Saralasin and indomethacin on the kidney function. The Saralasin infusion preceeds the indomethacin administration. The numbers on the abscissa give the successive 10-min periods. The percentage given represents the change in the last value, when the control was taken as 100%

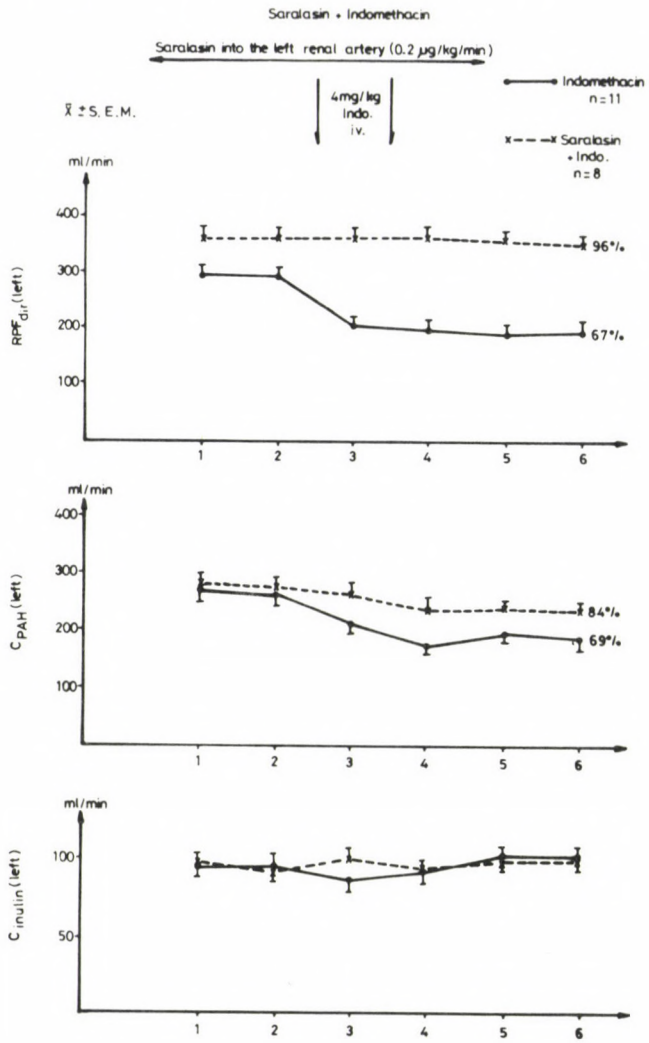

Fig. 4. The conjugated effect of Saralasin and indomethacin on the kidney function. The Saralasin infusion preceeds the indomethacin administration. The numbers on the abscissa give the successive 10-min periods. The percentage given represents the change in the last value, when the control was considered as 100%

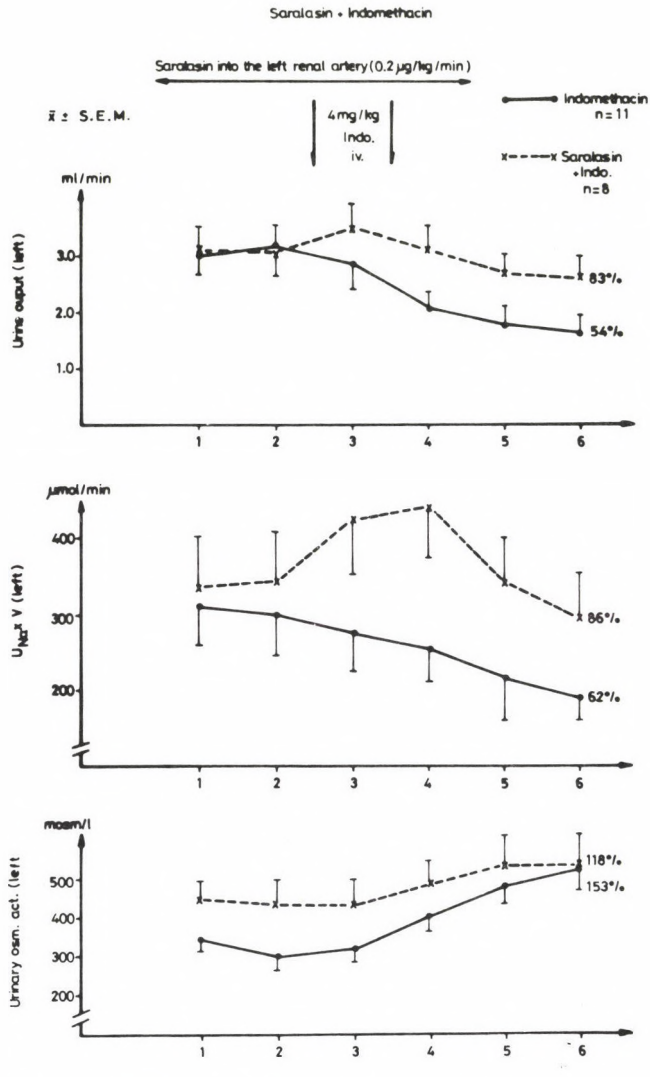

Fig. 5. The conjugated effect of Saralasin and indomethacin on the kidney function. The Saralasin infusion preceeds the indomethacin administration. The numbers on the abscissa represent the successive 10-min periods. The percentage given represents the change in the last value, when the control was considered as 100%

It is therefore evident, that the Salarasin pretreatment abolished or at least strongly attentuated the renal hemodynamic and antidiuretic effects of the indomethacin. The figures and tables give always the parameters of the left kidneys. In the case of the i.v. indomethacin infusion the changes were the same in both

kidneys. The Salarasin infused directly into the left renal artery attenuated also, but at a lesser extent the effects of the indomethacin on the right kidney function.

Discussion

Our experiments prove that indomethacin significantly increases renal vascular resistance and decreases water and sodium excretion in anesthetized dogs. Salarasin, an angiotensin II receptor blocking agent abolishes or at least strongly attenuates the renal effects of the indomethacin, when its infusion precedes the indomethacin administration.

Anti-inflammatory drugs can alter sodium excretion through a direct action, by removal the effects of PG-s on tubular sodium reabsorption [26, 51], by reduction of RBF with secondary effects on sodium excretion, or competition for mineralcorticoid receptors [17].

It is well known that PG synthesis inhibitors decrease RBF, renal water and sodium excretion in anesthetized dogs, but not in rats and hardly if at all in awake dogs [18, 27, 30, 33, 53, 55].

The species difference can be explained by the differences in receptors, vascular reactivity or on the basis of secondary effects [33, 53], but even in rats interaction between the PG-s and renin-angiotensin system could be demonstrated [37, 44, 47].

The difference in the reactivity to PG synthesis inhibitors in anesthetized and awake animals can mostly be explained by the presence and interaction of other hormones or humoral factors.

Now, it is widely accepted, that prostaglandins act mainly in a modulating manner, attenuating the effect of vasoconstrictor substances [10, 19].

In numerous experimental and clinical conditions, when vasoconstrictor systems are activated, prostaglandins play an important role in controlling renal hemodynamics [9, 12, 13]. Pressor hormones, including angiotensin II and norepinephrine stimulate the synthesis of vasodilator substances, among others prostaglandins (PGE_2, PGI_2). The vasodilator substances in turn influence the production and/or effect of the vasoconstrictors. For example, PGI_2 stimulates renin release, TXA_2 influences the effects of PG-s, and on the other hand PG-s facilitate the dephosphorylation of myosin in the vasoconstriction produced by angiotensin II [7].

In the case of anesthesia, surgical stress or volume depletion plasma levels of angiotensin II, norepinephrine and vasopressin are elevated [11, 25]. Burger et al. [6] clearly demonstrated, that in the drop of RBF during pentobarbital anesthesia, the angiotensin II plays a primary role.

It is well known that high doses of angiotensin II reduce RBF and GFR [42, 45]. Lower doses of angiotensin II selectively reduce RBF and increase filtration fraction [14], suggesting preferential vasoconstrictor action on the efferent arteriole [24], however there are several micropuncture studies indicating, that it can influence the tone of both afferent and efferent arterioles [36, 41]. It has also been demonstrated that papillary blood flow is selectively reduced in dogs by intrarenal infusions of angiotensin II [8, 16].

Angiotensin II is known to activate cellular responses through stimulation of inositols phospholipid metabolism. After receptor binding the response is initiated by a transient hydrolysis of phosphatidyl inositol 4-, 5-diphosphate (PIP_2) to inositol 1-, 4-, 5-triphosphate (IP_3) and 1-, 2-diacylglycerol (DG) [1, 22, 23, 49]. In the vascular smooth muscle angiotensin II dependent stimulation is associated with sustained increase in DG formation [23] providing source of arachidonate for the synthesis of bioactive eicosanoid metabolites. The increase in DG is followed by the release of arachidonic-acid presumably via activation of the DG lipase [3, 34]. The arachidonic-acid could either be reesterified into membrane phospholipids or might be rapidly oxidized via several enzyme systems such as cyclooxygenase, increasing the production of prostaglandins.

There is already a body of evidence showing that arachidonate metabolites of the lipooxygenase pathway play role in several angiotensin II dependent mechanisms [2, 15, 20, 38, 40]. Recently Stern and co-workers demonstrated a role of the lipooxygenase pathway in the angiotensin II induced vasoconstriction [50].

In our experiments, the angiotensin II receptor blocking Salarasin, when it was given before the indomethacin administration, almost abolished or at least strongly attenuated the renal effects of indomethacin.

In pentobarbital anesthetized animals under surgical intervention, the circulating level of vasoconstrictor substances is elevated [6, 11, 25] meaning an elevated level of not only the angiotensin II, but also of norepinephrine and vasopressin. All these vasoconstrictor substances may increase the production of vasodilator substances like prostaglandins and kinins attenuating the effect, and modulating the production of the vasoconstrictors. In our former, till unpublished experiments we could also decrease the renal action of indomethacin by pretreating the animal with the α-adrenerg receptor blocking agent phentolamine (Regitin) but the effect was less expressed. This observation is in good agreement with the findings of Burger et al. [6], that in the rather complex neurohormonal regulation of the renal circulation and function during pentobarbital anesthesia and surgical stress the angiotensin II and the related mechanisms might play a primary role.

In this situation the administration of cyclooxygenase inhibitors increase the effects of vasoconstrictors, this way also that of angiotensin II. That can be the reason

why blocking the angiotensin II receptors, one can strongly attenuate the renal effects of indomethacin in anesthetized dogs.

Our supposition is in a good agreement with the fact, that in intact animals, having lower circulating levels of vasoconstrictors indomethacin has no, or a less expressed effect on the renal function.

Acknowledgement

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REFERENCES

1. Alexander, R. W., Brock, T. A., Gimbrone, M. A., Rittenhouse, S. E.: Angiotensin increases inositol triphosphate and calcium in vascular smooth muscle. Hypertension Dallas 7, 447–451 (1985).
2. Antonopialli, I., Nadler, I., Horton, R.: Angiotensin feedback inhibition on renin is expressed via the lipoxygenase pathway. Endocrinology **122**, 1277–1281 (1988).
3. Beridge, M. J.: Inositol triphosphate and diglycerol as second messengers. Biochem. J. **220**, 345–360 (1984).
4. Blasingham, M. C., Nasgletti, A.: Differential renal effects of cyclooxygenase inhibition in sodium replete and sodium deprived dog. Am. J. Physiol. **239**, F360–F365 (1980).
5. Bolger, P. M., Fisher, G. M., Shea, P. T., Romwell, P. W., Slotkoff, L. M.: Effects of PGD_2 on canine renal function. Nature **267**, 628–630 (1977).
6. Burger, B. M., Hopkins, T., Tullock, A., Hollenberg, N. K.: The role of Angiotensin in the canine renal vascular response to barbiturate anesthesia. Circ. Res. **38**, 196–202 (1976).
7. Chatziantoniou, C., Arendshorst, W. J.: Prostaglandin interactions with angiotensin norepinephrine and thromboxane in rat renal vasculature. Am. J. Physiol. **262**, F68–F76 (1992).
8. Chou, S. Y., Faubert, P. F., Epstein, E. M., Blackstock, D. G., Porush, J. G.: Inner medullary hemodynamics in dogs with aortocaval fistula. Am. J. Physiol. **252**, F610–F618 (1986).
9. Di Bona, G. F.: Prostaglandins and non-steroidal anti-inflammatory drugs: effect on renal hemodynamics. Am. J. Med. Suppl. 1.A: 12–21 (1986).
10. Dunham, E. W., Zimmerman, B. G.: Release of prostaglandin-like material from dog kidney during nerve stimulation. Am. J. Physiol. **219**, 1279–1285 (1970).
11. Dunn, M. J.: Nonsteroidal antiflammatory drugs in renal function. Ann. Rev. Med. **35**, 411–428 (1984).
12. Dunn, M. J., Scharschmidt, L. A.: Prostaglandins modulate the glomerular actions of angiotensin II. Kidney Intern. **31**, S–95–S–101 (1987).
13. Epstein, M.: Renal prostaglandins and the control of renal function in liver disease. Am. J. Med. Suppl. 1.A: 46–55 (1986).
14. Fagard, R. H., Cowley, Jr. A. W., Navar, L. G., Langford, H. G., Guyton, A. C.: Renal responses to slight elevations of renal arterial plasma angiotensin II. concentration in dogs. Clin. Exp. Pharmacol. Physiol. **3**, 531–538 (1976).
15. Farber, H. W., Center, D. M., Rowards, S.: Bovine and human endothelial cell production of neutrophyl chemoattractant activity in response to components of the angiotensin system. Circ. Res. **57**, 898–902 (1985).

16. Faubert, P. F., Chou, S. Y., Porish, J. G.: Regulation of papillary plasma flow by angiotensin II. Kidney Int. **32**, 472–478 (1987).
17. Feldman, D., Loose, D. S., Tan, S. Y.: Nonsteroidal anti-inflammatory drugs cause sodium and water retention in the cat. Am. J. Physiol. **234**, F490–F496 (1978).
18. Feigen, L. P., Klainer, E., Chapnik, B. M., Kadowith, P. J.: The effect of indomethacin on renal function in pentothal-anesthetized dogs. J. Pharm. Exp. Ther. **198**, 457–463 (1976).
19. McGiff, J. C., Crowshaw, K., Terragno, H. A., Lonigro, A. J.: Renal prostaglandins: Possible regulators of the renal actions of pressor hormones. Nature **227**, 1255–1257 (1970).
20. Goetzl, E. J., Klichstein, L. B., Watt, K. W., Wintroub, B. V.: The preferential human mononuclear leucocyte chemotactic activity of the substituent tetrapeptides of angiotensin II. Biochem. Biophys. Res. Commun. **97**, 1097–1102 (1980).
21. Gornall, A. G., Bardawill, C. J., David, M. M.: Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**, 751–766 (1949).
22. Griedling, K. K., Berk, B. C., Ganz, F., Gimbrone, M. A., Alexander, R. W.: Angiotensin II stimulation of vascular smooth muscle phosphoinositide metabolism. Hypertension Dallas **9**, Suppl. III. 181–185 (1987).
23. Griedling, K. K., Rittenhouse, S. E., Brock, T. A., Ekstein, L. S., Gimbrone, M. A. Jr., Alexander, R. W.: Sustained diacyclglycerol formation from inositol phospholipids in angiotensin II – stimulated vascular smooth muscle cells. J. Biol. Chem. **261**, 5901–5906 (1986).
24. Hall, J. E.: Regulation of glomerular filtration rate and sodium excretion by angiotensin II. Federation Proc. **45**, 1431–1437 (1986).
25. Henrich, W. L., Berl, T., McDonald, R. M. et al.: Angiotensin II renal nerves and prostaglandins in renal hemodynamics during hemorrhage. Am. J. Physiol. **235**, F46–F51 (1978).
26. Iino, Y., Imai, M.: Effects of prostaglandins on NA transport in isolated collecting tubules. Pflügers Arch. **373**, 125–132 (1978).
27. Kirschenbaum, M. A., Stein, J. H.: The effect of inhibition of prostaglandin synthesis on urinary sodium excretion in the conscious dog. J. Clin. Invest. **57**, 517–521 (1976).
28. Kövér, G., Bálint, T., Tost, H.: Angiotensin and renal function. Intern. Urol. Nephrol. **3**, 271–282 (1971).
29. Kövér, G., Szemerédi, K., Tost, H.: Effect of indomethacin on renal function during different levels of surgical stress. Acta Physiol. Acad. Sci. Hung. **59/2**, 119–129 (1982).
30. Kövér, G., Tost, H.: The effect of indomethacin on kidney function: indomethacin and furosemide antagonism. Pflügers Arch. **372**, 215–220 (1977).
31. Lifschitz, M. D.: Prostaglandins in renal blood flow. In vivo studies. Kidney Int. **19**, 781–785 (1981).
32. Little, L. M.: Modified diphenylamine procedure for determination of inulin. J. Biol. Chem. **180**, 747–754 (1949).
33. Lonigro, A. J., Itskowitz, H. D., Crowshaw, K., McGiff, J. C.: Dependency of renal blood flow on prostaglandin synthesis in the dog. Circ. Res. **32**, 712–717 (1973).
34. Majeras, P. W., Connoly, T., Deckmyn, H., Rass, T. Ishii, H., Bansal, V., Wilson, D.: The metabolism of phosphoinositid-derived messenger molecules. Science Wash D. C. **234**, 1519–1526 (1986).
35. Miller, M. J. S, Quilley, J., McGiff, J. C.: Eicosanoid dependent mechanisms and the regulation of blood pressure. In: Handbook of Hypertension-Regulatory Mechanisms. eds A. Zanchetti and R. C. Tasozi, Elsevier, New York 1986 pp. 578–602.

36. Mitchell, K. D., Navar, L. G.: The renin-angiotensin-aldosteron systeme in volume control. In: Bailliere's Clinical Endocrinology and Metabolism. ed. by P. H. Baylis London: Harcourt Brace Joranovich vol. **3**, 393–430 (1990).
37. Murray, B. M.: Effect of protein intake on regional vascular resistance and reactivity to angiotensin II in the rat. Circ. Res. **67**, 440–447 (1990).
38. Nadler, J., Natarajan, R., Stern, N.: Specific action of the lipoxygenase pathway in mediating angiotensin II induced aldosteron synthesis in isolated adrenal glomerulosa cells. J. Clin. Invest. **80**, 1763–1769 (1987).
39. Nasjletti, A., Malik, K. U.: Interrelationship among prostaglandins and vasoactive substances. Med. Clin. North Am. **65**, 885–889 (1981).
40. Natarajan, R., Stern, N., Hseuh, W. Do, Y., Nadler, J.: Role of lypoxygenase pathway in angiotensin II mediated aldosterone biosynthesis in human adrenal glomerulosa cells. J. Clin. Endocrinol. Metab. **67**, 581–591 (1988).
41. Navar, L. G., Jirakulsomchok, D., Bell, P. D., Thomas, C. E., Huang, W. C.: Influence of converting enzyme inhibition on renal hemodynamics and glomerular hemodynamics in sodium restricted dogs. Hypertension Dallas **4**, 58–68 (1982).
42. Navar, L. G., Rosivall, L., Carmines, P. K., Oparie, S.: Effects of locally formed angiotensin II on renal hemodynamics. Federation Proc. **45**, 1448–1453 (1983).
43. Negus, P., Tannen, R. L., Dunn, M. J.: Indomethacin potentiates the vasoconstrictor actions of angiotensin II. Prostaglandins **12**, 175–180 (1976).
44. Paller, M. S., Hostetter, T. H.: Dietary protein increases plasma renin and reduces pressor reactivity to angiotensin II. Am. J. Physiol. **251**, F34–F39 (1986).
45. Rosivall, L., Navar, L. G.: Effects on renal hemodynamics of intra-arterial infusions of angiotensin I and II. Am. J. Physiol. **245**, F181–F187 (1983).
46. Rosivall, L., Youngblood, P., Navar, L. G.: The effects of infusions of angiotensin I and II on renal hemodynamics in the dog. Kidney International **21**, 247 (abs 5) (1982).
47. Schor, N., Ichikawa, I., Brenner, B. M.: Mechanism of intermittent glomerular perfusion in the rat. Clin. Res. **28**, 560A (1980).
48. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., Graber, M.: The renal clearances of substituted hippuric acid derivates and other aromatic acids in dog and man. J. Clin. Invest. **24**, 388–404 (1945).
49. Smith, J. B.: Angiotensin receptor signaling in cultured vascular smooth muscle cells. Am. J. Physiol. **250**, F759–F769 (1986).
50. Stern, N., Golum, M., Nozawa, K., Berger, M., Knoll, E., Yanagawa, N., Natarajan, R., Nadler, J. L., Tuck, M. L.: Selective inhibition of angiotensin mediated vasoconstriction by lipoxygenase blockade. Am. J. Physiol. **257**, H434–H443 (1989).
51. Stokes, J. B., Kokko, J. P.: Inhibition of sodium transport by prostaglandin E, across the isolated perfused rabbit collecting tubule. J. Clin. Invest. **59**, 1099–1104 (1977).
52. Tannenbaum, J., Splavinsky, J. A., Oates, J. A., Nies, A. S.: Enhanced renal prostaglandin production in the dog I. Effects on renal function. Circ. Res. **36**, 197–203 (1975).
53. Tost, H., Kövér, G.: Effect of indomethacin on the acutely isolated (denervated) kidney function Acta Physiol. Acad. Sci. Hung. **56**, 153–162 (1980).
54. Vander, A. J.: Direct effects of prostaglandin on renal function and renin release in anesthetized dogs. Am. J. Physiol. **214**, 218–221 (1968).
55. Zins, G. R.: Renal prostaglandins. Am. J. Med. **54**, 14–24 (1975).

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Effect of atrazine ingested prior to mating on rat females and their offspring

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The influence of the herbicide atrazine on the Fischer rat and their offspring was investigated. Six 120 mg doses of atrazine in paraffin oil per kg body weight were applied every 48 hours to the females in Group A. The females in the control group, Group C, received paraffin oil only. By the end of the intubation period the rats in Group A weighed less than those in Group C. Atrazine did not affect the estrus cycle, gestation and delivery, litter size and pup survival. Adult female offspring of Group A displayed increased activity in the activity cage and the male offspring avoided more electric shocks during the shuttle box acquisition trials. In addition Group A offspring had shorter latency time during extinction trials than Group C offspring.

Keywords: atrazine, s-triazine, reproduction, behavior, rat

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), a selective s-triazine herbicide, is one of the most widely used pesticides for treating corn fields. Therefore the extensive use of atrazine and its degradation in nature within 18 months [11] leads to its possible presence in raw and industrial food products as well as in agricultural watersheds [20].

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The chemical contamination of the environment presents risks not only to the current population and to unborn fetuses but is also a hazard to future generations through possible mutations affecting the reproductive system [16, 24]. Some chemicals directly affect conceptus while others, like atrazine, alter the reproductive function of neuroendocrine and target organs at different levels: hypothalamus [1, 13], anterior pituitary [1, 12], prostate [13, 23], uterus [25]. Furthermore atrazine is biotransformed in mammals [8] and the influence of its biodegradation products in reproductive organs can be expected. The indirect effect of atrazine on the pituitary-gonadal axis of male and female offspring was studied by treating rat females during pregnancy and/or during pregnancy and lactation. In the offspring of both sexes a slower maturation of the gonadotropic system was evident and as a consequence the modification of male and female 5-α-reductase activity was detected [14].

This study was encouraged by the previously mentioned data concerning the influence of atrazine on the gonadotropic feedback mechanisms as well as by the lack of knowledge of its possible long-term effects. The adult female rats were treated with atrazine by oral intubation before mating. The primary goal of this study was to investigate the influence of atrazine on the estrus cycle, the ability of copulation and conception, the course of pregnancy and delivery. In addition litter size, pup survival, and body weight of the treated animals and their offspring were analyzed. In adulthood the offspring were subjected to spontaneous activity and avoidance conditioning testing. Some consequences of atrazine treatment on the body weight of the treated females and on the behavior of their adult offspring were found.

Materials and methods

Animals. 75-day old Fischer strain female rats from our own breeding stock were used (mean weight 140 ± 3 g). They were raised under standard controlled laboratory conditions (temperature 22 ± 1 °C, lighting schedule 12 h light: 12 h dark, laboratory food and tap water ad libitum).

Procedure. Twenty females were divided into two groups (10 animals each). Group A (atrazine) received six 120 mg/kg b.w. doses of atrazine (2-chloro-4-ethyl-amino-6-isopropylamino-s-triazine, purchased from Herbos, Sisak, Croatia) in 0.5 ml of paraffin oil by oral intubation at 48 hour intervals. Before use the atrazine was purified, by recrystallization and TLC. Group C (control) received only paraffin oil. For investigating the effect of treatments on the rhythm of the estrus cycle vaginal smears were inspected microscopically twice in the first week and twice in the second week of treatment. After three days of rest the smear analysis resumed for 12 consecutive days. The smears were obtained between 9 and 10 a.m. The following estrus cycle stages were defined: proestrus, estrus, and diestrus. Thereafter the animals were left undisturbed for 2 weeks after which mating was started. The females were put in the same cage as males (3–4 females + 1 male). The next morning vaginal smears were inspected. The day when the smear was sperm positive was considered the first day of gestation. The animals with sperm negative smears were repeatedly mated over the course of 5 consecutive nights.

Body weights were checked weekly from the beginning of intubation to parturition. The offspring were weighed at the age of 1, 8, 15, 22, 29, 50, 70 and 90 days. About 24 hours after delivery the litters

were reduced to 4 female/4 male pups wherever possible. At weaning (30 days) 20 offspring from atrazine treated females and 20 from untreated females were randomly selected for behavioral testing. Thereafter the animals were numbered by ear-clipping, caged 5 per cage according to group and sex and left undisturbed except for cage cleaning, weighing and behavioral testing.

Behavioral observations. (I) Spontaneous activity. At age of 70 days spontaneous activity of the offspring was tested in a commercial automatic activity cage (Ugo Basile, Varese, Italy, No. 7400), described in detail in the literature [10]. The activity cage records spontaneous coordinate activity and variations of this activity in time. Briefly it consists of a 30 cm × 20 cm × 24 cm plexiglass box with a grid floor. A very weak electric current passing through the grid provides a count of the ambulatory activity of the animal. The testing of each individual animal lasted one hour. The number of impulses indicates the animal's spontaneous activity [10]. The mean activity of the total time (60 min) and of each of the four 15 min time blocks (1 – 15, 16 – 30, 31 – 45, 46 – 60 min successive measurement intervals) were noted and analyzed.

(II) Shuttle box conditioning. At age of 72 and 73 days the offspring were subjected to active avoidance conditioning in an automatic two-way shuttle box (Ugo Basile, Varese, Italy, No. 7501). This is a plexiglass box divided into two equal compartments separated by a 7 × 8 cm opening. The conditioned (light and buzzer, 90 db) and unconditioned (0.7 mA shock delivered through the elevated grid floor) stimuli were controlled by an automatic programmer described in the literature [10]. On the first day of testing (acquisition trials) each animal was allowed 2 min to adapt and then it was subjected to 30 avoidance conditioning trials. During each trial the conditioned stimulus preceded the shock by 3 sec. If the animal crossed to the opposite compartment within this period, no shock was received. If not the current remained on until the animal escaped to the safe compartment or for a maximum of 3 sec. The intertrial intervals lasted for 24 sec. On the second day the animals underwent the same 30 trials but without the shock (extinction trials). Each testing session consisted of 30 consecutive trials which were divided into three blocks of 10 trials each (runs 1 – 10, 11 – 20 and 21 – 30). The mean total latency as well as the total number of avoidance responses during acquisition and extinction trials were recorded [10]. The number of responses for each of these blocks were checked and submitted to analysis.

Statistics. Data on body weight, litter size and estrus cycle were analyzed using the Student's *t*-test and the behavioral data using the analysis of variance (ANOVA) as 2 × 2 factorials (Group x Sex) with Block included as a repeated measurement variable where appropriate. When significant interactions were found, subsidiary analysis (ANOVA) and post-hoc comparisons of the means of interest were carried out using the Duncan's multiple range test. The acceptable level of significance was established at $p < 0.05$. Results are represented as the means ± SE.

Results

Body weight. According to Table I atrazine reduced the body weight by the end of the intubation period. Females in Group A weighed less than females in Group C ($p < 0.05$) whereas the treatment did not affect the body weight of the offspring at any time.

Table I

Body weight (g) of rat females treated before mating with atrazine (A) in intervals of 48 h during 12 days, and of the control females (C)

Period	Week	Female rat body weight (g)	
		Atrazine treated group (A)	Control group (C)
IP	1	141.1 ± 2.9	146.0 ± 2.8
	2	140.4 ± 3.0*	149.2 ± 1.9
SAP	1	142.8 ± 3.6	151.0 ± 1.8
	2	159.8 ± 3.1	159.8 ± 1.9
PP	1	185.7 ± 6.0	179.7 ± 2.9
	2	201.6 ± 6.5	198.0 ± 3.1
	3	233.1 ± 7.6	231.5 ± 6.2

Periods: IP = intubation, SAP = smear analysis, PP = pregnancy. Mean ± standard error of the mean. N = 10 females for IP and SAP, N = 6 females for PP, respectively. *$p < 0.05$ vs. control.

Estrus cycle. The inspection of vaginal smears indicated that the atrazine treatment did not interfere with either the duration of the estrus cycle in days or with the frequency distribution of its individual phases.

Conception, pregnancy and litter size. Atrazine did not affect conception, course of pregnancy and delivery, or the litter size and the pup survival. Pregnancy occurred in 7 (Group A: 70%), and 6 (Group C: 60%) vaginal smear positive females. The only exception was found in one female from Group A which failed to deliver. An autopsy on the 23rd day revealed only one dead fetus in the uterus.

Behavior. (I) Spontaneous activity. A decline in the activity from the first to the last block of trials of all animals was evident. The significant Block effect, $F(3, 48) = 220.59$, $p < 0.001$, was qualified by the Group × Sex × Block interaction, $F(3, 48) = 9.95$, $p < 0.001$ (Fig. 1). The subsidiary analysis showed that during the first block the female offspring of Group A were more active than those of Group C, Group x Sex interaction, $F(3, 16) = 6.42$, $p < 0.05$. No differences between the two groups of male offspring were found (Fig. 1).

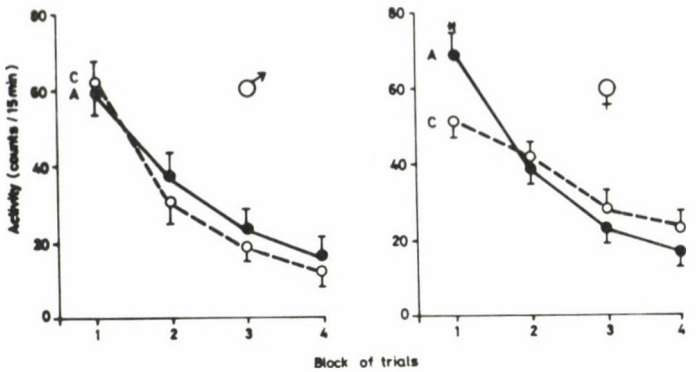

Fig. 1. Spontaneous activity (counts/15 min) in the activity cage of 70 day-old offspring of rat females treated before mating with atrazine (A) and those of the control (C) offspring. Each point represents the mean of 10 animals and the vertical lines the standard error of the mean. *p < 0.05 vs. C

(II) Shuttle box conditioning. The male offspring of Group A were more successful in acquisition trials than the male offspring of Group C indicated both by the shorter latency time and by the increased number of total shock avoidances, Group x Sex interaction, $F(3, 16) = 4.73$, $p < 0.05$, and $F(3, 16) = 11.42$, $p < 0.01$, respectively (Fig. 2).

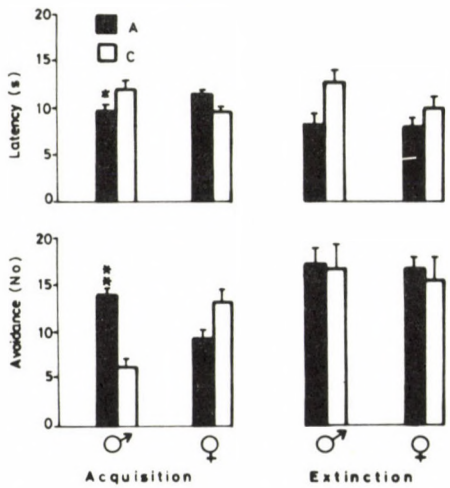

Fig. 2. Latency (s) and avoidance scores (No.) in the shuttle box during acquisition and extinction trials of 72 and 73 day-old offspring of female rats treated before mating with atrazine (A) and those of the control (C) offspring. The bars represent the mean of 10 animals and the vertical lines the standard error of the mean. *p < 0.05, **p < 0.01 vs. C, respectively

The difference in the avoidance scores appeared as Block effect, $F(2, 32) = 59, 57$, $p < 0.001$, Group x Sex interaction, $F(1, 16) = 9.24$, $p < 0.01$, and Group x Sex x Block interaction, $F(2, 32) = 3.90$, $p < 0.05$. The increase in the total number of active avoidance responses during three blocks of trials occurred in all offspring and was significant during the second and third block, Group x Sex interaction, $F(3, 16) = 7.70$, $p < 0.02$, and $F(3, 16) = 7.20$, $p < 0.02$, respectively (Fig. 3).

The mean total latency time of Group A offspring during extinction trials was shorter than that of Group C offspring, Group effect, $F(1, 16) = 6.12$, $p < 0.05$, but the number of active avoidance responses was the same in all comparisons (Fig. 2).

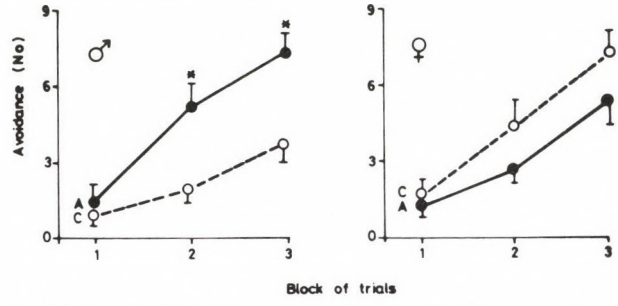

Fig. 3. Avoidance scores (No.) in the shuttle box during three blocks (10 runs each) of acquisition trials of 72 day-old offspring of rat females treated before mating with atrazine (A) and those of the control (C) offspring. Each point represents the mean of 10 animals and the vertical lines the standard error of the mean. *$p < 0.05$ vs. C

Discussion

Atrazine, an s-triazine herbicide, is a compound of low acute toxicity (group IV of toxicity). Oral LD50 values of 1500 and 2000 mg/kg b.w. have been reported in rats. Our previous results indicated that atrazine (120 mg/kg b.w.) affects the reproductive organs at the subcellular levels [2, 12, 13, 14, 15, 23, 25]. Although in the present study the treatment of female rats was terminated 14 days before mating several effects on the behavior of their adult offspring were found.

Since it has been shown that atrazine and its metabolite deethylatrazine affect the hypothalamus-pituitary-gonadal axis in females [15, 25] its interference with the estrus cycle, copulation and conception was also expected. But none of these functions were found to be disturbed. The recovery of the hypothalamus-pituitary-gonadal axis, on the basis of the inhibitory effect of atrazine on cytosol 5 α-dihydrotestosterone receptor complex formation in the prostate, occurs within 14 days in adult rats and within 22 days in younger rats [23]. In the present study the reproductive ability following treatment with atrazine in female rats was evaluated.

The estrus cycle was undisturbed by the treatment. However, according to our unpublished data, continuous 7-day atrazine treatment transiently prolonged the vaginal diestrus stage and reduced the mating ability if both parents were treated with atrazine prior to mating. Therefore the important factors might be dose, duration, frequency and treatment period [2, 14, 17, 27].

The negative effect of atrazine in the present study was indicated by the depressed body weight at the end of the treatment although recovery occurred soon. Infurna et al. [9] found that in rats the dose of ≥ 70 mg of atrazine/kg/day significantly decreased the body weight gain and the feed consumption on days 6 and 7 of gestation. The massive dose of 700 mg of atrazine/kg/day induced a high degree of mortality, increased the incidence of salivation, ptosis, bloody vulvas, swollen abdomen, drastic reduction in the feed consumption and body weight gain of the surviving females. The high incidence of mortality in the previous study suggests that pregnancy as well as multiple dosing may increase the rats' sensitivity to the lethal effects of atrazine in comparison with the LD50 doses between 1200 – 1500 mg/kg in nonpregnant rats [9]. Similarly triadomefon, a widely used triazole fungicide, also reduced body weight and body temperature in a dose-repsonse manner in both female and male rats [18].

In addition, in the present study no changes in the fetal and pup development as well as litter size were found. The discovery that in one female there was only one single dead fetus might be accidental. But the possibility cannot be excluded that in a more numerous sample the miscarriages might occur more frequently.

Although there were not too many effects of atrazine in the present study the fact that it reduced the body weight of the treated animals following the termination of the treatment and that it affected the behavior of the adult offspring of the females which had ingested the drug even before mating points to the dangers of atrazine and other so-called low toxicity agricultural chemicals. Offspring of the treated females were hyperactive to novelty in more or less stressful tasks. The long-term behavioral effect might be attributed to the alterations in motor capacities similar to those observed following the acute treatment of adult rats with triadimefon [4, 18, 19, 29]. In the present study both male and female offspring were used in order to examine possible sex related differences which are of particular interest in behavioral toxicology and teratology studies [18, 19]. The mild stressful testing in the activity cage affected the females only. The more stressful testing in the shuttle box affected only the males during acquisition trials and both sexes during extinction trials. Probably more complex stressful situations might increase the behavioral response and make it possible to detect latent neurotoxicity.

To our knowledge a similar observation has not been previously reported. Therefore we can only compare our behavioral results with those reported in adult animals immediately after treatment [28] or with those referring to other herbicides

of the triazine family [2, 17, 18, 19, 29]. Long-term atrazine treatment shortened the hexobarbital sleeping time but the absence of testing of behavioral alterations in the open field excludes possible neurological effects [28]. However it has been shown that triadimefon and its metabolite triadimenol increase the motor activity of male rats [4]. The stimulant like action of triadimefon on motor activity as a characteristic neurotoxic syndrome in rats appeared to be unique in pesticide research [18]. Recently it was observed that triadimefon increases locomotion and induces stereotyped behavior in rodents by the effects involving the central dopaminergic systems [29]. In experiments in which functional observational battery, motor activity and operant performance were used for neurobehavioral screening, the effects were tested on adult rats immediately after treatment. Recovery was evident the day after administration, but the effect of high doses of triadimefon (≥ 100 mg/kg) were prolonged for several days. In many measurements female rats were more sensitive than males [18, 19]. Prometryne and prometryne/simazine (s-triazines) combination administered to lactating rats affected the body weight, general somatic development and neuromuscular maturation of the pups before weaning [2, 17].

The activity of offspring in the activity cage was recorded in four successive 15-min intervals to analyze the habituation pattern of activity during the 60-min session. It was only affected in female offspring of the treated females. Their increased ambulation recorded by activity scores may be explained by the disruption of the habituation pattern indicating the increased sensitivity to novelty as a consequence of the maternal atrazine treatment.

Maternally mediated atrazine also affected the ability to avoid electric shock in the shuttle box both during the acquisition and the extinction trials. Shorter latency time accompanied by the increased number of active avoidance responses during the first day of testing indicate the superior learning ability of male offspring of Group A. In both sexes of Group A offspring atrazine facilitated extinction by shortening the latency time during the second day of testing. According to Boyd et al. [3] the learning ability of adult male rats was impaired if they were treated with a triazine herbicide 4-amino-6-tert-butyl-3-(methylthio)-as-triazine-5(4H)one (metribuzin) at weaning.

Atrazine exposure may be related to prepregnancy alterations but one can only speculate about the exact mechanisms involved. In any case atrazine, when present in the environment, can reach various mammalian tissues through the food chain [7, 11, 20, 24]. It is uncharged at physiological pH and its relative hydrophobicity suggests that atrazine may be a general membrane poison which can reach and interact with biomembranes as the primary target [26, 30] or within the target cells with specific hormone receptor complexes [1, 8, 12, 13, 14, 15, 23, 25]. In our previous experiments in which the rats were treated with 120 mg/kg b.w. of atrazine daily for 7 days the accumulation pattern for atrazine and its metabolite deethylatrazine was found to be

in the following order: kidney, liver, brain [8]. Deethylatrazine was detected in the same tissues as the parent molecule but in a higher quantity [8]. In another study on fish, atrazine was detected in the liver, muscle, heart, gonads and brain. The highest concentrations were recorded in the ovaries and liver [6]. Atrazine can reach and concentrate in various tissues [6, 8] and can be transferred to the offspring via the placental and/or the mammary route [2, 14, 17, 27]. The long-term effect in the offspring might be the consequence of a direct action of atrazine on the maternal genome and, as a gene activating agent [5], could produce DNA alterations via a specific hormone receptor complex. In any case a wide range of atrazine effects such as genotoxic [21], mutagenic [5], tumorigenic and carcinogenic effects [5, 22] require additional research. In addition, the persistence of atrazine [11] and its strong liposolubility [26, 30] indicate the possibility of the presence of atrazine in highly lipid-containing reproductive organs [6, 8]. An additional relevant fact is the strong synergistic action when s-triazines are used simultaneously in mammals [3, 17, 25].

In conclusion the presented results indicate that atrazine ingested by the female rats even prior to mating might affect the behavior of their offspring in adulthood.

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REFERENCES

1. Babic-Gojmerac, T., Kniewald, Z., Kniewald, J.: Testosterone metabolism in neuroendocrine organs in male rats under atrazine and deethylatrazine influence. J. steroid. Biochem. **33**, 141-146 (1989).
2. Benkwitz, F., Messow, A., Thriene, B.: Untersuchungen zur Toxizität von Prometryn in der Laktationsphase bei Ratten. Z. gesamte Hyg. **34**, 116-118 (1988).
3. Boyd, C. A., Weiler, M. H., Porter, W. P.: Behavioral and neurochemical changes associated with chronic exposure to low-level concentration of pesticide mixtures. J. Toxicol. Environ. Health **30**, 209-221 (1990).
4. Crofton, K. M., Boncek, V. M., MacPhail, R. C., Reiter, L. W.: Evidence for aminergic involvement in triadimefon-induced hyperactivity. Psychopharmacol. (Berlin) **97**, 326-330 (1989).
5. Donna, A., Betta, P. G., Bianchi, V., Ribotta, M., Bellingeri, D., Robutti, F., Marchesini, A.: A new insight into the histogenesis of 'mesodermas'-malignant mesotheliomas. Histopathology **19**, 239-244 (1991).

6. du Preez, H. H., van Vuren, J. H.: Bioconcentration of atrazine in the banded tilapia, Tilapia sparrmanii. Comp. Biochem. Physiol. **101**, 651–655 (1992).
7. Frank, R., Logan, L., Clegg, B. S.: Pesticide and polychlorinated biphenyl residues in waters at the mouth of the Grand, Saugeen, and Thames Rivers, Ontario, Canada, 1986–1990. Arch. Environ. Contam. Toxicol. **21**, 585–595 (1991).
8. Gojmerac, T., Kniewald, J.: Atrazine biodegradation in rats – a model for mammalian metabolism. Bull. Environm. Contam. Toxicol. **43**, 199–207 (1989).
9. Infurna, R., Levy, B., Meng, C., Yau, E., Traina, V., Rolofson, G., Stevens, J., Barnett, J.: Teratological evaluations of atrazine technical, a triazine herbicide, in rats and rabbits. J. Toxicol. Environ. Health **24**, 307–319 (1988).
10. Joffe, J. M., Peruzovic, M., Milkovic, K.: Progeny of male rats treated with methadone: physiological and behavioural effects. Mut. Res. **229**, 201–211 (1990).
11. Klassen, H. E., Kadoum, A. M.: Distribution and retention of atrazine and carbofuran in farm pond ecosystems. Arch. Environ. Contam. Toxicol. **8**, 345–353 (1979).
12. Kniewald, J., Mildner, P., Kniewald, Z.: Effects of s-triazine herbicides on hormone-receptor complex formation, 5α-reductase and 3α-hydroxysteroid dehydrogenase activity at the anterior pituitary level. J. Steroid. Biochem. **11**, 833–838 (1979).
13. Kniewald, J., Mildner, P., Kniewald, Z.: Effects of s-triazine herbicides on 5α-dihydrotesosterone receptor complex formation in hypothalamus and ventral prostate. In: Pharmacological Modulation of Steroid Action, eds Genazzani, E., DiCarlo, F., Mainwaring, W. I. P., Raven Press, New York 1980, pp. 159–169.
14. Kniewald, J., Peruzovic, M., Gojmerac, T., Milkovic, K., Kniewald, Z.: Indirect influence of s-triazines on rat gonadotropic mechanism at early postnatal period. J. steroid. Biochem. **27**, 1095–1100 (1987).
15. Kniewald, J., Tezak, Z., Šimic, B., Kniewald, Z.: Pesticide influence on pubertal female reproductive system. In: Proceedings of the Interdisciplinary Conference on "Effects of Food on the Immune and Hormonal Systems", Zürich, Switzerland, May 1991, pp. 170–174.
16. Longo, L. D.: Environmental pollution and pregnancy: Risks and uncertainties for the fetus and infant. Am. J. Obst. Gynec. **137**, 162–173 (1980).
17. Messow, A., Benkwitz, F., Hellwig, A.: Untersuchungen zur Toxicität einer Prometryn/Symazin-Kombination in der Laktationsphase bei Ratten. Z. gesamte Hyg. **36**, 170–173 (1990).
18. Moser, V. C., MacPhail, R. C.: Neurobehavioral effects of triadimefon, a triazole fungicide, in male and female rats. Neurotoxicol. Teratol. **11**, 285–293 (1989).
19. Moser, V. C., MacPhail, R. C.: Comparative sensitivity of neurobehavioral tests for chemical screening. Neurotoxicol. **11**, 335–344 (1990).
20. Muir, D. C. C., Yoo, J. Y., Baker, B. E.: Residue of atrazine and N-deethylated atrazine in water from five agricultural watersheads in Quebec. Arch. Environ. Contam. Toxicol. **7**, 221–235 (1978).
21. Pino, A., Maura, A., Grillo, P.: DNA damage in stomach, kidney, liver and lung of rats treated with atrazine. Mut. Res. **209**, 145–147 (1988).
22. Pintér, A., Török, G., Börzsönyi, M., Surján, A., Csík, M., Kelecsényi, Z., Kocsis, Z.: Long-term carcinogenicity bioassay of the herbicide atrazine in F 344 rats. Neoplasma **37**, 533–544 (1990).
23. Šimic, B., Kniewald, Z., Davies, J. E., Kniewald, J.: Reversibility of the inhibitory effect of atrazine and lindane on cytosol 5α-dihydrotestosterone receptor complex formation in rat prostate. Bull. Environ. Contam. Toxicol. **46**, 92–100 (1991).
24. Sullivan, F. M., Barbon, S. M.: Congenital malformations and other reproductive hazards from environmental chemicals. Proc. R. Soc. (Lond.) **205**, 91–100 (1979).

25. Tezak, Z., Šimic, B., Kniewald, J.: Effect of pesticides on oestradiol – receptor complex formation in rat uterus cytosol. Fd. Chem. Toxic. **30**, 879–885 (1992).
26. Tanfani, F., Ambrosini, A., Albertini, G., Bertoli, E., Curatola, G., Zolese, G.: Interaction of the herbicide atrazine with model membranes. I: Physico-chemical studies on dipalmitoyl phosphatidylcholine liposomes. Chem. Phys. Lipids **55**, 179–189 (1990).
27. Ugazio, G., Bosio, A., Nebbia, C., Soffietti, M. G.: Age- and sex-related effects on hepatic drug metabolism in rats chronically exposed to dietary atrazine. Res. Commun. Chem. Pathol. Pharmacol. **73**, 231–243 (1991).
28. Ugazio, G., Bosio, A., Burdino, E., Ghigo, L., Nebbia, C.: Lethality, hexobarbital narcosis and behavior in rats exposed to atrazine, bentazon or molinate. Res. Commun. Chem. Pathol. Pharmacol. **74**, 349–361 (1991).
29. Walker, Q. D., Lewis, M. H., Crofton, K. M., Mailman, R. B.: Triadimefon, a triazole fungicide, induces stereotyped behavior and alters monoamine metabolism in rats. Toxicol. Appl. Pharmacol. **102**, 474–485 (1990).
30. Zolese, G., Ambrosini, A., Bertoli, E., Curatola, G., Tanfani, F.: Interaction of the herbicide atrazine with model membranes. II. Effect of atrazine on fusion of phospholipid vesicles. Chem. Phys. Lipids **56**, 101–108 (1990).

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The effect of ß-aminopropionitrile on bleomycin-induced lung injury in rats

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The effect of ß-aminopropionitrile on collagen cross-links, lysyl oxidase and prolyl hydroxylase and particular collagen type content in rat lungs after bleomycin treatment was investigated.

It was stated, that ß-aminopropionitrile significantly diminishes elevated dihydroxylysinonorleucine to hydroxylysinonorleucine ratio, prevented increase of lysyl oxidase activity and increase in type I collagen content in the lungs.

It is suggested, that ß-aminopropionitrile may be useful in the treatment of lung fibrosis.

Keywords: ß-aminopropionitrile, lung fibrosis, collagen cross-linking

Fibrotic lung diseases, regardless of the cause, are characterized by a excessive accumulation of collagen in the lung [13, 56, 60]. Collagen molecules are long, stiff rods consisting of a triple helix of three polypeptide chains, called α-chains. This fibrous protein is cross-linked by an unique mechanism, based on aldehyde formation from lysine and hydroxylysine side chains. There are intramolecular cross-links in which two α-chains within the same molecule may be covalently linked and intermolecular cross-links that involve the formation of covalent bridges between chains in different molecules.

Intermolecular, interchain cross-links are formed, when two residues of allysine in terminal non-helical peptides of two α-chains combine in an aldol-type condensation product [6]. Intramolecular covalent bands have been identified only between amino-terminal telopeptides so far [9].

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The aldehyde-derived cross-links of collagen can conveniently be segregated into two classes: one based on lysine aldehyde (allysine pathway), the other one on hydroxylysine aldehydes (hydroxyallysine pathway).

Numerous studies have demonstrated, that the metabolic functions of the lung fibroblasts responsible for collagen synthesis and deposition are affected by various factors including inflammation [35], proteases [29], lipid [46], nutritional [36] and immunologic [21] status.

Studies on the development of an animal model of pulmonary fibrosis using bleomycin have employed chronic subcutaneous, intraperitoneal, or intravenous dosing over several weeks in a variety of species, including mice [49], dogs [47], baboons [31] and pheasants [4].

Recently, the prevalent model of bleomycin-induced lung injury exposed animals via intratracheal instillation [11, 17, 52].

Lysyl oxidase is the enzyme required for cross-link formation in collagen. This enzyme catalyzes the oxidative deamination of ε-amino groups of lysyl and hydroxylysyl residues to the corresponding aldehydes, called allysine and hydroxyallysine, respectively [42]. This is a copper-containing metalloenzyme, and may require pyridoxal phosphate as cofactor [18]. This enzyme catalyzes a critical post-translational modification of collagen molecule [48].

Prolyl hydroxylase catalyzes the hydroxylation of specific prolyl residues in collagen to hydroxyproline, a process that appears to be essential for the active secretion of the collagen molecule [7]. This processing step is very important in the regulation of collagen accumulation [28].

Previously, we have described bleomycin-induced changes in antioxidant enzyme activities in rat lung [23] (the phospholipid composition of lung surfactant [24], and hydroxyproline content and collagenase and elastase activities [25]).

ß-aminopropionitrile is the active principle of the sweet pea (*Lathyrus odoratus*), which produces the syndrome of osteolathyrism, inhibiting intra- and intermolecular cross-linking of collagen [30] and elastin [38].

For this reason, we became interested in the effects of ß-aminopropionitrile (BAPN) on dihydroxylysinonorleucine to hydroxylysinonorleucine (DHLNL:HLNL) ratio, and on lysyl oxidase and prolyl hydroxylase activities in bleomycin-injured rat lungs.

Materials and methods

Male Sprague Dawley rats weighing 200 – 250 g were used throughout this study, housed in a laminar flow cabinet, three in a cage. They received pelleted rat chow and water *ad libitum*. After an adaptive period of 4 weeks the animals were divided as follows: One group (A, n = 10) was instilled intratracheally with 0.2 ml of 0.9% saline, whereas a second group (B, n = 40) received one single dose

of bleomycin sulfate (Bleocine, Nippon Kayaku Co., Ltd., Tokyo, Japan, 10 mg/kg). Intratracheal instillation of bleomycin was accomplished *via* a stainless steel cannula inserted into the trachea under intraperitoneal chloral hydrate anaesthesia. To achieve adequate anaesthesia, x ml of 6% solution was used, where x = body weight:150 [55]. One half of group B was coincidently supplemented with BAPN (100 mg/kg every 24 hours). After 7 days from the start of bleomycin and BAPN supplementation 20 animals (10 bleomycin − and 10 bleomycin + BAPN treated) were killed, and after 14 days another 20 animals were sacrificed.

The left lungs were minced and washed with 5 mM phosphate buffer containing 0.9% NaCl, pH = 7.4 to remove blood and soluble proteins. After centrifugation, pieces of about 40 mg wet weight of lung tissue were incubated in 3 ml of 0.1 M sodium phosphate buffer, pH = 7.4 for 4 h at room temperature. Then, $NaB(^3H)_4$ (142 Ci/mole, Amersham, Aylesbury, Bucks., England) was added at a ratio of 1:30 (based on dry weight) to the sample. After 1 h reduction was stopped by the addition of 1 ml glacial acetic acid. The tissues were then rinsed with water, hydrolyzed in 6 M HCl for 18 h at 110 °C in sealed ampoules, evaporated and filtered. Hydroxyproline content in hydrolyzates were determined by colorimetric procedure of Woessner [59].

The reduced difunctional cross-links DHLNL and HLNL were analyzed by high performance liquid chromatography of lung hydrolyzate aliquots, containing about 50 μg of hydroxyproline on a C_{18} reversed phase column (Ultrasphere 0.4 × 5 cm, Altex, Berkeley, USA). The elution buffer consisted of 22.5% propanol in 100 mM phosphate, pH = 2.8, containing 0.3% sodium dodecyl sulfate. Flow rate was 0.8 ml/min. Amino acids and cross-links in the effluent were visualized by their fluorescence (excitation 360 nm emission 455 nm, Gilson Spectra Glo spectrofluorimeter, Gilson, Middleton, USA) after postcolumn derivatization with o-phthalaldehyde. Fractions (1.3 ml) were collected from the effluent every minute for the determination of radioactivity by liquid scintillation counting.

The right lungs were homogenized in a Potter-Elvehjem homogenizer in 0.015 M potassium phosphate + 0.154 M NaCl, pH = 7.7 using 3 ml of buffer per 1 g of tissue. The homogenate was centrifuged at 15 000 g for 20 min. The supernatant was discarded and the pellet was subsequently resuspended in buffer, homogenized and centrifuged again. The resulting pellet was finally extracted twice with 4 M urea buffered with 15 mM potassium phosphate, pH = 7.7. The urea-soluble supernatants were dialyzed against 0.1 M sodium borate, (pH = 8.0) overnight to remove the urea. Lysyl oxidase activity was quantified by the standard tritium release assay [42]. Prolyl hydroxylase was quantified by the method of Hutton et al. [14]. Quantitation of type I and type III collagen in lungs was made by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method of Bellon and Borel [5].

Results

The results of difunctional collagen cross-links analysis in lungs of bleomycin-treated rats and those with BAPN supplementation, are presented in Table I. The most striking difference between control and bleomycin-injured lungs was in their total amount of cross-links, which was elevated severalfold and in elevated DHLNL:HLNL ratio. BAPN administration caused a significant decrease in DHLNL:HLNL ratio and in total amount of cross-links both in 7 and 14 days after bleomycin administration.

Table I

Collagen cross-links in bleomycin-injured rat lungs after β-aminopropionitrile treatment (cpm/μg hydroxyproline)

	DHLNL	HLNL	DHLNL:HLNL
Control	113 ± 15	50 ± 6	2.3 ± 0.4
Bleomycin 1 week	451 ± 27	101 ± 15	4.5 ± 0.6
Bleomycin 2 weeks	893 ± 43	183 ± 19	4.9 ± 0.7
Bleomycin + BAPN 1 week	382 ± 22	76 ± 9	5.0 ± 0.4
Bleomycin + BAPN 2 weeks	644 ± 35	151 ± 16	4.2 ± 0.4

Mean values ± S.D. BAPN – ß-aminopropionitrile, DHLNL – dihydroxylysinonorleucine. HLNL hydroxylysinonorleucine

Table II shows the effect of BAPN on lysyl oxidase and prolyl-hydroxylase activity in bleomycin-injured rat lungs. Seven days after bleomycin instillation, a 6.5-fold increase in lysyl oxidase and 3.6-fold increase in prolyl hydroxylase was observed. Fourteen days after bleomycin instillation, the activities of these enzymes were 12-fold and 7-fold greater than in control lungs, respectively. Administration of BAPN prevented much of the increase in lysyl oxidase activity, without effect on prolyl hydroxylase.

Table II

Effect of β-aminopropionitrile on lysyl oxidase and prolyl hydroxylase activities in bleomycin-injured rat lungs (cpm/mg protein × 10^{-3})

	Prolyl hydroxylase	Lysyl oxidase
Control	8.4 ± 0.6	11.1 ± 0.9
Bleomycin 1 week	32.5 ± 1.0	72.5 ± 2.4
Bleomycin 2 weeks	61.2 ± 2.7	131.4 ± 4.6
Bleomycin + BAPN 1 week	30.4 ± 1.1	34.2 ± 1.2
Bleomycin + BAPN 2 weeks	57.5 ± 3.4	66.5 ± 2.5

Mean values ± S.D. BAPN – ß-aminopropionitride

Table III shows collagen types in bleomycin- and bleomycin + BAPN-treated rats. Bleomycin administration causes an increase in type I collagen content, and a decrease in type III collagen. BAPN administration diminishes these changes. Type I/type III collagen ratio increases in bleomycin-injured rat lungs from 1 (control) to 3 (2 weeks after bleomycin administration), in BAPN-treated rats this increase reaches only 1.5.

Table III

Collagen types in bleomycin-injured rat lungs after β-aminopropionitrile treatment

	Type I	Type III	Type IV	Type V	Type I/Type III ratio
Control	41.2 ± 2.0	38.6 ± 1.8	14.1 ± 0.6	6.1 ± 0.4	1.07 ± 0.07
Bleomycin 1 week	56.4 ± 2.8	22.5 ± 1.6	13.6 ± 0.7	7.5 ± 0.4	2.51 ± 0.16
Bleomycin 2 weeks	61.3 ± 3.0	20.1 ± 1.5	13.7 ± 0.8	4.9 ± 0.4	3.05 ± 0.22
Bleomycin + BAPN 1 week	44.2 ± 2.1	31.3 ± 1.9	14.4 ± 0.7	10.1 ± 0.7	1.41 ± 0.10
Bleomycin + BAPN 2 weeks	48.3 ± 2.2	33.4 ± 1.7	13.5 ± 0.8	4.8 ± 0.5	1.45 ± 0.12

Mean values ± S.D. Total collagen in each gel is assumed to be 100% and the different collagen types are calculated as relative percentages. BAPN – ß-aminopropionitrile

Discussion

In the early stages of tissue repair there is an increase in the synthesis of type III collagen [3, 20]. As this repair progresses, there is a return to a marked predominance of type I collagen [58].

Changes in the amount of collagen in acquired diseases and repair processes are, however, more apparent than changes in the types of this protein [53, 54]. In most repair processes the increase in the amount of collagen synthesis is reflected by an increase in the activity of prolyl and lysyl hydroxylases, an increase in the rate of accumulation of soluble collagens and an increase in reducible cross-links in the tissues [19, 44, 45, 50].

Our knowledge regarding the mechanisms that regulate collagen metabolism remains incomplete. However, our understanding of the biochemistry of collagen biosynthesis, deposition and degradation is sufficiently advanced to suggest that it may be possible to modulate selectively those biochemical processes caused by drugs to prevent the development of pulmonary fibrosis.

Collagen α-chains are synthesized as precursors which contain large extension peptides at each end of the molecule [10]. These pro-chains are extensively modified prior to their association and folding into the triple helical conformation characteristic of the collagen molecule. During these posttranslational reactions, about 100 residues of peptidyl-proline and 5–20 residues of peptidyl-lysine, are enzymatically converted to 4-hydroxyproline and hydroxylysine, respectively. These hydroxylation reactions are catalyzed by prolyl- and lysyl hydroxylases, which require oxygen, iron, α-ketoglutarate and ascorbate as cofactors [7]. These post-translational modifications are crucial to the stability of the collagen molecule and to the strength of the collagen fibers. A collagen molecule that cannot form a stable triple helix is functionally useless since it cannot be incorporated into collagen fibrils.

Inhibition of collagen cross-linking, should have a selective effect on collagen maturation since they prevent the formation of the covalent cross-links which stabilize the fibrillar structure of collagen and which are responsible for the high tensile strength of mature fibers. With the exception of elastin, such cross-links are not found in any other protein.

BAPN, is one of the earliest agents known to have an effect on collagen deposition. However, in Syrian hamsters, BAPN was found to cause emphysematous lung alterations in a $CdCl_2$ model of lung fibrosis [37]. BAPN occurs naturally in *Lathyrus odoratus*, the common sweet pea. In growing animals, addition of the sweet pea to the diet results in a form of lathyrism, usually called osteolathyrism. In 1955 the toxic principle was isolated and identified as ß-aminopropionitrile [2, 57].

The acute LD_{50} of BAPN in the mouse is approximately 6.5 g/kg orally and 1.25 g/kg intravenously. Adult rats were administered BAPN (0.4–400 mg/kg/day) for 5–7 weeks [15]. Animals receiving 160 mg/kg/day had no significant changes in urinary hydroxyproline excretion.

BAPN was first administered to humans for the treatment of scleroderma [16]. When patients were treated with 1–3 g/day for 22–67 days, clear effects on collagen metabolism were observed.

Most clinical experience with BAPN has been reached in the treatment of exuberant surface scars either alone or in a combination with colchicine [39, 40]. There is a considerable interest in the application of ophthalmic preparations of BAPN to control wound healing in the eye [32, 33, 34].

Large increases in both prolyl hydroxylase and lysyl oxidase activities were demonstrated in the rat lung after bleomycin administration. Similar changes were observed for increase in prolyl hydroxylase activity in paraquat poisoning, thus suggesting a common mechanism of fibrosis [50].

The dose of BAPN used in this study effectively inhibited lung lysyl oxidase. Similar results were obtained by Arem et al. [1] for the skin enzyme. It was also been shown that BAPN reduces collagen deposition in several other models of lung fibrosis [8, 27, 41].

Cross-linking is affected by BAPN only in collagen undergoing synthesis: there is little or no effect on molecules fully formed [26]. BAPN administration reduced the number of aldehydes at lysyl and hydroxylysyl residues available for cross-linking [12]. Kuhn and Starcher [22] have shown impaired cross-linking of elastin by BAPN after elastase-induced lung injury in hamsters. It is likely that total protein was reduced by BAPN in some fashion in addition to an effect on cross-linking of fibrous proteins [43]. It has been shown that lysyl oxidase has broad substrate specificity to lysyl residues, because the enzyme deaminates simply nonpeptidyl amines in addition to its effect on lysyl residues in collagen and elastin [51].

The results of this work strongly suggest, that BAPN may be the useful agent in the treatment of bleomycin-induced lung fibrosis, diminishing the amount of cross-links in collagen molecules, and lovering the lysyl oxidase activity in the lungs.

REFERENCES

1. Arem, A., J., Misiorowski, R., Chvapil, M.: Effects of low dose BAPN on wound healing. J. Surg. Res. **27**, 228 – 232 (1979).
2. Bachhuber, T. E., Lalich, J. J., Schilling, E. D., Strong, F. M.: Lathyrus factor activity of beta-aminopropionitrile. Fed. Proc. **14**, 175 (1955).
3. Bailey, A. J., Sims, T. J., Le Lous, M., Bazin, S.: Collagen polymorphism in experimental granulation tissue. Biochem. Biophys. Res. Commun. **66**, 1160 – 1165 (1975).
4. Bedrossian, C. M. W., Greenburg, S. D., Yawn, D. H., O'Neal, R. M.: Experimentally induced bleomycin sulfate pulmonary toxicity. Arch. Pathol. Lab. Med. **101**, 248 – 254 (1977).
5. Bellon, G., Borel, J. P.: Quantitative evaluation of collagen fractions separated by acrylamide gel electrophoresis: its application for collagen typing in normal and pathological tissues. J. Chromatogr. **433**, 81 – 94 (1988).
6. Bornstein, P., Piez, K. A.: The nature of the intramolecular cross-link. Biochemistry **5**, 3460 – 3473 (1966).
7. Cardinale, G. J., Udenfriend, S.: Prolyl hydroxylases. Adv. Enzymol. **41**, 245 – 300 (1974).
8. Chichester, C. O., Palmer, K. C., Hayes, J. A., Kagan, H. M.: Lung lysyl oxidase and prolyl hydroxylase: increases induced by cadmium chloride inhalation and the effect of ß-aminopropionitrile in rats. Am. Rev. Resp. Dis. **124**, 703 – 713 (1981).
9. Eyre, D. R., Paz, M. A., Gallop, P. M.: Cross-linking in collagen and elastin. Ann. Rev. Biochem. **53**, 717 – 748 (1984).
10. Fessler, J. H., Fessler, L. I.: The biosynthesis of procollagen. Annu. Rev. Biochem. **47**, 129 – 162 (1978).
11. Giri, S. N., Hyde, D. M.: Increases in severity of lung damage and mortality by treatment with cyclo- and lipoxygenase inhibitors in bleomycin and hyperoxia model of lung injury in hamsters. Pathology **19**, 150 – 158 (1987).
12. Goldstein, G. R., Haddad, R., Hamosh, P.: Effect of ß-aminopropionitrile (BAPN) on the elastic behaviour of the rat lung. Clin. Res. **18**, 89A (1970).
13. Heppelston, A. G.: Pulmonary toxicology of silica, coal and asbestos. Environ. Health Perspect. **55**, 111 – 127 (1984).
14. Hutton, J. J., Tappel, A. L., Udenfriend, S.: A rapid assay for collagen proline hydroxylase. Anal. Biochem. **16**, 384 – 394 (1966).
15. Keiser, H. R., Harris, E. D., Sjoersdma, A.: Studies on beta-aminopropionitrile in animals. Clin. Pharmacol. Ther. **8**, 587 – 592 (1967).
16. Keiser, H. R., Sjoersdma, A.: Studies on beta-aminopropionitrile in patients with scleroderma. Clin. Pharmacol. Ther. **8**, 593 – 602 (1967).
17. Kelley, J., Newman, R. A., Evans, J. N.: Bleomycin induced pulmonary fibrosis in the rat. J. Lab. Clin. Med. **96**, 954 – 959 (1980).
18. Kagan, H. M., Sullivan, K. A.: Lysyl oxidase: preparation and role in elastin biosynthesis. Meth. Enzymol. **82**, 637 – 650 (1982).
19. Kivirikko, K. I., Risteli, L.: Biosynthesis of collagen and its alterations in pathological states. Med. Biol. **54**, 159 – 186 (1976).
20. Knapp, T. R., Daniels, J. R., Kaplan, E. N.: Pathologic scar formation. Am. J. Pathol. **86**, 47 – 70 (1977).

21. Kravis, T. C., Ahmed, A., Brown, T. E., Fulmer, J. D., Crystal, R. D.: Pathogenic mechanisms in pulmonary fibrosis:collagen-induced migration inhibition factor production by cytotoxicity mediated by lymphocytes. J. Clin. Invest. **58**, 1223 – 1232 (1976).
22. Kuhn, C., Starcher, B. C.: The effect of lathyrogens on the evolution of elastase-induced emphysema. Am. Rev. Resp. Dis. **122**, 453 – 460 (1980).
23. Ledwożyw, A.: Protective effect of liposome-entrapped superoxide dismutase and catalase on bleomycin-induced lung injury in rats. I. Antioxidant enzyme activities and lipid peroxidation. Acta Vet. Hung. **39**, 215 – 224 (1991).
24. Ledwożyw, A.: Protective effect of liposome-entrapped superoxide dismutase and catalase on bleomycin-induced lung injury in rats. II. Phospholipids of the lung surfactant. Acta Physiol. Hung. **78**, 157 – 162 (1991).
25. Ledwożyw, A., Ruciński, T., Synowiec, R.: The influence of endotracheally administered bleomycin on hydroxyproline content and collagenase and elastase activity in rat lungs. Pol. Arch. Wet. (in press).
26. Levene, C. I.: Lathyrism. In: Molecular pathology of connective tissue (Perez-Tamayo, R., Rojkind, M. eds) Marcel Dekker, New York 1973, 175 – 228.
27. Levene, C. I., Bye, I., Saffiotti, U.: The effect of ß-aminopropionitrile on silicotic pulmonary fibrosis in the rat. Br. J. Exp. Pathol. **49**, 152 – 159 (1968).
28. Mann, S. W., Fuller, G. C., Rodil, J. V., Viding, E. I.: Hepatic prolyl hydroxylase and collagen synthesis in patients with alcoholic liver disease. Gut **20**, 825 – 832 (1979).
29. Manner, G., Goldring, I. P., Kuleba, M.: Effect of proteolytic enzymes on collagen and noncollagen protein synthesis in human lung fibroblasts. Connect. Tiss. Res. **2**, 283 – 290 (1974).
30. Martin, G. E., Piez, K. A., Lewis, M. S.: The incorporation of ^{14}C-glycine into the subunits of collagen from normal and lathyric animals. Biochem. Biophys. Acta **69**, 472 – 479 (1963).
31. McCullough, B., Collins, J. F., Johanson, W. G., Grover, F. L.: Bleomycin-induced diffuse interstitial pulmonary fibrosis in baboons. J. Clin. Invest. **61**, 79 – 88 (1978).
32. Moorhead, L. C.: Inhibition of collagen cross-linking: a new approach to ocular scarring. Curr. Eye Res. **1**, 77 – 83 (1981).
33. Moorhead, L. C.: Effects of beta-aminopropionitrile after posterior penetrating injury in the rabbit. Am. J. Ophthalmol. **95**, 97 – 109 (1983).
34. Moorhead, L. C., Carroll, J., Constance, G., Jenkins, D. E., Ameniades, C. D.: Effects of BAPN on rabbit cornea after radial keratotomy. ARVO Abstr. Invest. Ophthalmol. **22**, 29 (1982).
35. Narayanan, A. S., Page, R. C.: Biochemical characterization of collagens synthesized by fibroblasts derived from normal and diseased human gingiva. J. Biol. Chem. **251**, 5464 – 5471 (1976).
36. Narayanan, A. S., Page, R. C.: Serum modulates collagen types in human gingiva fibroblasts. FEBS Lett. **80**, 221 – 224 (1977).
37. Niewoehner, D. E., Haidal, J. R.: Lung fibrosis and emphysema: divergent responses to a common injury. Science **217**, 359 – 360 (1982).
38. O'Dell, B. L., Elsden, D. F., Thomas, J., Partridge, S. M., Smith, R. H., Palmer, R.: Inhibition of the biosynthesis of the cross-links in elastin by a lathyrogen. Nature **209**, 401 – 402 (1966).
39. Peacock, E. E.: Pharmacologic control of surface scarring in humans. Ann. Surg. **193**, 592 – 597 (1981).
40. Peacock, E. E.: Control of wound healing and scar formation in surgical patients. Arch. Surg. **116**, 1325 – 1329 (1981).
41. Percarpio, B., Fischer, J. J.: Beta-aminopropionitrile as a radiation reaction preservative agent. Radiol. Biol. **121**, 737 – 740 (1976).
42. Pinnel, S. R., Martin, G. R.: The cross-linking of collagen and elastin: enzymatic conversion of lysine in peptide linkage to α-aminoadipid-δ-semialdehyde (allysine) by an extract of bone. Proc. Natl. Acad. Sci. USA **61**, 708 – 716 (1968).
43. Riley, D. J., Kerr, J. S., Berg, R. A., Janni, B. D., Pietra, G. G., Edelman, N. H., Prockop, D. J.: Beta-aminopropionitrile prevents bleomycin-induced pulmonary fibrosis in the hamster. Am. Rev. Resp. Dis. **125**, 67 – 73 (1982).

44. Risteli, J., Kivirikko, K. I.: Intracellular enzymes of collagen biosynthesis in rat liver as a function of age and in hepatic injury induced by dimethylnitrosamine: changes in prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase activities. Biochem. J. **158**, 361–367 (1976).
45. Risteli, J., Tuderman, L., Kivirikko, K. I.: Intracellular enzymes of collagen biosynthesis in rat liver as a function of age and in hepatic injury induced by dimethylnitrosamine: purification of rat prolyl hydroxylase and comparison of changes in prolyl hydroxylase activity with changes in immunoreactive prolyl hydroxylase. Biochem. J. **158**, 369–376 (1976).
46. Ronnemaa, T., Juva, K., Kulnen, E.: Effect of hyperlipidemic rat serum on the synthesis of collagen by chick embryo fibro blasts. Atherosclerosis **21**, 315–324 (1975).
47. Schaeppi, U., Phelan, R., Stadnicki, S. W., Fleishman, R. W., Heyman, I. A., Ilievski, V., Redding, R. A.: Pulmonary fibrosis following multiple treatment with bleomycin (NCS–125066) in dogs. Cancer Chemother. Rep. **58**, 301–310 (1974).
48. Siegel, R. C.: Lysyl oxidase. Int. Connect. Tissue Res. **8**, 73–118 (1972).
49. Sikic, B. I., Young, D. M., Mimnaugh, E. G., Gram, T. E.: Quantification of bleomycin pulmonary toxicity in mice by changes in lung hydroxyproline content and morphometric histopathology. Cancer Res. **38**, 787–792 (1978).
50. Thompson, W. D., Patrick, R. S.: Collagen prolyl hydroxylase levels in experimental paraquat poisoning. Br. J. Exp. Pathol. **59**, 288–291 (1978).
51. Trackman, P. C., Kagan, H. M.: Nonpeptidyl amine inhibitors are substrates of lysyl oxidase. J. Biol. Chem. **254**, 7831–7836 (1979).
52. Tryka, A. F., Godleski, J. J., Brain, J. D.: Differences in effects of immediate and delayed hyperoxia exposure on bleomycin-induced pulmonary injury. Cancer Treat. Rep. **68**, 759–764 (1984).
53. Tuderman, L., Kivirikko, K. I.: Immunoreactive prolyl hydroxylase in human skin, serum and synovial fluid: changes in the content and components with age. Eur. J. Clin. Invest. **7**, 295–299 (1977).
54. Uitto, J.: Collagen biosynthesis in human skin: a review with emphasis on scleroderma. Ann. Clin. Res. **3**, 250–258 (1971).
55. Veninga, T. S., Vriesendorp, R., Bloom-Muilwijk, M. C., Sleyfer, D. T., Konings, A. W. T.: Absence of an additional fibrotic response caused by oxygen in the lungs of rats after the intratracheal administration of bleomycin. Br. J. Anaesth. **61**, 413–418 (1988).
56. Watters, L. C., King, T. E., Schwartz, M. I., Waldron, J. A., Stanford, R. E., Chernick, R. M.: A clinical, radiographic, and physiologic scoring system for the longitudinal assessment of patients with idiopathic pulmonary fibrosis. Am. Rev. Resp. Dis. **133**, 97–103 (1986).
57. Wawzonek, S., Ponseti, J. V., Shepard, R. S., Wiedemann, L. G.: Epiphyseal plate lesions, degenerative arthritis, and dissecting aneurysms of the aorta produced by aminonitriles. Science **121**, 63 (1955).
58. Weber, L., Meigel, W. N., Spier, W.: Collagen polymorphism in pathologic human scars. Arch. Dermatol. Res. **261**, 63–71 (1978).
59. Woessner, J. F.: The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. Arch. Biochem. Biophys. **94**, 440–447 (1961).
60. Ziskind, M., Jones, R. M., Weill, H.: Silicoses. Am. Rev. Resp. Dis. **113**, 643–665 (1976).

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Effects of vitamin D_3 administration on the levels of serum calcium and inorganic phosphorus in the smooth water snake, Enhydris Enhydris (Schneider)

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Effect of vitamin D_3 administration (12,000 IU/100 g body wt.) on levels of serum calcium and inorganic phosphorus levels in the smooth water snake, *Enhydris enhydris* was investigated. Hypercalcemia and hyperphosphatemia was observed from day one till the end of the experiment (day 14). Maximum values were recorded on the 4th day followed by a steady decline.

Keywords: vitamin D_3, calcium, inorganic phosphorus, *Enhydris enhydris*

The role of Vitamin D in calcium regulation in the lower vertebrates appears to be the same as in mammals. However, the metabolism of Vitamin D has not been well documented in reptiles [3, 11], especially in snakes. In the present study, an attempt has therefore been made to record the changes in calcium and inorganic phosphorus levels in *Enhydris enhydris* after administration of Vitamin D_3 (12,000 IU/100 g body wt.).

Materials and methods

Adult specimens of both sexes of *E. enhydris* were collected from the seacoast near Bombay. These were acclimatized for a week prior to use. The snakes were divided into control and experimental groups. The experimental group was injected intraperitoneally with vitamin D_3 ('Arachitol' Dupar –

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Interfran Ltd. Bombay Batch No. B 2313) at a dose of 12,000 IU/100 g body wt. The control specimens were injected with the vehicle arachic oil at a dose of 0.01 ml/100 g body wt. by the same route. The animals of both the groups were sacrificed 4 h after the last injection on the 1st, 4th, 7th and 14th day. Blood samples were collected by cardiac puncture and the sera separated and analysed for serum calcium and serum phosphate according to Trinder's [12] and Gomori's method [5] respectively. The animals were not fed during the period of experimentation. The injections were given at approximately the same time throughout the course of the experiment. Differences in the values of serum calcium and inorganic phosphorus were tested for statistical significance using Student's *t*-test for group comparison.

Results and discussion

The administration of vitamin D_3 in *E. enhydris* resulted in hypercalcemia and hyperphosphatemia (Table I). The serum calcium level increased steadily from day 1 and reached its maximum on day 4. Thereafter a gradual fall in the serum calcium level was noticed, but the values still remained above normal calcium level on the 14th day. The serum inorganic phosphorus level also followed the same trend. These results clearly show that vitamin D_3 is effective in inducing hypercalcemia and hyperphosphatemia in *E. enhydris* and concur with similar results in other groups of vertebrates [1, 4, 6, 7, 9, 10]. The hypercalcemia response is attributed to the mobilization of calcium from its stores [2] since the snakes were unfed. The decrease in hypercalcemia observed on the 14th day may be attributed to the possible stimulation of the secretion of calcitonin, a hypocalcemic factor from the ultimobranchial gland.

Table I

Effect of vitamin D_3 on serum calcium and inorganic phosphorus levels (mg/100 ml) of Enhydris enhydris

Days	Serum calcium 100 mg/100 ml		Serum inorganic phosphorus 100 mg/100 ml	
	Control	Experimental	Control	Experimental
1	11.19 ± 0.80	12.71 ± 0.30a	4.14 + 0.50	4.48 + 0.56
4	11.53 ± 0.39	14.43 ± 0.36b	4.54 ± 0.15	5.38 ± 0.13b
7	11.19 ± 0.08	13.92 ± 0.24b	4.22 ± 0.33	4.65 ± 0.10
14	11.21 ± 0.26	13.12 ± 0.44a	4.06 ± 0.33	4.14 ± 0.26

The values are mean ± SD of six determinations;
a & b indicate significant responses: $p < 0.01$ and $p < 0.001$, respectively

The hyperphosphatemic response in the snake under study after administration of vitamin D_3 may also be due to mobilization of phosphorus from the bones or soft tissue. Similar results were also observed by Srivastav and Rani [8] after vitamin D_3 treatment in the snake, *Natrix piscator*.

REFERENCES

1. Bentley, P. J.: Urinary loss of calcium in an anuran amphibian (*Bufo marinus*) with a note on the effects of calcemic hormones. Comp. Biochem. Physiol. **76 B**, 717–719 (1983).
2. Bentley, P. J.: Calcium metabolism in the Amphibia. Comp. Biochem. Physiol. **79 A**, 1–5 (1984).
3. Dacke, C. G.: Calcium regulation in sub-mammalian vertebrates. Academic Press, London (1979).
4. Das, V. K., Srivastava, M., Das, S.: Vitamin D_2 induced hypercalcemia and hyperphosphatemia in unfed male catfish *Rita rita* (Ham). J. Adv. Zool. **11**, 13–18 (1990).
5. Gomori, G.: A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. Clin. Med. **27**, 955–959 (1942).
6. Robertson, D. R.: Effects of ultimobranchials and parathyroid glands and Vitamin D_2, D_3 and dihydrotachysterol$_2$ on blood calcium and intestinal calcium transport in frog. Endocrinology **96**, 934–940 (1975a).
7. Robertson, D. R.: The *in vitro* transport of calcium by the frog intestines and the effect of Vitamin D_3. Comp. Biochem. Physiol. **51**, 705–710 (1975b).
8. Srivastav, A. K., Rani, L.: Phosphocalcic response to Vitamin D_3 treatment in fresh water snake, *Natrix piscator*. Zoological Science **5**, 893–895 (1988).
9. Swarup, K., Srivastav, S. P.: Vitamin D_3 induced hypercalcemia in male catfish, *Clarias batrachus*. Gen. Comp. Endocrinol. **46**, 271–274 (1982).
10. Swarup, K., Norman, A. W., Srivastav, A. K., Srivastav, S. P.: Dose-dependent Vitamin D_3 and 1,25-dihydroxy vitamin D_3 induced hypercalcemia and hyperphosphatemia in male catfish, *Clarias batrachus*. Comp. Biochem. Physiol. **78**, 553–555 (1984).
11. Taylor, C. W.: Calcium regulation in vertebrates: an overview. Comp. Biochem. Physiol. **82 A**, 249–255 (1985).
12. Trinder, P.: Colorimetric micro determination of calcium in serum. Analyst. **85**, 889–894 (1960).

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Experimental study on the kinetics of mucociliary activity in rabbit airways

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Noninvasive radioaerosol technique was applied to investigate the mucociliary activity of bronchial mucosa using ^{99m}Tc-labelled homologous erythrocytes for inhalation. Radioactivity was continuously measured for 60 min with a gamma camera connected online to the computer. Time-activity kinetic parameters were calculated by a monoexponential model. Test substance azelastine and bromhexine were used to demonstrate the validity of experimental method discussed.

Keywords: mucociliary clearance, radiolabelled erythrocytes, elimination kinetics, azelastine, bromhexine

Mucociliary clearance is a non-specific defence mechanism, which continuously removes inhaled particles and cellular debris from the airways. Clearance function includes two major cooperative mechanisms, i.e. ciliary transport and mucous secretory system. Pulmonary clearance may be affected by several factors, including lung diseases and pharmacological agents. Human and animal models have been used to study these effects. Experimental methods are mainly directed to investigate the mucus production, rheology of the mucus, as well as the ciliary beating activity.

Estimation of tracheobronchial mucus secretion has been already proposed in early literature [9]. The main disadvantage of methods lies in the low amount of mucus (60–80 μl/kg per hour) collected during several hours in control experiments [2]. This fact induced to develop novel methods for estimation of the bronchial secretion by use of fluorescein [6] or phenol red [4].

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Ciliary beating frequency has been investigated by both in vitro and in vivo techniques. Ciliary motility can be measured on bronchial explants in vitro with photoelectric method [7]. The tracheal-window test is used for in vivo studies performed in anesthetized animals. The control ciliary beat frequency is approximately 16 Hz (1000 beats per min) in mammals. This function can be studied by high-speed cinematography or recently by laser light scattering spectroscopy [11].

Another group of techniques gives information on the linear speed of mucus transport. Standardized particles (charcoal, teflon discs, radiolabelled-albumin microspheres) are introduced to the mucus membrane of trachea or bronchi and the rate of the transport is measured by bronchoscopy, fiberoptics [10] or scintillation detector [14]. Mean mucus transport rates vary between 5–18 mm per min in anesthetized animals and normal subjects.

Modern methods have been proposed to study the complex function of the mucociliary system. These methods are based on the inhalation of monodisperse particles of appropriate size (2–8 μm), that reach even the peripheral respiratory pathways. The clearance rate of particles is an appropriate measure of mucociliary activity of the whole lung. The basic condition of the noninvasive methods is the selection of the radiolabelled substance, which can be detected from outside of the thoracic wall. For this purpose iron oxide, sulphur colloid [8], teflon particles labelled with ^{99m}Technetium have been generally applied. More recently, ^{99m}Tc-labelled erythrocytes ([^{99m}Tc] RBC) having homogenous particle size proved to be an excellent marker in clinical diagnostics [13].

The aim of present study is to demonstrate a method for estimating the mucociliary activity in rabbit airways using inhalled [^{99m}Tc] RBC for inhalation. The expectorant bromhexine [3], and a novel antiasthmatic drug, azelastine [1, 12] were used as test substances.

Materials and methods

New Zealand rabbits were anesthetized by iv. pentobarbital (25 mg/kg); and polyethylene cannula was introduced into trachea and a jugular vein. Drugs and vehicle were injected iv. The labelled RBC aerosol were inhaled through the tracheal cannula.

The [^{99m}Tc] RBC was prepared freshly [5] with an activity of 250–270 MBq/ml. A sample of 0.6 ml was nebulized to fill up a reservoir of 4 litre volume. The tracheal cannule of rabbit was immediately connected to the reservoir and aerosol was administered in 3 minutes. This process was repeated twice. After inhalation has been stopped, radioactivity was measured for 60 minutes over the chest of animal by a gamma camera connected online to the computer. In this period 60 images were successively obtained in one-minute frames and stored for evaluation.

By means of computer program, regions of interest were selected over the peripheral zones of lung to estimate the bronchial mucociliary activity. Time-activity curves were generated from the regions and the elimination half-life ($t_{1/2}$eff) of the marker was calculated by fitting a monoexponential function to the experimental data. After correction for the natural decay of the radioisotope, the elimination rate

constant and biological half-life ($t_{1/2}$biol) of inhaled RBC were determined. The fractions of [^{99m}Tc] RBC eliminated from the region were calculated.

Mucociliary clearance was expressed as the percentage of inhaled marker cleared during a 1-hour period of time. The increase of mucociliary clearance indicates the enhancement of mucociliary activity elicited by previous drug treatment.

Results

Control experiment

All experiments were carried out in five-member groups of rabbits. The control group was treated with physiological saline (1 ml/kg, iv.). Figure 1 shows the elimination rate of [^{99m}Tc] RBC as a function of time. The calculated biological half-life of the marker was 498.2 min in average. With regard to the mucociliary clearance, 8.71% per hour mean value was measured (Table I).

Table I

Biological half-life and mucociliary clearance values of radiolabelled RBC after azelastine treatment

Treatment (iv.)	Dose (mg/kg)	$t_{1/2}$ biol. (min)	Clearance (%/hour)
Control	-	498.2 ± 70.4	8.71 ± 2.43
Azelastine	1	289.7 ± 63.6	13.86 ± 2.65*
	2	231.4 ± 75.2	18.02 ± 6.01*
	5	172.6 ± 39.0	22.25 ± 4.70*

* Significance related to control value

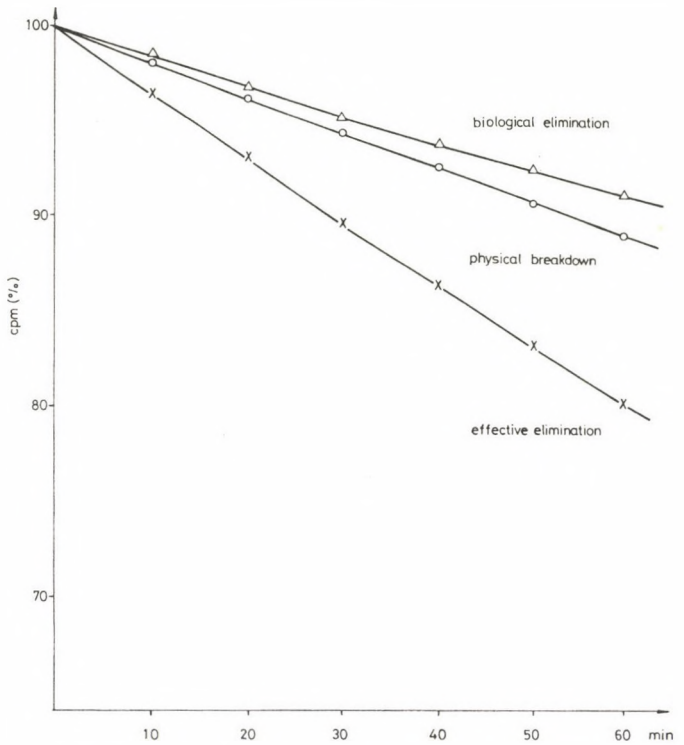

Fig. 1. Elimination curves of ^{99m}Tc-labelled erythrocytes

Effect of azelastine on the mucociliary activity

Three groups of rabbits were pretreated with increasing dose of azelastine iv., 30 min prior to inhalation of radioaerosol. A dose-dependent increase was found in elimination rate of [^{99m}Tc] RBC (Fig. 2); the biological half-life of the marker has been simultaneously shortened. Following the administration of 1, 2 and 5 mg/kg doses, the mucociliary clearance increased up to 13.86, 18.02 and 22.25% per hour, respectively (Table I). The change was significant related to control.

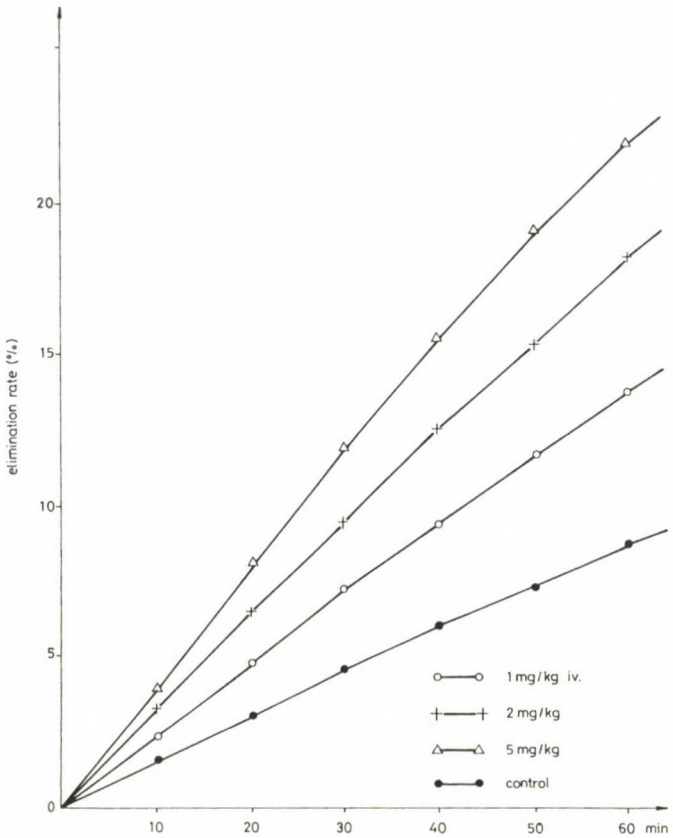

Fig. 2. Effect of azelastine on the mucociliary activity in rabbit airways

Effect of bromhexine on the mucociliary activity

Another three groups of animals have been selected for bromhexine administration. The drug was dissolved in solution of glycerin-water (1:5) and administered as a 20-minute iv. infusion before inhalation of marker.

It was found that bromhexine in a dose-range of 20, 28 and 40 mg/kg decreased the biological half-life of [^{99m}Tc] RBC in a significant manner, related to the control treated with vehicle. Consequently, the mucociliary clearance was enhanced up to 13.36, 17.13 and 19.73% per hour, respectively (Table II).

Table II

Biological half-life and mucociliary clearance values of radiolabelled RBC after bromhexine treatment

Treatment (iv.)	Dose (mg/kg)	$t_{1/2}$ biol. (min)	Clearance (%/hour)
Vehicle	–	397.8 ± 65.03	10.15 ± 1.62
Bromhexine	20	296.5 ± 48.38	13.36 ± 2.04*
	28	239.2 ± 67.28	17.13 ± 5.22*
	40	198.9 ± 50.83	19.73 ± 4.23*

* Significance related to control value

Comparing the effect of two test substances, azelastine proved to be about 10 times more effective than bromhexine. The dose-effect relation of both substances is demonstrated in Fig. 3.

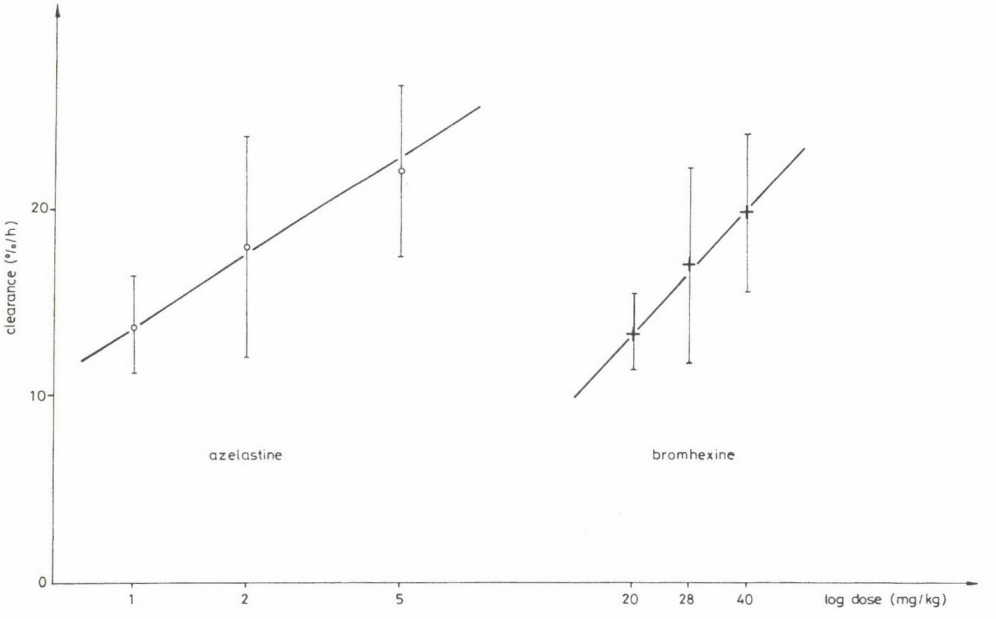

Fig. 3. Dose-dependent mucociliary clearance effect of azelastine and bromhexine

Discussion

Drugs used in the treatment of respiratory diseases often influence the mucociliary activity of the airways. Clearance may be enhanced by drugs such as

beta-2-agonists, theophylline and mucolytics; on the contrary, anticholinergic agents appear to depress this function.

Moreover, stimulant and depressant effects on the mucociliary activity have been attributed to a number of other pharmacological agents. Cholinergic drugs, histamine and bradykinin increase the mucociliary transport, too. This effect seems to be independent of bronchoconstrictor activity of these agents. On the other hand, general anesthetics, opioids, benzodiazepines are also able to depress the mucociliary function.

These examples indicate the importance of appropriate methods for evaluation of the influences on the mucociliary activity.

REFERENCES

1. Achterrath-Tuckermann, U., Saano, V., Minker, E., Stroman, F., Arny, I., Joki, S., Nuutinen, J., Szelenyi, I.: Influence of azelastine and some selected drugs on mucociliary clearance. Lung, **170**, 201–209 (1992).
2. Adams, G. K., Aharonson, E. F., Reasor, M. J., Proctor, D. F.: Collection of normal canine tracheobronchial secretions. J. Appl. Physiol., **40**, 247–249 (1976).
3. Boyd, E. M., Sheppard, P.: On the expectorant activity of Bisolvon. Arch. int. Pharmacodyn., **163**, 284–295 (1966).
4. Engler, H., Szelenyi, I.: Tracheal phenol red secretion, a new method for screening mucosecretolytic compounds. J. Pharmacol. Methods, **11**, 151–157 (1984).
5. Hamilton, R. G., Alderson, P. O.: A comparative evaluation of techniques for rapid and efficient in vivo labelling of red cells with 99mTc-pertechnotate. J. Nuclear Med., **18**, 1010–1013 (1977).
6. Kogi, K., Konishi, K., Saito, T.: Pharmacological and toxicological studies on Domiodol, a new mucolytic agent and expectorant. Arzneim. Forsch., **33**, 1281–1286 (1983).
7. Mercke, U., Häkansson, C. H., Toremalm, N. G.: A method for standardized studies of mucociliary activity. Acta Otolaryngol. (Stockholm), **78**, 118–123 (1974).
8. Müller, M., Konietzko, N., Adam, W. E., Matthys, H.: Die mukociliare Klarfunktion der Lunge, untersucht mit radioaktiv markierten Schwefelkolloid. Klin. Wochenschr., **53**, 815–822 (1975).
9. Perry, W. F., Boyd, E. M.: A method for studying expectorant action in animals by direct measurement of the output of respiratory tract fluids. J. Pharmacol. exp. Ther., **73**, 65–77 (1941).
10. Sackner, M. A., Rosen, M. J., Wanner, A.: Estimation of tracheal mucous velocity by bronchofiberscopy. J. Appl. Physiol., **34**, 495–499 (1973).
11. Svartengren, K., Wiman, L. G., Thyberg, P., Rigler, R.: Laser light scattering spectroscopy: a new method to measure tracheobronchial mucociliary activity. Thorax, **44**, 539–547 (1989).
12. Szelenyi, I.: Pharmacological profile of azelestine. Drugs Today (Suppl), **6**, 3–8 (1989).
13. Vastag, E., Matthys, H., Köhler, D.: In vivo measurement of mucociliary clearance by 99mTc-labelled human erythrocytes (in Hungarian). Pneumologia Hungarica, **38**, 61–72 (1985).
14. Yeates, D. B., Aspin, N., Levinson, H., Jones, M. T., Bryan, A. C.: Mucociliary tracheal transport rates in man. J. Appl. Physiol., **39**, 487–495 (1975).

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The porphyrins in cancer and virus research

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Compounds with extended conjugated electron-system are under constant study in view of their numerous biological functions. Among them natural and synthetic porphyrins are of particular interest because of their activity in photodynamic treatment of cancer and virus infection. Recently, many modifications in tetrapyrrole ring system have been reported, which are briefly reviewed. Among the tetracationic porphyrins, TΘOPP, a compound having four electron-sending tentacle side chains, exhibits large aggregation tendency in the presence of DNA of different base-sequence. The aggregation tendency of TΘOPP can be attributed to its relatively basic core (pK_a is ~ 4.6) which is also responsible for the existence of a TΘOPP–DNA adduct where the core is protonated. This protonation, observed even at physiological pH, alters TΘOPP binding mode on the surface of the DNA.

Keywords: porphyrin, DNA-binding, aggregation

The group of porphyrins includes compounds of vital importance. Chlorophyll, e.g., in photosynthesis, haemoglobin, in transporting of oxygen, or cytochromes, in biological redox processes are well known representatives having tetrapyrrole ring system. Many porphyrins are currently under extensive investigation in view of their potential use in solar-energy conversion [7].

In addition, porphyrins or their metallo-derivatives have other biological effects, even medical applications. They have been found to be active against certain cancers [2, 3] and viruses [4, 9, 11]. These effects are based on a selective uptake of porphyrins by the tumor cell followed by photodynamic treatment.

As far as the mechanism of their action is concerned, only some details have been revealed. The interaction of the porphyrins with DNA is a possible pathway [10, 12] but it must be emphasized that not only cationic porphyrins but neutral, even anionic, porphyrins are also active [4]. Furthermore, it is not clear if there is any

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relationship between the properties of porphyrins and their activity [12]. Another problem is that the chemistry and photochemistry of the porphyrins is not completely understood, e.g. a fundamental question about monomeric or dimeric state of the well-known TMpyP(4) (*meso*-tetrakis[4-methylpiridyl]porphine, see Fig. 1, I) is still unresolved [13].

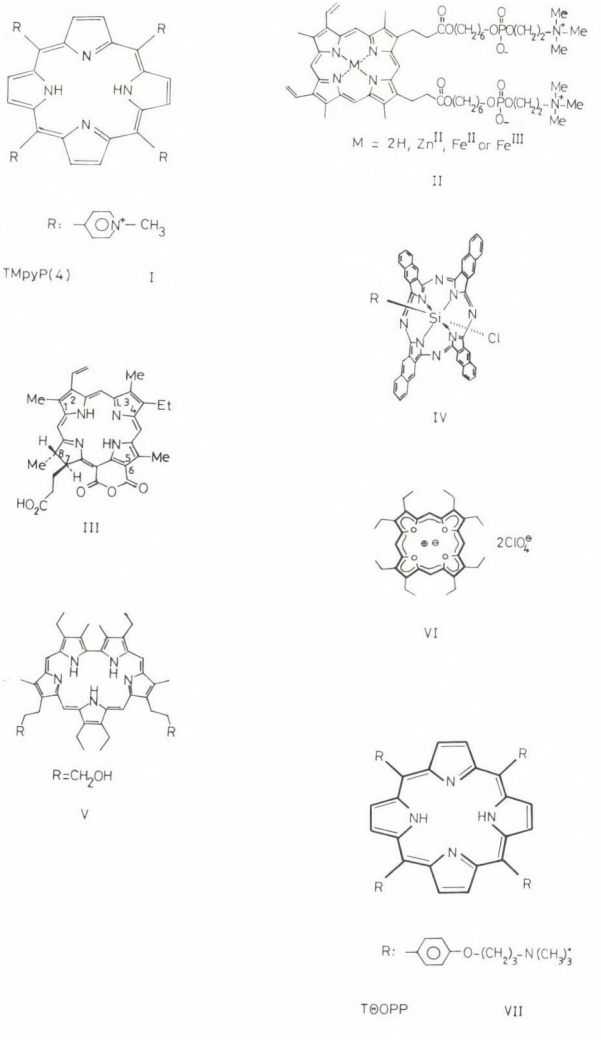

Fig. 1. Structures of porphyrins and related compounds synthesized recently

Recently, extensive studies have been carried out on new tetrapyrrole compounds or related molecules. The substituents on the porphyrin ring have been modified, e.g. compounds with alkylphosphocholine groups (Fig. 1, II) [15] or a series from a chlorophyll derivative (Fig. 1, III) [8]. Other structures, closely related to the porphyrin system, are also prepared and studied (Fig. 1, IV, V, VI) ([1, 6, 16] respectively), searching for molecules useful for photodynamic therapy. (Porphyrins are good candidates also for magnetic resonance imaging [14]).

In order to get a better understanding of the porphyrin-DNA interaction, we synthesized a new tetrapositive porphyrin, TΘOPP (*meso*-tetrakis[4-[(3-trimethylamino-propyl)oxy]-phenyl]porphine, Fig. 1, VII). This compound has two new features, compared to those studied previously: it bears four *"tentacle"* side chains and has a relatively basic center on nitrogens with an apparent pK_a of ~ 4.6 measured at low (< 10 mM) ionic strength. In this paper two major conclusions are pointed out, both presumably due to the basic characteristic of TΘOPP: 1. TΘOPP is unable to intercalate into the Guanine-Cytosine region of DNA under any experimental condition and 2. it forms a DNA adduct when TΘOPP core is protonated even at physiological pH.

Method

The preparation of TΘOPP is described elsewhere [17]. Calf thymus DNA was purchased from SIGMA, $[poly(dG\text{-}dC)]_2$ and $[poly(dA\text{-}dT)]_2$ were obtained from PHARMACIA. DNA samples were prepared in 10 mM NaCl and stored frozen. All other chemicals were used as received from SIGMA. The typical pH of 7 was adjusted with a 10 mM PIPES (piperazine-N,N'-bis(2-ethane-sulfonic acid)) buffer solution.

Visible spectra were taken on a VARIAN Cary3 spectrophotometer. A JASCO 600 spectropolarimeter was used for taking circular dichroism (CD) spectra. Viscosimetric studies were performed on an Ostwald capillary viscosimeter at 30 °C. For both spectroscopic investigations 1 cm cuvette was used and the temperature was adjusted to 25 °C.

Results and Discussion

As a typical change in *vis* spectra when 10 mM PIPES was present, the Soret band of TΘOPP (418 nm at neutral pH) was red shifted by 6–7 nm, regardless of the *a*) ratio of porphyrin: DNA base pairs (R throughout), *b*) NaCl concentration and *c*) the nature of DNA. This suggested an outside binding mode for any base-sequence since at least 15 nm red shift occurs in the case of intercalation [10]. The CD spectra were in accordance to the *vis* results: exciton type curves were induced under any circumstances. (For intercalation, small negative CD band is expected.) Based on the exciton curves, stacking of TΘOPP on the DNA surface was suggested, a binding mode similar to that found previously [5]. Figure 2 shows typical spectra for TΘOPP

– $[poly(dA\text{-}dT)]_2$ solutions; essentially the same spectra were found for CT DNA and for the GC polymer. The viscosimetric results (Fig. 3) were supportive in binding mode: TMpyP(4), a typical intercalator is able to unwind the duplex hence the viscosity increases. For an outside binder no such effect is expected.

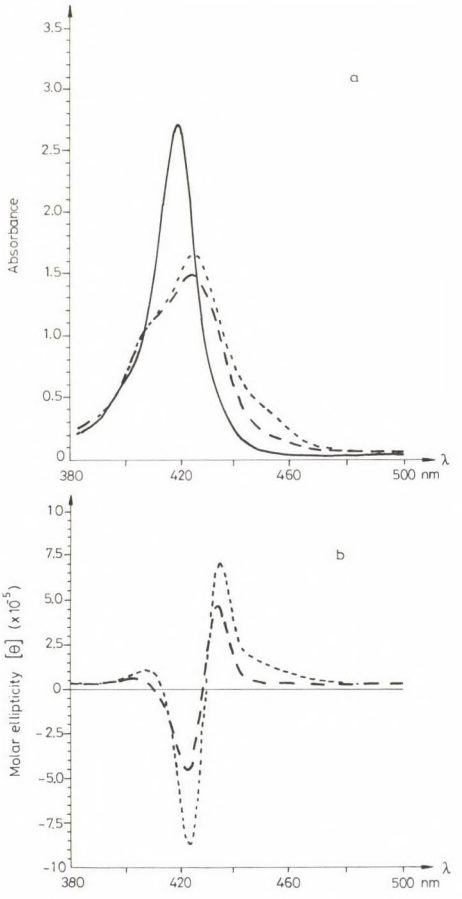

Fig. 2. Visible (a panel) and CD (b panel) spectra of 5 μM TΘOPP without DNA (——) and with DNA at R = 0.05 (– – –) and at R = 0.01 (- - -). PIPES = 10 mM (pH = 7), NaCl = 10 mM. [Θ] unit is deg • cm² • decimol⁻¹

When the TΘOPP–DNA solutions did not contain PIPES, at large DNA excess (R = 0.01) a shoulder developed around 450 nm on the *vis* spectrum; simultaneously the original red-yellow color changed to greenish. The R = 0.01 solution was stored in dark for one day and the spectrum was taken again. The

intensity of the 450 nm band increased further and the green became more intense. Since the protonated form of TΘOPP with no DNA is green and displays a 445 nm maximum, we attributed this new band observed in the presence of DNA to a species in which the TΘOPP is bound to DNA and is protonated. Generally, from a decrease in pH protonation of higher degree can be anticipated, thus we repeated the experiment at pH 6 (Fig. 4). Even at R = 0.5, the shoulder developed around 450 nm and a large 451 nm band appeared at R = 0.01; simultaneously the 424 nm band (nonprotonated bound TΘOPP) decreased in intensity. In such a protonated species stacking is impossible. Indeed, the CD spectra (Fig. 4) displayed a combination of an exciton signal and a small positive band when R = 0.5 but only a single positive band could be observed at R = 0.01, indicating an outside binding mode with no porphyrin stacking for this low R value [10]. Further decrease of pH to 5.5 had only a slight effect on the 451 nm band, suggesting that protonation is dominantly taking place in the pH 6 solution. This observation was unexpected with regard to the pK_a of ~ 4.6; a careful spectrophotometric pH-titration gave an apparent pK_a of ~ 6.4–6.6 at R = 0.01 for all three DNAs, i.e. a large increase in basicity of TΘOPP was advanced by the presence of polymers.

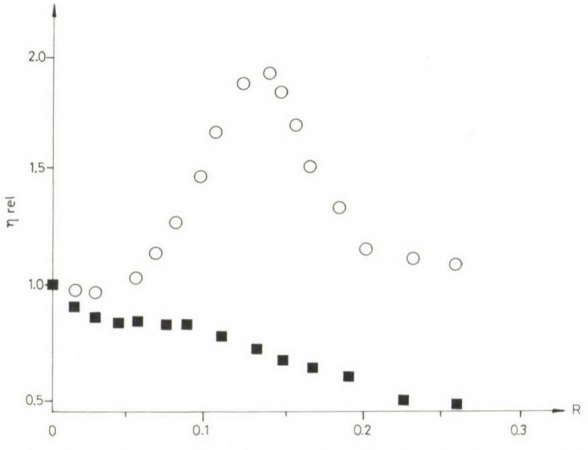

Fig. 3. Relative viscosity (η_{rel}) vs. [porphyrin]/[base-pair] ratio for titration of a DNA solution with TMpyP(4) (o) and with TΘOPP (▪)

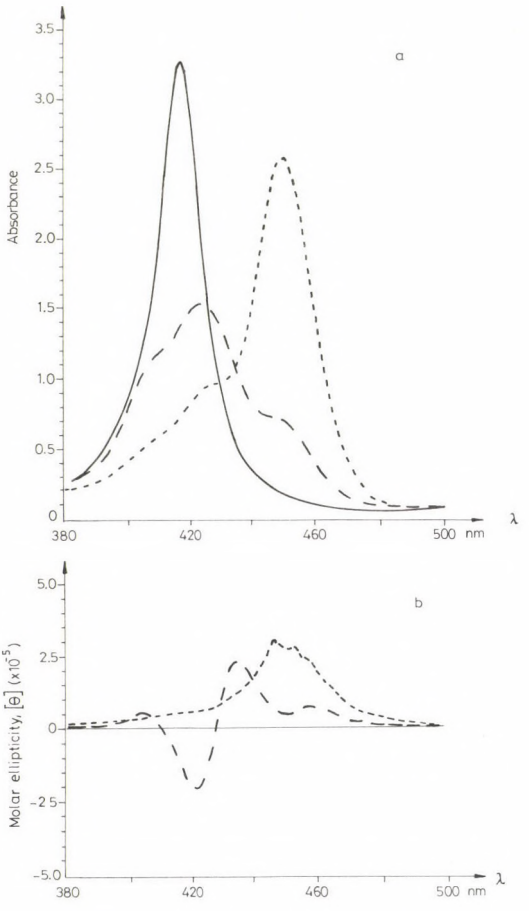

Fig. 4. Visible (a panel) and CD (b panel) spectra of 5 μM TΘOPP without DNA (——) and with DNA at R = 0.05 (– – –) and at R = 0.01 (- - -). pH = 6 (no buffer), NaCl = 10 mM. For [Θ] unit see Fig. 2

Conclusions

The relatively basic TΘOPP has a large stacking tendency in the presence of DNA with either Guanine–Cytosine or Adenine–Thymine base-pairs. Binding mode of TΘOPP (outside binding with porphyrin stacking) is illustrated as a small (6–7 nm) red shift of the Soret band in vis spectra and an exciton type CD. At pH 6 in large DNA excess, an intense new band evolves at 451 nm band attributable to a protonated bound porphyrin species, which can observed even at pH 7 but on a lower extent. The observed increase of ca. 2 log unit in apparent pK_a of TΘOPP can be explained by the large negative environment of DNA duplex.

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REFERENCES

1. Brasseur, N., Nguyen, T.-L., Langlois, R., Ouellet, R., Marengo, S., Houde, D., van Lier, J. E.: Synthesis and photodynamic activities of silicon 2,3-naphtalocyanine derivatives. J. Med. Chem., **37**, 415 – 420 (1994).
2. Diamond, I., Granelli, S. G., McDonagh, A. F.: Biochem. Med., **17**, 121 – 127 (1977).
3. Ding, L., Casas, C., Etemad-Moghadam, G., Meunier, B., Cros, S.: Synthesis of water-soluble, cationic functionalized metalloporphyrins having a cytotoxic activity. New J. Chem., **14**, 421 – 431 (1990).
4. Dixon, D. W., Kim, M. S., Kumar, V., Obara, G., Marzilli, L. G., Schinazi, R. F.: Amino- and hydroxytetraphenylporphyrins with activity against the human immunodeficiency virus. Antiviral Chem. Chemother., **3**, 279 – 282 (1992).
5. Gibbs, E. J., Tinoco, I. Jr., Maestre, M. F., Ellinas, P., Pasternack, R. F.: Self-assembly of porphyrins on nucleic acid templates. Biochem. Biophys. Res. Commun., **157**, 350 – 358 (1988).
6. Iverson, B. L., Shreder, K., Král, V., Sessler, J. L.: Phosphate recognition by sapphyrin. A new approach to DNA binding. J. Am. Chem. Soc., **115**, 11022 – 11023 (1993).
7. Kalyanasundaram, K.: Photochemistry and sensitized evolution of hydrogen from water using water-soluble cationic porphyrins. J. Chem. Soc., Faraday Trans., **79**, 1365 – 1374 (1983).
8. Lee, S-J. H., Jagerovic, N., Smith, K. M.: Use of the chlorophyll derivative, purpurin-18 for syntheses of sensitizers for use in photodynamic therapy. J. Chem. Soc., Perkin Trans. 2369 – 2377 (1993).
9. Levere, R. D., Gong, Y-F., Kappas, A., Bucher, D. J., Wormser, G. P., Abraham, N. G.: Heme inhibits human immunodeficiency virus 1 replication in cell cultures and enhances the antiviral effect of zidovudine. Proc. Natl. Acad. Sci. USA, **88**, 1756 – 1759 (1991).
10. Marzilli, L. G.: Medical aspects of DNA-porphyrin interactions. New J. Chem., **14**, 409 – 420 (1990).
11. Matthews, J. L., Newman, J. T., Sogandares-Bernal, F., Judy, M. M., Skiles, H., Leveson, J. E., Marendo-Rowe, A. J., Chanh, T. C.: Photodynamic therapy of viral contaminants with potential for blood banking applications. Transfusion, **28**, 81 – 83 (1988).
12. Munson, B., Fiel, R. J.: DNA intercalation and photosensitization by cationic meso-substituted porphyrins. Nucleic Acids Res., **20**, 1315 – 1319 (1992).
13. Pasternack, R. F., Bustamante, C., Collings, P. J., Giannetto, A., Gibbs, E. J.: Porphyrin assemblies on DNA as studied by a resonance light-scattering technique. J. Am. Chem. Soc., **115**, 5393 – 5399 (1993).
14. Sessler, J. L., Mody, T. D., Hemmi, G. W., Lynch, V., Young, S. W., Miller, R. A.: Gadolinium(III)texaphyrin: A novel MRI contrast agent. J. Amer. Chem. Soc., **115**, 10368 – 10369 (1993).
15. Tsuchida, E., Komatsu, T., Toyano, N., Kumamoto, S.-I., Nishide, H.: Lipid-porphyrin fibres: Morphology and incorporation into phospholipid vesicle. J. Chem. Soc., Chem. Commun. 1731 – 1733 (1993).
16. Vogel, E., Dörr, J., Herrman, A., Lex, J., Schmickler, H., Walgenbach, P., Gisselbrecht, J. P., Gross, M.: Octaethyltetraoxaporphyrin dication-counterpart of octaethylporphyrin in the tetraoxaporphyrin series. Angew. Chem. Int. Ed. Engl., **32**, 1597 – 1600 (1993).
17. Mukundan, N. E., Pethő, G., Dixon, D. W., Kim, M. S., Marzilli, L. G.: Interactions of an electron-rich tetracationic tentacle porphyrin with Calf Thymus DNA. Inorg. Chem., in press.

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Comparison of two mechanical carotid baroreceptor stimulation techniques

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We compared two mechanical carotid baroreceptor stimulation techniques, the phase related external suction (PRES) method and the conventional neck suction techniques concerning their effects on blood pressure and heart rate responses in a group of 10 normotensive men. The cuff pressure using the PRES method was phase-locked in time to the R-wave of the ECG. During the conventional neck suction technique the cuff pressure changes were not related to the cardiac cycle, it was either negative or positive. Blood pressure was measured in four of the patients both invasively and noninvasively to compare the two baroreceptor stimulating methods.

The results have indicated that (1) both mechanical carotid baroreceptor stimulation technique showed a significant heart rate deceleration to baroreceptor stimulation. (2) The heart rate changes were more pronounced during the continuous neck cuff technique, and the heart rate recovered sooner to the baseline. The variation of baroreceptor activity as induced by the PRES method seems to prevent habituation much more than the continuous neck suction method. (3) The systolic blood pressure decrease was significant both during PRES and continuous neck suction stimulation. A higher decrease in systolic blood pressure was shown during continuous neck suction stimulation compare to the PRES stimulation. (4) The diastolic blood pressure changes showed the same alteration for baroreceptor stimulation as compared to the control condition but there was no difference between the two stimulation methods. (5) The noninvasive Finapres blood pressure device measures blood pressure reliably.

Keywords: PRES method, continuous neck suction, baroreceptor stimulation, non-invasive blood pressure measurement

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The regulation of systemic blood pressure represents a complex physiological process involving neuronal and endocrine mechanisms, which, in turn, have short- and long-term effects on this regulation. An important mechanism in this regulatory system is represented by the arterial baroreceptors, the most prevalent of which are found in the aortic arch and the internal carotid artery. By inhibiting sympathetic vasoconstrictor outflow, the baroreceptors play an important role in compensating rapid changes in blood pressure induced by orthostatic and other stress-related factors. There also appear to be differences in the relative contributions of the aortic and carotid baroreceptors in inhibiting sympathetic outflow. Stimulation of the baroreceptors induces a deceleration in heart rate and a decline in mean arterial blood pressure [10].

For studying the arterial baroreceptors, a variety of methods to manipulate their activation has been developed. Mechanical stimulation with surgically implanted balloons into the region of the carotid sinus has been used in dogs [8] in order to study the physiology of the baroreceptors. Pharmacological blood pressure manipulation under conditions of intact and destroyed baroreceptors have been used in rats [2] to study effects of baroreceptor activation on central nervous processes like pain perception. Orthostatic stimulation via body tilt has been used in both animals and humans [6, 7] to study classical conditioning of the baroreceptor reflex and the impact of baroreceptor activation on epileptic phenomena.

The carotid neck cuff technique has been developed in order to allow non-invasive stimulation and inhibition of arterial baroreceptors in humans and animals [4, 15]. Psychophysiologists are becoming more an more interested in the field of baroreceptor research since the theories by Lacey & Lacey [9] who postulated influences of baroreceptor activation directly onto the brain. Toon et al. [16] used the neck cuff technique in combination with a reaction time task and predicted slower reaction times during periods in which the baroreceptors were stimulated compared to inhibition trials. The negative cuff pressure condition (stimulation) is perceived as being much more aversive compared to the positive cuff pressure condition (inhibition). This unspecific effect could mask possible "real" effects. In order to have the possibility of a more controlled baroreceptor activation, Rau et al. [12] have recently developed a modified neck cuff method following an idea by Dworkin [1], which overcame the disadvantage of having no adequate control condition. The principle of this method is that the application of short (about 200 ms) intervals of negative pressure affects the reflexive heart rate responses depending on the location of this pressure burst within the cardiac cycle [3]. Since the arterial baroreceptors are stretch receptors located in the arterial wall, they detect transmural pressure changes independent from the source of pressure changes: Increasing the intravasal pressure and thus dilating the vessel has similar stimulating effects on the baroreceptors as decreasing the surrounding pressure, e.g. within a cuff around the neck. The

baroreceptors are more sensitive to changes in the pressure than to static pressure levels. For this that PRES not only decelerates the heart rate, but also decreases the arterial blood pressure, measured invasively or with the Finapres device [11]. The aim of the present experiment was to compare the PRES technique and the conventional neck suction method, which applies negative or positive pressure to the neck for some heart cycles.

Experiment I

In this experiment, only heart rate responses were measured to the baroreceptor stimulation. It was hypothesized that heart rate would decelerate in response to the baroreceptor stimulation trials compared to the control trials.

The main question regards possible differences in the size and/or shape of these heart rate responses between the two baroreceptor stimulation methods PRES and the continuous neck suction technique.

Methods I

Subjects

Ten male subjects aged from 21 to 32 years (mean = 27.1) and free of any cardiovascular disorders and drug application participated in the present experiments. The subjects were students from the Freiburg University or staff members from the Cardiovascular Rehabilitation Center, Bad Krozingen. Systolic blood pressure as measured before the experiment ranged from 110 to 130 mm Hg (mean = 117.5), diastolic from 60 to 80 mm Hg (mean = 76). All subjects were informed about every part of the experiment and they were told that they could finish the experimental session at every time they wanted. All subjects received a monetary reward of 35 German Marks for their participation.

Ethical approval

Prior to investigation, a research proposal was submitted to the state ethical committee for approval, which was subsequently granted. Some days prior to the examination, the subjects were requested to participate in the study. At the time of examination, they were given a written explanation of the general research aims and possible hazards. At this time they were asked to sign a statement of informed consent, which was complied to in all cases. Emphasis, however, was placed on the voluntary nature of their participation, as well as on the possibility to discontinue their participation at any time.

Baroreceptor stimulation

A comprehensive description of PRES including the construction of the valves and the computer algorithms for the valve switching is given by Rau et al. [12]. Shortly, a malleable neck cuff, fashioned from lead plate, which was coated to prevent contact with the skin, was designed according to the description given by Eckberg et al. [4]. A dense, foam rubber strip was attached to the edge surfaces of the cuff to insure the subjects' comfort and to maximize the air-tightness of the cuff. The cuff was positioned on the subject's neck and secured with an elastic band and Velcro tape. The local relative air

pressure inside the cuff was recorded during the experiments using piezo-electric pressure transducer. The PRES neck-suction technique was implemented by a specially designed device. Its basis is an electrical motor which elicits a continuous air current in a closed chamber (20 cm width, 30 cm long, 15 cm high). This current of air is directed to the neck cuff by opening and closing four different valves, which regulate air flow into and out of the chamber. Four electromagnetic couplers, under control of the computer, opened and closed the valves so that the changes in air flow to the cuff could be synchronized to the heart cycle. Pressure changes in the cuff are triggered upstroke of the R-wave of the electrocardiogram. Each time a R-wave is detected, a cardiotachometer coupler generates a square wave which is fed into the computer (via a TTL circuit to the digital input) which then determines the time (to within 0.5 ms) of the R-wave appearance. For stimulation (systolic negative pressure/diastolic positive pressure) the cuff pressure begins to drop 100 ms after the detection of a R-wave. About 2/3 of the final pressure is achieved 180 ms later. The negative pressure remains for an interval which lasts as long as half of the trial's mean inter beat interval minus 100 ms. Then, a positive cuff pressure is applied for exactly the same duration. After this interval, the valves are opened and atmospheric pressure persists until the next R-wave triggers the valves switches.

The whole sequence is started over and over again, resulting in a continual wave of bipolar pressure pulses. In the present experiment, this continual wave has been presented for periods of 6 s each. For control trials, the sequence of negative and positive pressure pulses is reversed in time: each R-wave triggers a positive cuff pressure (again for 50% of the time of the last trial's mean minus 100 ms). This period is followed by a negative pressure period of equal duration. If the next R-wave occurred prior to the completion of the bipolar pressure pulse, 100 ms later the current pulse was interrupted and the next one was initiated.

The continuous neck suction technique was used similar as described in [13]: During stimulation trials, a 6 s lasting negative pressure was applied to the neck. During control trials, on the other hand, a 6 s lasting positive pressure was applied. The negative pressure reached about −30 mm Hg, whereas positive pressure reached only about +10 mm Hg.

Physiological recordings

The electrocardiogram was measured in all subjects in order to trigger the valves and to compute heart rate responses to the manipulation. Three Beckman Ag/AgCl electrodes were attached to the thorax using the position which produced clear R-waves. One lead was amplified with a time constant of 0.3 s and a high frequency cut off 30 Hz. The amplified signal was fed into a modified cardiotachometer coupler which detects the R-waves and generates a square wave each time it detects an R-wave. The square wave impulses were fed into the digital input channel of a input-output board (Data Translation 2821) in an IMB−AT compatible computer. The computer measured the interval between successive R-waves with a precision of 0.5 ms. The computer triggered the valves and stored the time information about the occurrence of R-wave for the off line calculation of heart rate.

Experimental rrocedure

All subjects underwent a clinical investigation in order to exclude subjects who showed any cardiovascular risk. The electrodes and the neck cuff were attached to the subjects. Additionally a finger tip electrode was placed to the left middle finger in order to present weak electric stimuli for testing detection thresholds. The results involving this part are described elsewhere and are not reported in the present paper. The subjects were instructed to sit quietly during the experiment. First the PRES technique was carried out: 128 trials each lasting 9 s, were presented the subjects. Half of these trials were baroreceptor stimulation trials (systolic negative/diastolic positive pressure), the remaining half

were control trials (systolic positive/diastolic negative pressure). The sequence of the trials were pseudo-randomized. Intervals between trials has a variable duration between 9 and 14 s. After the PRES phase, a break between 30 and 45 min was held. Then, the continuous neck suction phase was presented with the same design as the PRES phase with the exception that the stimulation trials consisted of a 6 s negative pressure period and the control trials of a 6 s positive pressure period.

Statistical analyses

Heart rate data were averaged, separated for subjects and conditions. For the resulting averages, scores were built as the mean change in heart rate during the 6 s stimulation period referred to the 2 s pre-trial baseline. The data were entered into an ANOVA consisting of the factors METHOD (PRES vs. continuous neck suction technique) and CONDITION (stimulation vs. control trial).

Results I

The ANOVA revealed a significant CONDITION effect $F(1, 9) = 57.7$; $p < 0.0001$) indicating a much more pronounced heart rate deceleration during the stimulation (−5.9 bpm) compared to the control trials (−0.6 bpm). Additionally, the interaction between CONDITION and METHOD was significant ($F(1, 9) = 13.0$; $p < 0.01$). Post hoc tests revealed that heart rate responses were highly significantly different between stimulation and control trials for both methods. There was a borderline significant difference between both methods in regards to the heart rate deceleration elicited by the stimulation trials ($F = 4.7$; $p < 0.1$) indicating a slightly larger deceleration in response to the continuous neck suction trials (−6.3 bpm) compared to the PRES stimulation trials (−5.5 bpm). Both methods were substantially different in regard of the heart rate responses elicited by the control trials ($F = 8.6$; $p < 0.05$): The continuous neck suction control trials did not changed heart rate (−0.0 bpm), whereas the PRES control trials reduced heart rate slightly (−1.2 bpm) (Fig. 1).

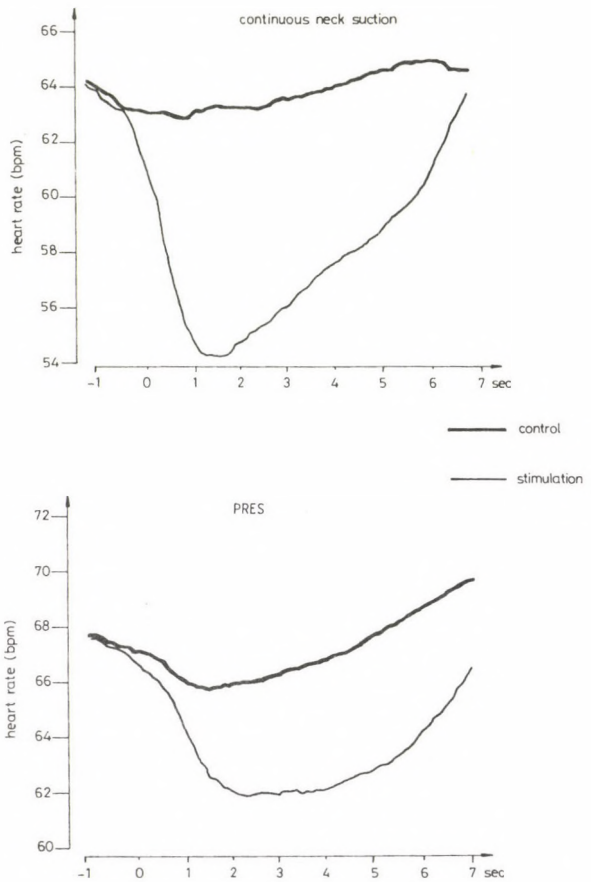

Fig. 1. The heart rate responses separated for both methods and conditions are presented. There was significant difference between the two methods regarding the heart rate decelerations elicited by the stimulation trials indicating a slightly larger deceleration in response to the continuous neck suction trials compared to the PRES stimulation trials. The continuous neck suction control trials did not change heart rate, whereas the PRES control trials reduced heart rate

Discussion I

Both baroreceptor stimulation methods had clear effects on heart rate. heart rate decelerates for more than 5.5 bpm in response to the baroreceptor stimulation trials. There was a small advantage for the continuous neck suction technique over the PRES method. Especially in the control condition, the continuous neck suction method had the advantage of producing no heart rate changes. The PRES control condition, on the other hand, produces slight heart rate decreases. Inspection of Figure 1, however, shows that both methods produce different shapes in heart rate

response: The continuous neck suction method produces a strong initial decrease in heart rate followed by a rapid recovery within the ongoing stimulation period. At the end of the stimulation, the baseline level has been reached. The PRES stimulation method, on the other hand, produces less rapid decreases with a smaller maximum decrease compared to the continuous neck suction technique. The heart rate decrease, however, remained more stable throughout the whole period of stimulation. This might be due to the fact that rhythmical stimulation within the heart cycle as applied with the PRES method is a more natural stimulus as compared to the longer lasting changes as applied with the continuous method. The variation of baroreceptor activity as induced by the PRES method seems to prevent habituation much more than the other method.

Experiment II

The aim of Experiment II was to compare both baroreceptor manipulation methods regarding their influence on heart rate responses as well as regarding their impact on arterial blood pressure. In order to study blood pressure responses to both methods, blood pressure was measured continuously intraarterially and non-invasively using the Finapres 2300 device.

Methods II

Subjects

All four subjects were male staff members in the age range from 32 to 51 years (mean = 39.7). In these subjects, systolic blood pressure ranged from 110 to 130 mm Hg (mean = 122.5), diastolic blood pressure from 70 to 80 mm Hg (mean 77.5). The subjects received a honorarium of 300 German Marks for their participation.

Baroreceptor stimulation

As in Experiment I, the PRES method and the continuous neck suction method were presented to the subjects.

Physiological recordings

In order to screen subjects who could be punctured for the invasive arterial blood pressure measurement, an Allen test evaluating the efficacy of the collateral of the hand was performed. Subjects with positive results were excluded. In the remaining subjects, the arteria radialis of the left forearm was punctured: the site for the puncture was the most proximal point where the artery was easily palpable. Local anesthetic solution (1 cc 0.5% lidocain sc.) was applied. The a. radialis was punctured and the cannula was introduced with Seldinger technique. A combitrans one way pressure transducer (BRAUN) was connected to the cannula. The signal was amplified by a HELLIGE polygraph. The amplified signal was analogue to digital converted with a sampling rate of 100 Hz and stored on the computer's hard disc for off line analysis. Additionally to the invasive blood pressure measurement, a non-invasive estimate of

finger arterial blood pressure was performed using the OHMEDA Finapres 2300 device [14]. Systolic, diastolic, and mean blood pressure were off line calculated from the digitized signals: The mean of the last 20 ms before the detection of the R-wave in the electrocardiogram was calculated as diastolic blood pressure. The maximum value within one heart cycle was taken as systolic blood pressure. The mean of the blood pressure curve within one heart cycle was calculated as mean blood pressure. These values were calculated for every cardiac cycle and averaged, separately for the baroreceptor manipulation conditions.

Procedure

The procedure was comparable to that in Experiment I with the exception that the subjects had no task during the experiment and there was no electrical stimulus presented to them.

Statistical analyses

Scores from heart rate and the blood pressure variables were built on a second by second basis beginning with second 0 (the first second after beginning of the stimulation) going to second 5 (the last second within the stimulation interval). The data were entered into an ANOVA consisting of the factors SECONDS (second 0 to second 5), METHOD BARO (PRES vs. continuous neck suction), CONDITION (stimulation vs. control trials), and METHOD BP (intraarterial blood pressure vs. Finapres). Where appropriate, Geisser-Greenhouse epsilon values were applied to correct for lack of sphericities in the covariance matrices involving within-subject factors with levels exceeding two (here SECONDS).

Results II

Heart rate

The ANOVA calculated a significant CONDITION × SECONDS interaction ($F(5, 15) = 2.9$; $p < 0.05$) which is illustrated in the upper part of Figure 2: Heart rate decreases during the stimulation trials but remained on baseline level during the control trials.

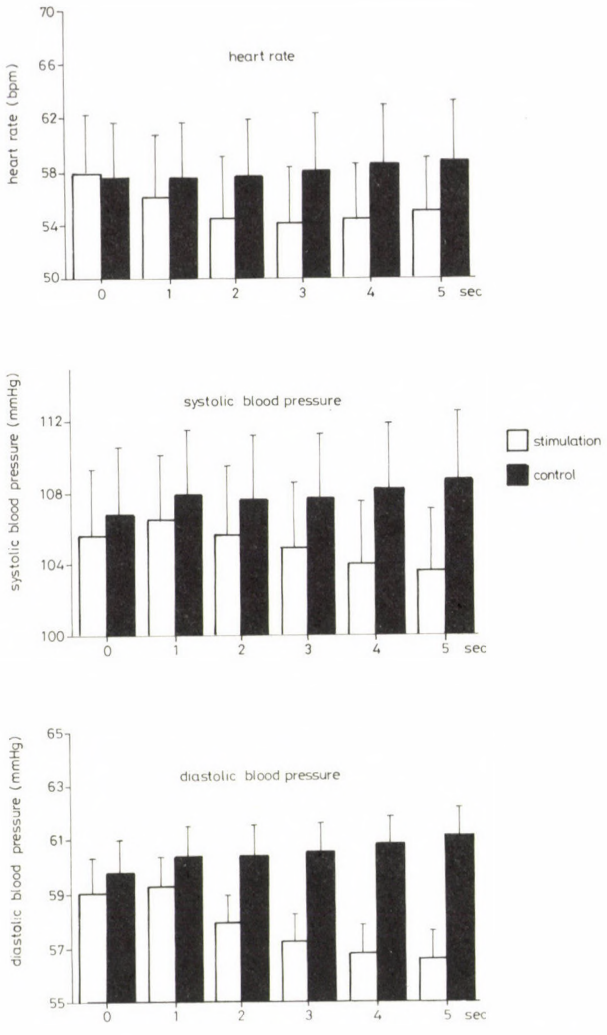

Fig. 2. Heart rate, systolic and diastolic blood pressure responses are shown. The heart rate decreased during the stimulation trials but remained on baseline level during the control trials. Systolic blood pressure was significantly lower during the stimulation as compared to the control trials. The interaction between the stimulation methods and conditions proved to be significant indicating a more pronounced differentiation in systolic blood pressure between the stimulation and control conditions using the continuous neck suction technique as compared to PRES. Diastolic blood pressure decreases significantly over the seconds as response to the stimulation trials and increases slightly in response to the control trials. There were no differences between the two blood pressure measurements techniques

Systolic blood pressure

The ANOVA confirmed a significant CONDITION effect ($F(1, 3) = 16.2$; $p < 0.05$): Systolic blood pressure was significantly lower during the stimulation as compared to the control trials. The interaction between METHOD BARO and CONDITION proved to be significant, too ($F(1, 3) = 11.2$; $p < 0.05$), indicating a more pronounced differentiation in blood pressure between both CONDITIONs using the continuous neck suction technique as compared to PRES. Finally, the interaction between METHOD BARO, SECONDS, and CONDITION was significant ($F(5, 15) = 5.1$; $p < 0.05$).

This effect is described in Fig. 3: The blood pressure curves differentiation across SECONDs differs more between the CONDITIONs in the continuous neck suction technique as compared to the PRES method. There were no ANOVA effects including the factor METHOD BP.

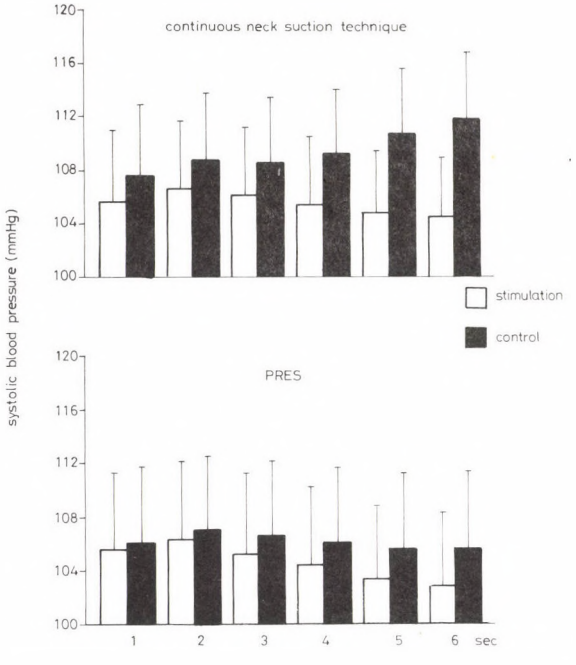

Fig. 3. The interaction between the baroreceptor manipulation methods, stimulation time, and conditions was significant regarding the systolic blood pressure. The blood pressure curves differentiation across SECONDs differs more between the stimulation and control conditions in the continuous neck suction technique as compared to the PRES method. There were no differences between the two blood pressure measurement techniques

Diastolic blood pressure

For the diastolic blood pressure, the ANOVA calculated a significant CONDITION effect ($F(1, 3) = 9.4$; $p = 0.05$) due to the fact that the stimulation trials were associated with lower diastolic blood pressure compared to the control trials. The CONDITION × SECONDS interaction was significant, too ($F(5, 15) = 4.4$; $p = 0.05$). The lower part of Figure 2 describes this effect: diastolic blood pressure decreases over the SECONDS as response to the stimulation trials and increases slightly in response to the control trials. There were no ANOVA effects including the factor METHOD BP.

Discussion II

Despite the fact that the number of subjects who have been investigated was quite low, the results of the present experiment verified that both methods, the PRES method and the continuous neck suction method, had a reliable impact on both, heart rate and blood pressure responses. Neither for heart rate nor for diastolic blood pressure responses, could be found a significant difference between the two baroreceptor manipulation methods. There was, however, a difference between both methods on the systolic blood pressure: The continuous neck suction technique evoked clear decreases in systolic blood pressure in response to the stimulation trials and, opposite to the PRES technique, it evoked a substantial increase in systolic blood pressure in response to the control trials.

Thus, it seems to be clear that the control trials in the continuous neck suction technique were not real control trials but inhibition trials: The inhibitory effect on the baroreceptor activity evoked blood pressure increases. Systolic blood pressure responses in the PRES condition were only unidirectional: Whereas the control trials do not evoke significant changes in blood pressure stimulation trials elicit blood pressure decreases. The slight, but not significant increase in both diastolic and systolic blood pressure observed in the first post-stimulation second can be due to the delay of the vasodepressor effect in response to baroreceptor stimulation.

In the PRES method, the control trials seemed to be real control trials without any substantial effects on systolic blood pressure. The number of subjects is, however, too small to draw final conclusions from this experiment. Further investigations seem to be necessary to evaluate whether the effects reported here remain stable. If this would be the case, the application of PRES could be recommended if the application of a real control of placebo condition is to be achieved. The continuous neck cuff technique, on the other hand, seems to be advantageous if a real baroreceptor inhibition is necessary.

Additionally, the absence of any ANOVA effects including the factor METHOD BP confirm the non-invasive Finapres blood pressure method: Invasive recordings of phasic blood pressure responses are not substantially different from non-invasive recordings.

General discussion

Data from Experiment I suggest that the PRES method and the continuous neck suction method have different impact on the shape of heart rate responses: The control trials did not reliably influence heart rate in any of the methods. Stimulation trials, on the other hand, elicited sudden and strong heart rate decreases in the continuous neck suction technique.

After the second or third heart beat, however, heart rate tended to return to baseline due to habituation of the baroreceptors and/or counter regulation of the unloaded aortic baroreceptors. At the end of the 6 s stimulation period heart rate has nearly approached baseline level. The stimulation trials in the PRES method, however, seem to produce a slow but consistent heart rate deceleration which is less than that in the continuous technique. The heart rate remains low during the whole period of stimulation. The pulsating technique seems to prevent habituation. Data from Experiment II suggest that both, the PRES and the continuous neck suction technique, evoke blood pressure responses which indicate effective baroreceptor manipulation. Systolic blood pressure responses indicate that the continuous neck suction control trials inhibit baroreceptors, whereas the PRES control trials have no effect on systolic blood pressure. Finally, the absence of ANOVA effects including the factor "METHOD blood pressure" indicate that the non-invasive Finapres blood pressure device measures blood pressure reliably and shows no dramatic differences to blood pressure values measured invasively.

REFERENCES

1. Dworkin, B.: Hypertension as a learned response: The baroreceptor reinforcement. In: T. Elbert Langosch, W., Steptoe, A., Vaitl, D. eds., Behavioural medicine in cardiovascular disorders. Chichester: John Wiley & So. 1988, pp. 17–47.
2. Dworkin, B., Filewich, R. J., Miller, N. E., Craigmyle, N., & Pickering, T. G.: Baroreceptor activation reduces reactivity to noxious stimulation: Implications for hypertension. Science, **205**, 1299–1301 (1979).
3. Eckberg, D. L.: Temporal response patterns of the human sinus node to brief carotid baroreceptor stimuli. Journal of Physiology, **258**, 769–782 (1976).
4. Eckberg, D. L., Cavanaugh, M. S., Mark, A. L., & Abboud, F. M.: A simplified neck suction device for activation of carotid baroreceptors. Journal of Laboratory and Clinical Medicine, **85**, 167–173 (1975).

5. Furedy, J., Rau, H. & Roberts, L.: Physiological and psychological differentiation of bidirectional baroreceptor carotid manipulation in humans. Biological Psychology (in press).
6. Furedy, J. J., & Poulos, C. X.: Heart rate decelerative Pavlovian conditioning with tils as UCS: Towards behavioural control of cardiac dysfunction. Biological Psychology, **4**, 93–106 (1976).
7. Gellhorn, E., Yesinick, L., Kessler, M. & Hailman, H.: Carotid sinus reflexes and convulsions. American Journal of Physiology, **137**, 396–403 (1942).
8. Koch, E. B.: Die irradiation der pressorezeptorischen Kreislaufreflexe. Klinische Wochenschrift, **2**, 225–227 (1932).
9. Lacey, B. and Lacey, J.: Two-way communication between the heart and the brain: Significance of time within the cardiac cycle. American Psychologist, **33**, 99–113 (1978).
10. Mancia, G.: Reflex control of circulation in normotensive and hypertensive humans. In: Ganten, D., Pfaff, D. eds. Central Cardiovascular Control. Spriger-Verlag, Berlin 1983, pp. 63–86.
11. Rau, H., Brody, S., Droste, C. & Kardos, A.: Blood pressure changes validate phase related external suction, a controlled method for stimulation of human baroreceptors. European Journal of Applied Physiology, **67**, 26–29 (1993).
12. Rau, H., Elbert, T., Geiger, B. & Lutzenberger, W.: PRES: The controlled noninvasive stimulation of the carotid baroreceptors in humans. Psychophysiology, **29**, 165–172 (1992).
13. Rau, H., Elbert, T., Lutzenberger, W., Eves, F., Rockstroh, B., Larbig, W. & Birbaumer, N.: Pavlovian conditioning of peripheral and central components of baroreceptor reflex. Journal of Psychophysiology, **2**, 119–128 (1988).
14. Settels, J. J. & Wesseling, K. H.: Noninvasive finger arterial pressure waveform registration. In: Orlebeke, J. F., Mulder, G., van Doornen, L. J. P. eds: Psychophysiology of cardiovascular control. Models, Methods, and Data. Plenum Press, New York 1985 pp. 267–284.
15. Thron, H. L., Brechmann, W., Wagner, J. & Keller, K.: Quantitative Untersuchungen über die Bedeutung der Gefäßdehnungs-rezeptoren im Rahmen der Kreislaufhomöostase beim wachen Menschen. Pflügers Arhiv, **293**, 68–99 (1967).
16. Toon, P. D., Bergel, D. H. & Johnston, D. W.: The effect of modification of baroreceptor activity on reaction time. Psychophysiology, **21**, 486–494 (1984).

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Gas chromatographic procedure for simultaneous determination of Selegiline metabolites, amphetamine, methamphetamine and demethyl-deprenyl in pig plasma

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A sensitive assay for the simultaneous quantitative determination of amphetamine, methamphetamine and demethyl-deprenyl in pig plasma is described. NP-Gas chromatography is used to determine the extracted plasma concentrations of the three target compounds as their N-penta-fluoro-benzoyl derivatives. Quantitation is performed using 1-phenyl-2-pentylamine as internal standard. The derivatives are separated on a phenyl-methyl-silicone capillary column. Quantitation limit for each target compound was 1.2 ng ml^{-1}. Levels of amphetamine, methamphetamine and demethyldeprenyl have been determined in plasma of pigs treated with Selegiline in different formulations and doses.

Keywords: selegiline, amphetamine, methamphetamine, demethyl-deprenyl, GC-NPD procedure

Selegiline [(−)-deprenyl HCl; N-methyl-N-2-propynyl-1-phenyl-2-propylamine hydrochloride) is a selective monoamine oxidase type B inhibitor that has been proven effective in the treatment of Parkinson's disease [1–3] and has shown considerable promise in alleviating the symptoms of Alzheimer-type dementia [4–6]. In vivo metabolic studies [7, 8] demonstrate that the main metabolic pathway of Selegiline is N-dealkylation. It is mainly and efficiently metabolised to demethyl-

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deprenyl (DMD; 1-phenyl-2-(N-2-propynyl)amino propane) and methamphetamine (MA; 1-phenyl-2-methyl-aminopropane) by oxidative demethylation and depropynylation, respectively. DMD and MA are then further metabolised to amphetamine (A; 1-phenyl-2-aminopropane) by oxidative depropynylation and demethylation, as summarised in Figure 1.

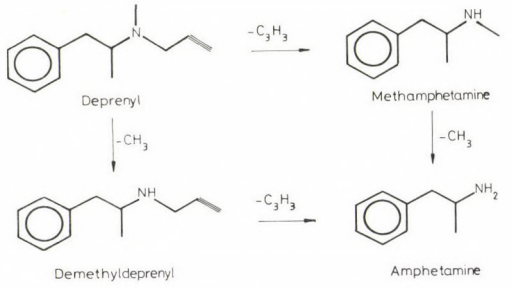

Fig. 1. Metabolites formed by N-dalkylation of Selegiline

Each of these metabolites, which are excreted in the urine, has some biological activity. Determination of metabolite concentrations after Selegiline administration would provide information on their possible contribution on the observed drug effects. Previous pharmacological, pharmacokinetic and clinical studies have indicated that the favourable clinical effect of Selegiline in Parkinson's disease is not exclusively due to the selective inhibition of MAO-B but the inhibition of the uptake of biogenic amines and potential neurotoxins also plays an important role. This action could be related to the uptake inhibitor potential of metabolites [9–11]. While a single peak concentration of the parent compound is enough to produce irreversible MAO-B inhibition i.e. a long lasting (and species dependent) decrease in MAO-B activity, the reversible uptake inhibition requires a steady-state concentration of Selegiline metabolites, primarily methamphetamine and amphetamine. So as to fulfil both requirements simultaneously it seemed appropriate to develop a biphasic dosage form. The probable plasma concentrations of A, MA, and DMD are to be expected in the ng ml^{-1} concentration range. For this reason, a sensitive and specific method was required to assess the bioavailability of novel Selegiline formulations accurately. Previously, several procedures were developed for the quantitation of amphetamine and its analogues, including immunoassay techniques [12], GC with nitrogen-phosphorus detection [13], GC with mass spectrometric (MS) detection [14] high performance liquid chromatography [15], as well as a variety of derivatizing procedures for GC with ECD detection [16–17]. Methods describing the determination of A and MA in biological fluids are numerous, although only few can claim limits of quantitation below 5 ng ml^{-1}. Many

of these methods previously employed have insufficient selectivity and/or sensitivity for simultaneous analysis in body fluids of these metabolites. MS procedures, although highly sensitive and specific, are often outside the financial limits of small laboratories and require highly trained personnel. In the present report we describe a GC-NPD procedure for the simultaneous determination of plasma levels of the N-dealkylated metabolites of Selegiline (A, MA and DMD) as their N-pentafluorobezoyl derivatives. Plasma was taken from pigs treated orally with Selegiline in a traditional tablet form and with a new dosage forms, capsule containing two sorts of pellets (minigranules providing immediate and sustained release of Selegiline). Pig was chosen because according to relevant studies only this species has MAO activity (their platelets contain measurable level of MAO-B activity [18]) and a physiology very similar to that of humans.

Materials and Methods

Materials

Standard materials, (−)amphetamine hydrochloride, (−)methamphetamine hydrochloride, (−)demethyl-deprenyl hydrochloride and 1-phenyl-2-pentyl-amine hydrochloride (ISTD – internal standard) were obtained from Synthetic Research Laboratory IV, Chinoin (Budapest). 2,3,4,5,6-penta-fluoro-benzoyl-chloride (PFBCl was obtained from Fluka Chemie AG). Further chemicals were of the highest purity available.

Sample preparation and extraction

To 1 ml plasma 40 μl of ISTD solution (0.5 ng μl^{-1} in bidistilled water), 500 μl of 10 N NaOH solution, 700 μl saturated solution of NaCl, and finally 7 ml n-hexane were added then rotated for 20 min. with a Glas-Col rotator. After centrifugation at 3000 rot min^{-1} until 10 min the organic phase was transferred into a conically shaped 15 ml test tube and pentafluoro-benzoyl derivatives were prepared by adding 100 μl of 0.5% PFBCl in dichloromethane. The reaction was complete in one hour at room temperature. After the end of the reaction time, the solvent was evaporated under reduced pressure, and the samples were reconstituted with 40 μl toluene by vortexing for 1 min and 3 μl was injected into the gas chromatograph.

Gas chromatography

GC/NPD analyses were performed using an HP 5890 gas chromatograph (Hewlet Packard), equipped with NP selective detector. Samples were introduced by splitless injection (3 μl, delaying time 1 min) from an HP 7673A auto injector onto a fused silica HP−2 capillary column (25 m × 0.2 mm; 0.11 μm film of a cross-linked 5% Ph-Me-silicone as stationary phase; Hewlet Packard, USA). The initial oven temperature was 90 °C for 0.95 min. First heating up to 100 °C at 10 °C min^{-1} was followed immediately by a second heating up to 190 °C at 5 °C min^{-1} and it was maintained for 6 min. A third heating up to 280 °C at 70 °C min^{-1} was followed by a waiting time of 3 min. The injector port and detector temperatures were maintained at 250 °C and 300 °C respectively. Both the carrier and make-up gas was high purity nitrogen at a column head pressure of 15 psi, and 20 ml min^{-1}, respectively. Detector

gases were hydrogen at a flow rate of 3.8 ml min^{-1} and air at a flow rate of 100 ml min^{-1}. Under the above conditions the compounds eluted with the following retention times: amphetamine at 21.4 min, methamphetamine at 21.6 min, demethyldeprenyl at 24.0 min and internal standard at 25.6 min after injection. Seven separate sets of calibration standards (1.5, 3, 5, 10, 30, 50 and 70 ng ml^{-1}) were analysed on seven days for determination of inter-day accuracy and precision. The intra-day validation was performed then a set of calibration standards and five replicate control samples at six levels was analysed. The peak height ratios of the test compounds and the corresponding ISTD were calculated for the quantitation. The concentrations of metabolites were determined for each individual standard data point using the slope and intercept of the associated calibration curve. The calibration curve was obtained after a logarithmic transformation of the calibration data, peak height ratio (metabolite/ISTD) versus known metabolite plasma concentration, and was fitted to the straight lines the calibration points using the least squares method. This assay method has been applied to determination of A, MA, DMD in plasma of pigs treated with oral dose of Selegiline in the form of traditional tablet and with the same oral dose in the form of capsule, containing pellets providing immediate and sustained release form of Selegiline.

Animal experiments

Female "Hungarian Big White" Kahyb pigs were used for the experiments in three groups (3 animals each, one group as control). The weight of the animals were in the range of 20–22 kg, age approx. 10–12 weeks. Pigs were housed individually in separated boxes and the animal-room was climatized (20 °C±3, 50–70% humidity). For measurement of target metabolites cca. 5 ml blood samples – through v. cava cranialis – were taken into centrifuge tubes containing 500 IU heparin for analytical measurement. Plasma was separated after centrifugation at 3000 rpm for 10 min, and was ampoulled and stored at −20 °C till the measurement of metabolites. Following a blank sample, blood samples were taken at 1, 2, 4, 8, 10, 12 and 14 hours after oral administration of Selegiline. Plasma levels of the metabolites were compared after a single 0.5 mg·kg^{-1} oral dose of Selegiline tablets (Chinoin) and after single 0.5 mg·kg^{-1} oral dose – 0.25 mg·kg^{-1} in immediate release and 0.25 mg·kg^{-1} in sustained release form Selegiline (Sanofi-Winthorp, D'Ambares) in capsule.

Results and Discussion

The method is selective for the three metabolites in pig plasma because no other metabolites of Selegiline, or endogenous compounds interfered with the test compounds. The intra-day precision of the three test materials, showed by the average CV% values proved to be 12.60%, 12.32% and 8.11% for amphetamine, methamphetamine and demethyldeprenyl, respectively and even the highest CV% value at the lowest calibration point did not reach 20%. The inter-day precision for the three test compounds showed by the average CV% values, proved to be 12.6%, 12.32% and 8.11% for amphetamine, methamphetamine and demethyldeprenyl, respectively and even the highest CV% value at the lowest calibration point did not reach 20%. The average deviation % values for inter-day accuracy determination were 4.11% for amphetamine, 4.18% for methamphetamine, and 3.02% for demethyl-deprenyl and not even the highest values reached 10%. The average

deviation % values for intra-day accuracy were 4.14%, 3.93% and 4.62% in case of amphetamine, methamphetamine and demethyl-deprenyl, respectively and even the highest values hardly exceeded 10%. Based on values of precision and accuracy the limit of quantitation of the method is 1.5 ng ml^{-1} calculated for hydrochloride form of the test materials which corresponds to 1.2 ng ml^{-1} base of the compounds. The range of the calibration curves for 1 ml plasma was 1.5 to 70 ng ml^{-1} for the hydrochlorides of the test materials which corresponds to 1.2 to 55–57 ng ml^{-1} base. Figure 2 depicts plasma concentrations of methamphetamine (the main metabolite) versus time profile. These data were obtained by the analysis of pig plasma after administering the single 0.5 mg kg^{-1} oral dose of Selegiline applied in traditional tablet form and in capsule containing the two kinds of traditional Selegiline formulations (immediate and sustained release). As Figure 2 shows, the capsule provided prolonged active ingredient release, two concentration maximums (the tablet had one C_{max}), a somewhat lower peak concentration, than the tablet, and steady plasma levels over a 10-hour period.

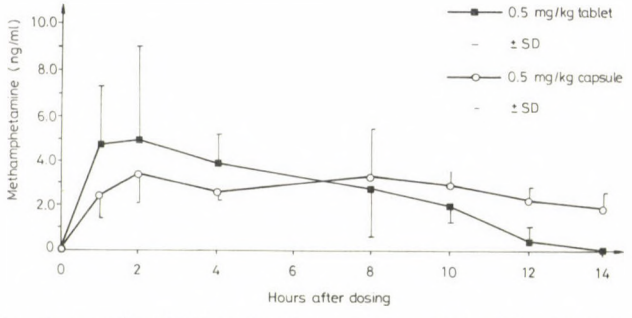

Fig. 2. Concentration-time profile of MA in the plasma of pigs given a 0.5 mg/kg oral dose of Selegiline applied in traditional tablet form and in capsule containing the two formulations of Selegiline (immediate and sustained release)

In Table I we present the pharmacokinetic parameters obtained from non-compartimental analysis of the data.

After administration of the tablet C_{max} was higher, than after administration of the capsule, whereas the T_{max} values (first value of the capsule) did not differ. In the case of the capsule the concentration level of the metabolite reached a second maximum at 8 hours after the administration. The AUC values showed significant difference neither in the 0–14 hour nor in the 0-infinity periods. Significant differences could be observed though in the case of terminal elimination rate constant, and half life. The MRT_{0-inf} values were found to be different, but these differences were not significant. From the results described above we can conclude, that with the tested capsule (a combination of immediate and sustained release formulations) it was possible to obtain long-lasting concentration levels of

methamphetamine. This measurement demonstrate the potential of the assay for mapping the Pharmacokinetic distribution of Selegiline metabolites in pigs in case of the ng ml^{-1} concentration range of metabolites in plasma.

Table I

Mean values of pharmacokinetic parameters (n = 3, ±SD) calculated by Non-Compartmental analysis of concentration data obtained for MA in the plasma of pigs given a 0.5 mg/kg oral dose of Selegiline applied in traditional tablet form and in capsule containing the two formulations of Selegiline (immediate and sustained release)

Selegiline Dose	0.5 (mg/kg)	0.25 (mg/kg) in imediate release and 0.25 (mg/kg) in sustained release formulations
Formulation	tablet	capsule
Mean values of pharmacokinetic parameters (n = 3, ±SD)		
C_{max} (ng/ml)	4.9±3.8	3.4±0.7
T_{max} (hour)2.0±1.5	2.0±1.2	
Term. elim. rate (lambda) (1/hour)	0.216±0.03	0.122±0.04
$t_{1/2}$ 3.2±1.0	5.7±2.4	
$AUC_{(0-14)}$ (ng*hour/ml)	37.1±22.7	30.9±9.5
$AUC_{(0-inf)}$ (ng*hour/ml)	39.2±27.6	36.1±15.9
$MRT_{(0-inf)}$ (hour)	5.8±1.2	8.4±2.6

REFERENCES

1. Ambrozi, L., Birkmayer, W., Reiderer, P. L., Youdim, M. B. H.: Implications of combined treatment with Medopar and l-deprenyl in Parkinson's disease. Lancet, **1**, 439 (1977).
2. Elsworth, J. D., Kohout, L. J., Lees, A. J., Shaw, K. M., Sandler, M., Stern, G. M., Youdim, M. B. H.: Deprenyl in Parkinson's disease. Lancet, **2**, 791 (1977).
3. Giovanni, P., Martignoni, E., Piccolo, I. et al.: (−) Deprenyl in Parkinson's disease: a two year study in the different evolutive stages. Journal of Neural Transmission, **22** (Suppl.), 235–246 (1986).
4. Cohen, R. M., Mellow, A. M., Mueller, A. A., Murphy, D. L., Newhouse, P. A., Tariot, P. N., Sunderland, T., Weingartner, H., Yount, D.: L-deprenyl in Alzheimer's disease. Preliminary evidence for behavioural change with monoamino oxidase B inhibition. Arch. Gen. Psychiatry, **44**, 427 (1987).
5. Finali, G. C., Piccinin, G. L., Piccirilli, M.: Neuropsychological effects of L-deprenyl in Alzheimer's type dementia. Clin. Neuropharmacol., **13**, 147–163 (1990).
6. Frattola, L., Grassi, M. P., Mangoni, A.: Effects of a MAO-B inhibitor in the treatment of Alzheimer's disease. European Neurology, **31**, 100–107 (1991).

7. Dobó, R., Hollósi, G., Kalász, H., Kerecsen, L., Knoll, J., Pucsok, J.: Studies on the metabolism of (−) Deprenyl by gas chromatography mass spectrometry, Chromatography' 87, Kalász, H. and Ettre, L. S. (Eds.), Akadémiai Kiadó, Budapest, 1988, pp. 275–283.
8. Szatmári, I., Tóth, K.: Pharmacokinetics and metabolism of Selegiline. Acta Pharmaceutica Hungarica, **62**, 243–248 (1992).
9. Magyar, K.: Monoamine Oxidases and their Selective Inhibition ed. Magyar, K., Pergamon Press, Akadémia Kiadó, Budapest 1980, p. 11–21.
10. Hársing, L. G., Magyar, K., Tekes, K.: Inhibition by deprenyl of dopamine uptake in rat striatum: a possible correlation between dopamine uptake and acetylcholine release inhibition. Polish Journal of Pharmacology, Biochemistry and Behaviour, **15**, 297–307 (1979).
11. Elsworth, J. D., Sandle, M., Lees, A. J.: The contribution of amphetamine metabolites of (−)deprenyl to its antiparkinsonian properties. Journal of Neural Transmission, **54**, 105–110 (1982).
12. Ebert, M. H., Schmidt, D. E.: Neuromethods, Vol. 10, Analysis of Psychiatric Drugs, eds Boulton, A. A., Baker, G. B., Coutts, R. T., Humana Press Clifton, NJ 1988, p. 241.
13. Jones, G. R., Singer, P. O.: Neuromethods, Vol. 10, Analysis of Psychiatric Drugs, eds Boulton, a. A., Baker, G. B., Coutts, R. T., Humana Press Clifton, NJ 1988, p. 303.
14. Mamer, O. A., Reimer, M. L. J.: Determination of amphetamine, methamphetamine and demethyldeprenyl in human plasma by gas chromatography/negative ion chemical ionisation mass spectrometry. Biol. mass Spectrum., **22**, 235–242 (1993).
15. Jane, I.: Determination of methamphetamine, amphetamine and piperidine in human urine by HPLC with chemiluminescence, J. Chromat. Chromatogr., **31**, 459–466 (1990).
16. Salonen, J. S.: Determination of the amine metabolites of selegiline in biological fluids by capillary gas chromatography, J. of Chromatogr. Biomed. Appl., **527**, 163–168 (1990).
17. Baker, G. B., Caffaro, L. E., Coutts, T. R., Greenshaw, A. J., Paetsch, P. R., Rauw, G. A.: Electron-capture gas chromatographic procedure for simultaneous determination of amphetamine and N-methamphetamine. J. of Chromatogr. Biomed. Appl., **573**, 313–317 (1992).

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Distribution and elimination of RGH-5002 in rats

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In the present study distribution and elimination of RGH–5002 – a new centrally acting muscle relaxant – were investigated in rats by using ^{14}C-labelled compound. Whole-body autoradiography and quantitative determination of the radioactivity in various organs following single and repeated oral administration of [^{14}C]RGH–5002 demonstrated extensive distribution of the drug with high levels in the gastrointestinal tract, kidneys, liver, endocrine and exocrine glands and lungs. Minimal accumulation was observed after repeated (8 days) administration. The same distribution characteristics were observed in both sexes. In pregnant rats radioactivity appeared in the placenta and fetal tissues. Elimination was investigated by measuring radioactivity in 24 h fractions of urine and faeces after single dose administration of the drug. The larger portion of radioactivity was excreted in the urine (81.67 ± 1.61% of the dose). The faecal recovery was 11.12 ± 1.19% of the administered dose. Approximately 80% of the excreted radioactivity was recovered within the first 24 hours.

Keywords: RGH–5002, rat, distribution, excretion

RGH–5002 (N-/[dimethyl-4-fluor-benzyl-silil]-methyl/-piperidine hydrochloride) is a new muscle relaxant acting in the central nervous system. It is being developed by Chemical Works of Gedeon Richter Ltd. On the basis of pharmacological studies muscle relaxant activity of RGH–5002 is similar to tolperisone but has a longer duration of action and much better oral activity. It presumably will have less CNS depressant and motor side effects in human patients as compared to other central muscle relaxant drugs [1].

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The aim of the present study was to investigate the distribution and elimination of the drug in rats and to obtain information on possible accumulation, difference between sexes and penetration through the blood-brain barrier and placenta. Investigations involved single and repeated dose whole-body autoradiography studies on both sexes and a single dose study on pregnant dams, single and repeated dose tissue distribution studies based upon quantitative determination of radioactivity in various organs and tissues on male rats, and a single dose mass balance study on male rats.

Materials and Methods

The radiolabelled compound was prepared by Central Research Institute for Chemistry, Hungarian Academy of Sciences with specific activity of 607 MBq/mmol, and chemical and radiochemical purity > 98% (Fig. 1).

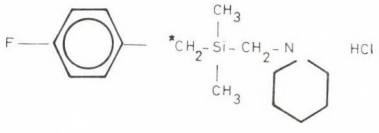

Fig. 1. Chemical structure of [^{14}C]RGH-5002

Healthy Wistar rats were used in the studies:
in whole-body autoradiography:
5 males (200-260 g)
5 females (120-160 g)
2 pregnant females (250-260 g)
(obtained from LATI Ltd., Gödöllő, Hungary)
in tissue distribution study:
12 males (220-320 g),
in mass balance study:
4 males (160-180 g)
(obtained from Lab-Tech Ltd., Charles River Laboratories, Budapest, Hungary)
Animals were fed by 'Altromin' standard rodent diet (LATI Ltd.), and had free access to water.

The dose administered to rats was 30 mg/kg (the highest dose without toxic effects). The radioactive dose was 11.2 MBq/kg in whole-body autoradiography, 6.0 MBq/kg in tissue distribution study, and 2.4 MBq/kg in mass balance study. Rats were treated orally with an aqueous solution of [^{14}C]RGH-5002 by gavage. In repeated dose studies animals were administered the compound once a day on 8 consecutive days. Pregnant rats were treated on the 19th day of gestation.

Whole-body autoradiography was performed in male and female rats at 1, 4, 8 and 24 h after single and 24 h after multiple administration, and in pregnant dams at 1 and 4 h after single dosing. At scheduled time points animals under chloroform anaesthesia were freezed in n-hexan cooled with solid CO_2 (-70 °C) and were embedded in carboximethylcellulose gel. Sagittal 40 μm whole-body sections were taken by PMW 450 MP cryomicrotom (LKB, Sweden). Freeze-dried sections were left in contact with X-ray film (AGFA CURIX XP, Belgium). The exposure time was 5 weeks. The relative density of

organs and tissues observed in autoradiographic films was scored from 0 to 5. The high density in autoradiograms represents high level of radioactivity [3].

In *tissue distribution study* at 1, 6 and 24 h after single and 24 h after repeated dose administration 3 rats were sacrificed. Animals were exsanguinated under ether anaesthesia, then selected organs and tissues were taken by dissection. The samples were freeze-dried and homogenized. Two aliquots from each sample were combusted in a sample oxidizer and measured for radioactivity by liquidscintillation counting (1219 Rackbeta, LKB, Wallac, 'coctail A' = 5 g PPO + 0.1 g POPOP in 1 l toluol). Radioactivity of samples was expressed as dpm/mg of wet tissue. Compound related material (CRM) concentrations of organs were derived from the radioactivity values. Accumulation indexes were calculated as the ratio of 24 h-value after multiple administration to that of single dose treatment [2].

In the *mass balance study* animals were housed individually in stainless steel metabolic cages, which permitted the separate collection of urine and faeces. Collections were made in every 24 h up to 168 h post-dose. Cages were rinsed after each collection period and the cage-wash was added to the respective urine samples. Radioactivity of 5 aliquots was measured directly in 'coctail B' ('coctail A': abs. ethanol, 8:2 V/V). Faeces samples were freeze-dried and homogenized. Five aliquots were combusted in sample oxidizer and measured for radioactivity in 'coctail A'. After the last sample collection animals were killed and the carcasses were dissolved in 98% sulphuric acid. 5*80 – 100 µl aliquots were taken and measured for radioactivity after the addition of 300 µl of 30% H_2O_2, 300 µl isopropanol, 6 ml ethylcellosolve and 10 ml 'coctail C' (1 l 'coctail A' + 6 g PPO).

Results

Whole-body autoradiograms and quantitative determination of the radioactivity in various organs after single per os dose demonstrated extensive distribution of the compounds (Fig. 2). Table I presents the mean CRM concentrations of tissues and organs 1, 6 and 24 h after a single dose.

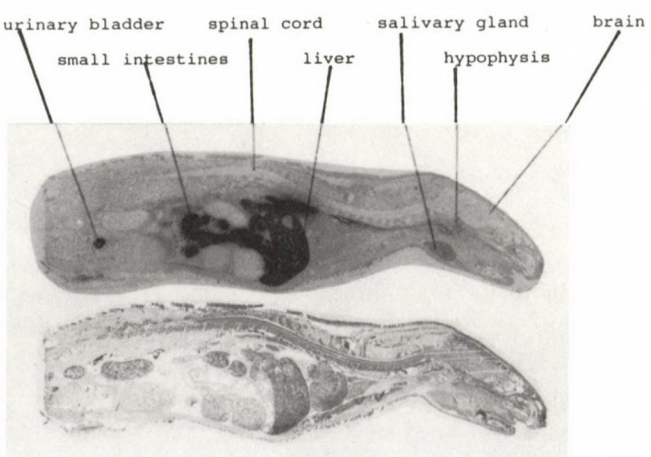

Fig. 2. Whole-body autoradiogram (up) and histological section (down) of a male rat 1 h after 30 mg/kg single oral dose of [^{14}C]RGH – 5002

Table I

*Mean CRM concentrations (ng equivalent/mg) at 1, 6 and 24 h after a single 30 mg/kg dose of [^{14}C]RGH – 5002 *one animal's value*

1 h		6 h		24 h	
organ	conc.	organ	conc.	organ	conc.
stomach	107.96 ± 3.58	liver	23.81 ± 1.23	liver	13.3 ± 3.07
small int.	98.91 ± 77.05	kidney	15.42 ± 5.14	large intestine	7.41 ± 5.69
kidney	62.55 ± 34.02	small int.	7.63 ± 3.33	kidney	6.67 ± 1.84
liver	61.44 ± 13.47	large int.	7.25 ± 5.45	small int.	4.77 ± 3.77
large int.	51.48 ± 60.60	pancreas	4.68 ± 0.79	thyroid	2.55*
thyroid	31.88 ± 23.57	salivary gl.	4.28 ± 3.62	pancreas	1.93 ± 0.21
lung	29.79 ± 8.95	stomach	3.39 ± 1.05	prostate	1.61 ± 0.66
prostate	28.52 ± 23.07	prostate	2.93 ± 1.51	adrenal	1.53 ± 0.17
adrenal	17.18 ± 1.51	plasma	2.74 ± 0.61	plasma	1.29 ± 0.26
pancreas	16.53 ± 2.75	adrenal	2.55 ± 1.04	salivary gland	0.98 ± 0.45
salivary gl.	15.90 ± 7.62	epididymis	2.55 ± 0.51	epididymis	0.92 ± 0.14
epididymis	15.34 ± 11.56	testis	2.43 ± 1.16	skin	0.80 ± 0.12
spleen	11.75 ± 4.44	lung	2.01 ± 0.14	stomach	0.75 ± 0.40
sem. ves.	11.51 ± 10.68	blood	1.68 ± 0.15	lung	0.63 ± 0.05
fat	10.07 ± 6.74	fat	1.66 ± 0.49	blood	0.61 ± 0.10
heart	8.41 ± 2.19	skin	1.41 ± 0.37	fat	0.50 ± 0.06
brain	8.00 ± 3.57	sem. ves.	1.28 ± 0.14	sem. ves.	0.43 ± 0.16
thymus	6.28 ± 2.29	spleen	1.04 ± 0.18	spleen	0.43 ± 0.07
plasma	5.98 ± 0.49	brain	0.89 ± 0.45	testis	0.37 ± 0.04
skin	5.79 ± 1.22	thyroid	0.86 ± 0.23	bone	0.34 ± 0.02
blood	4.89 ± 0.35	thymus	0.83 ± 0.16	heart	0.33 ± 0.04
muscle	4.78 ± 0.92	heart	0.82 ± 0.11	thymus	0.31 ± 0.01
testis	4.68 ± 2.76	muscle	0.59 ± 0.11	muscle	0.22 ± 0.04
bone	4.15 ± 1.16	eye	0.59 ± 0.05	eye	0.22 ± 0.05
eye	2.95 ± 0.65	bone	0.58 ± 0.04	brain	0.21 ± 0.02

sem. ves.: seminal vesicle

One hour after administration the highest level of radioactivity could be detected in the gastrointestinal tract and excretory organs: liver, kidneys and in urinary bladder. In endocrine and exocrine glands (hypophysis, thyroid, salivary gland, adrenal, pancreas, prostate) as well as in lungs considerable radioactivity could be detected, too. Relatively low levels were detected in blood, plasma and CNS.

There was no significant change in the 1-hour pattern of distribution at 4, 6 and 8 h after dosing, but the level of CRM decreased considerably. Relatively high concentration remained in the liver and kidneys and in the gastrointestinal tract.

Twenty-four h after treatment considerable radioactivity was detected only in the liver, gastrointestinal tract (first of all in the large intestine) and urinary tract.

The level of CRM in the central nervous system was similar to that of plasma at 1 h, but it was lower at 6 and 24 h after the treatment.

There was no significant difference between sexes in the distribution pattern at the examined time-points.

The distribution of CRM in pregnant dams was very similar to that of nonpregnant animals. The radioactivity penetrated through the placenta and there was a weak uptake in the fetuses.

The repeated applications did not cause any specific cumulation of radioactivity. Significant levels could be detected in liver, gastrointestinal tract, kidneys and bladder. Other tissues showed weak or background density on autoradiograms (Fig. 3).

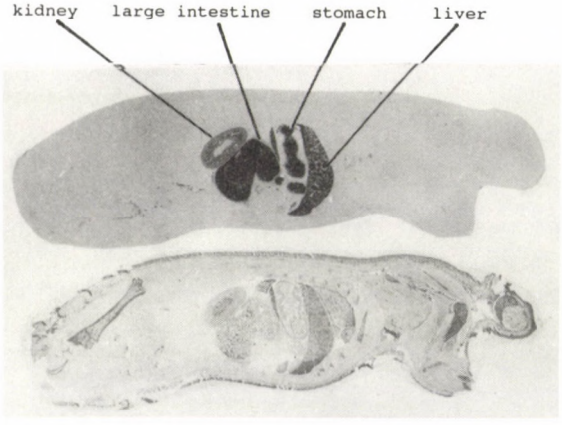

Fig. 3. Whole-body autoradiogram (up) and histological section (down) of a male rat 24 hours after repeated oral treatment with [^{14}C]RGH – 5002

Figure 4 presents the comparison of mean CRM concentrations 24 h after single and repeated administration. The quotient of CRM concentration of an organ at 24 h after multiple to single administration is given as the accumulation index of the drug for the tissue (Fig. 4). The accumulation index of 1.6 can be estimated from the indexes of individual organs.

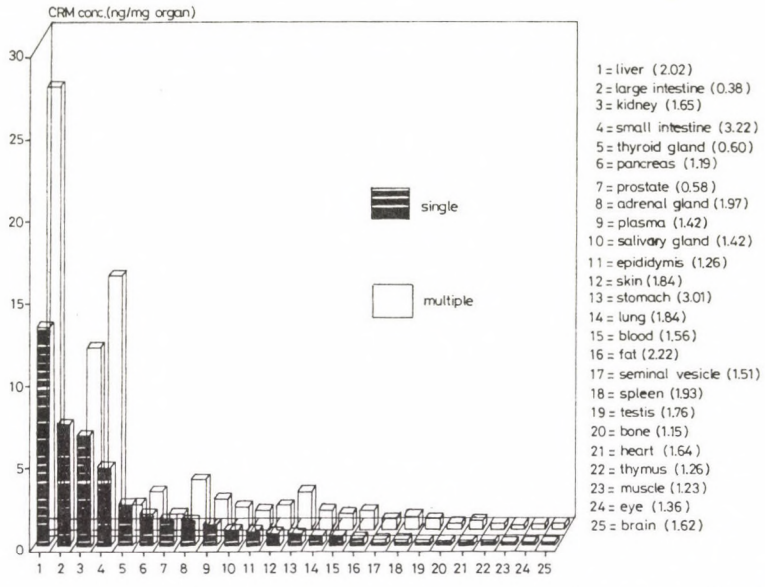

Fig. 4. CRM concentrations 24 h after single and multiple dose administration of [^{14}C]RGH – 5002 (accumulation indexes in (1))

Radioactivity was eliminated mainly via the urine: 82.67 ± 1.61% of the radioactive dose was recovered in urine. The mean faecal recovery was 11.12 ± 1.19%. The radioactivity remained in the carcass was negligible (0.6 ± 0.15%). Excretion of CRM was fairly rapid since the majority (approximately 80%) of the excreted radioactivity was recovered within the first 24 h after the administration of the drug (Table II and Fig. 5).

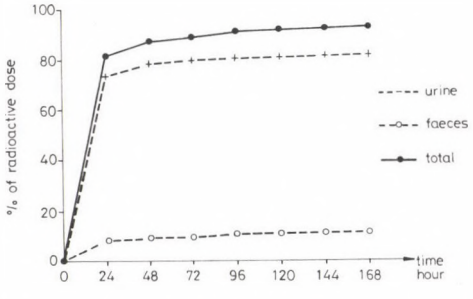

Fig. 5. Recovery in urine and faeces following a single 30 mg/kg per os dose of RGH – 5002

Table II

Excretion of radioactivity in urine and faeces expressed as % of the administered dose

Time period	Excretion in urine	Excretion in faeces
0 – 24 h	73.500 ± 1.79	8.078 ± 1.42
24 – 48 h	4.780 ± 1.41	0.923 ± 0.27
48 – 72 h	1.370 ± 0.40	0.433 ± 0.09
72 – 96 h	0.878 ± 0.31	1.205 ± 1.94
96 – 120 h	0.503 ± 0.14	0.193 ± 0.08
120 – 144 h	0.408 ± 0.14	0.175 ± 0.08
144 – 168 h	0.235 ± 0.02	0.113 ± 0.02
Total	81.67 ± 1.61	11.12 ± 1.19
Cage-wash		1.50 ± 0.50
Total recovery		94.30 ± 0.84

Discussion

The fate of [^{14}C]RGH – 5002 in rat can be characterized by rapid absorption, distribution and elimination from the body.

The distribution studies indicated that RGH – 5002 and its metabolites were distributed mostly in the gastrointestinal tract and the organs of excretion: liver and kidneys. Endocrine and exocrine glands and lungs must be highlighted as well, due to the relatively high CRM concentration. CRM level in the CNS, the target organ, was similar to that of plasma at 1 h after administration, indicating the penetration of the compound through the blood-brain barrier.

The compound passes through the placenta and appears in the fetus.

There was no significant accumulation after repeated administration. The estimated accumulation index of the drug indicates, that at the dose regimen used in the study the C_{min} values of the CRM in steady state are about 1.6 times higher the corresponding values obtained after a single dose.

The total recovery of the single 30 mg/kg dose in urine and faeces was 94.30 ± 0.84%. Radioactivity was eliminated mainly via the urine (81.67%). Complete elimination of the CRM can be demonstrated by the very low residual ^{14}C-levels measured in the carcasses (0.6%).

REFERENCES

1. Farkas, S., Kárpáti, E.: The pharmacology of RGH – 5002, a new centrally acting muscle relaxant with a long duration of action and less motor side-effects. Pharmacological Research., Vol 25. Supplement 2, 25 – 26 (1992).
2. Rowland, M. N., Tozer, T.: Drug Accumulation. In: Clinical Pharmacokinetics, Lea & Febiger, Philadelphia, 1989, pp. 78 – 83.
3. Ullberg, S., Larsson, B.: Whole-body autoradiography. In: Progress in Drug Metabolism, Vol. 6, John Wiley & Sons, Chichester, 1981, pp. 249 – 288.

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Effects of chlorpromazine on the rate of synthesis of various glycerolipids from [^{3}H]glucose in the human primordial placenta

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The in vitro effect of chlorpromazine (CPZ) on the biosynthesis of various neutral and phospholipids of the 8–10 week old human placenta from [^{3}H]glucose has been studied. Time course (with 0.5 mM CPZ) and dose-response (with 0.1–1.0 mM CPZ) experiments were performed. In order to investigate the significance of the cationic nature of CPZ, the effects were compared with those of 0.05% (v/v) Triton X-100, a nonionic amphiphile, and with the effects of the combined treatments with CPZ and Triton. The results provide evidence that (I) CPZ and Triton stimulate the formation of phosphatidic acid (PA) from glucose, (II) CPZ but not Triton inhibits the activity of phosphatidate phosphohydrolase (PPH), (III) both compounds inhibit the activity of diacylglycerol (DAG)-acyltransferase with an attendant rise of the levels of DAG and phosphatidylcholine (PC), (IV) the combined treatment decreases PC formation presumably due to inhibition of citidylyl transferase activity, (V) CPZ treatment leads to accumulation of labelled phosphatidylinositol (PI) irrespective of the presence or absence of Triton, and (VI) CPZ cannot promote the formation of PI from PA accumulating in response to Triton. The cationic nature of CPZ seems to play specific roles in the inhibition of PPH activity and in the activation of phosphatidyltransferase, both of which direct intermediates toward PI. On the other hand, the amphipathic nature of CPZ and Triton appears sufficient to account for the inhibited DAG-acyltransferase and citidylyl transferase activities.

Keywords: chlorpromazine, Triton X-100, phosphatidic acid, phosphatidylinositol, phosphatidylcholine, diacylglycerol, triacylglycerol, [^{3}H]glucose, primordial placenta (human)

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Phenothiazines form one of the most potent family of major tranquillizers, and chlorpromazine $(CPZ)^+$ is one of the oldest but still widely used member of this family. In addition to antidopaminerg activity, the amphipathic nature of this drug may also be an important property without which penetration through the blood-brain barrier and access to cerebral sites of action could not be possible. Moreover, CPZ is a cationic amphiphile which can exert a multitude of effects on the activity of enzymes of lipid metabolism [3, 5, 10, 12–14, 16–18, 23], and on the signal-transducing [10, 15] or carrier [8, 19] function of membrane-bound proteins. The question whether these interactions contribute or not to the pharmacological effects or to the side effects of this and structurally related drugs is not yet unequivocally answered.

The inhibitory effect of CPZ on the reaction catalyzed by Mg^{2+}-dependent phosphatidate phosphohydrolase (PPH) has been demonstrated in several papers [3, 5, 18, 23]. The increased rate of synthesis and accumulation of PI is a characteristic response in many tissues to CPZ treatment [1, 3, 6, 7, 18, 24]. This is believed to stem from a change in the direction of PA metabolism resulting in an enhanced rate of formation of PI [1, 5–7, 18, 24]. The concomitant decrease in the rate of synthesis of TG and PC is consistent with this view, since the formation of 1,2-DAG, the immediate precursor of these lipids in the de novo synthetic pathway, is generated from PA by Mg^{2+}-dependent PPH.

Despite efforts to elucidate the mechanism of action of CPZ on lipid metabolism, little information has accumulated for human tissues. Although CPZ is still a drug of choice for the treatment of seizure of pregnant women with ecclampsia, data concerning the effects of CPZ in the human placenta are still lacking. Previously we have demonstrated marked stimulatory effects of Triton X-100, a nonionic detergent, on the accumulation of PA and DAG in minced first trimester placenta tissues [11, 20, 21]. The aim of the present study was to examine the effects of CPZ on the formation of various glycerolipids from [^{3}H]glucose. Furthermore we wished to evaluate the significance of the cationic nature of CPZ by comparing its effects with those of Triton and by studying the effects of combined CPZ-Triton treatments.

Materials and Methods

Radioactive isotope and chemicals

[2–^{3}H]glucose (20 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, UK). Chlorpromazine, Silicagel H, and diolein were from Sigma (St. Louis, MO), various phospholipids from Serva (Heidelberg, Germany). HEPES was from Calbiochem (La Jolla, CA) Triton X-100, triolein and oleic acid and other chemicals were obtained from Reanal (Budapest, Hungary).

Tissue – Primordial human placenta fragments were collected during legal instrumental interruption of normal 8–10 week old pregnancies at the 2nd Department of Obstetrics and Gynecology,

Semmelweis University of Medicine, Budapest. The tissue was placed in ice cold 0.9% NaCl/40 mM HEPES–Na (pH 7.4), 1 mg/ml glucose solution and transported without delay to the biochemical laboratory where the experiment started immediately.

Incubations and lipid extraction – The tissue fragments were rinsed with ice cold physiological saline, chopped up with a pair of scissors and 500 or 600 mg tissue mince (in the same experiment always identical amounts) was incubated in 2.0 ml glucose-deficient Hanks medium buffered with 40 mM HEPES–Na (pH 7.4). The incubates contained 10 μCi [^{3}H]glucose and CPZ and/or Triton X-100 as indicated in the captions to figures or tables. The incubations were conducted in open vials shaken continuously in a 37 °C water bath for 60 min. Where specified 30 min long incubations were also included in the experiment. When added, various concentrations of CPZ (0.1–1.0 mM) were present from the zero time of incubation. Triton X-100 was always added at 30 min of incubation and in 0.05% (v/v) final concentration. Incubations were terminated by adding 5 ml ice-cold 0.9% NaCl/0.1 M acetic acid solution; the tissue was collected by low speed centrifugation and homogenized in methanol. Lipids were extracted using a modified method [20] of Bligh and Dyer [4]. Extracted lipids were dissolved in 5.0 ml chloroform/methanol (1:1, v/v) and 2×0.5 ml portions were withdrawn to determine the total extracted radioactivity. The solvent from the residual 4 ml extract was evaporated in N_2 stream, the lipids were dissolved in 200 μl chloroform/methanol (2:1, v/v) and aliquots were used for TLC analysis.

Radio TLC of phospholipids and neutral lipids – For chromatography 0.25 mm thick Silica-gel H thin layers and various solvent systems were employed [11, 20–22]. PA was separated from other phospholipids and from acylglycerols (AGs) using chloroform/pyridine/formic acid = 100:60:14 (v/v/v) solvent mixture. For separation of PC and PI chloroform/methanol/acetic acid/water = 100:50:14:6 (v/v/v/v) solvent system was applied. Using a two-dimensional phospholipid TLC procedure [2], PS and PE were found to contain negligible radioactivity. Therefore, data referring to these phospholipids are not documented in the paper. 1,2(sn)–DAG was separated from TGs using petrolether/diethylether/acetic acid = 70:30:1 (v/v/v) solvent system. PA and DAG standards were routinely run together with the samples in the respective TLC separation. For identification of various lipids, standards were chromatographed in separate lanes as well. Extracted (PI, PC and TGs) and standard lipids were made visible by brief exposure of the gels to iodine vapor. After localization of separated lipids and evaporation of iodine, gel portions were scraped off into counting minivials. After addition of 2 ml scintillation solution [20], the radioactivity was counted with 32% efficiency using a Beckman LS 7800 liquid scintillation spectrometer.

Calculation of results. From the cpm associated to silica-gel scrapings, the total cpm along the lane of migration was determined, and the percentage distribution of radioactivity among the separated neutral and phospholipids was calculated. Finally, from the the percentage value and the total extracted cpm, the cpm associated with a particular neutral or phospholipid was computed as cpm/500 mg tissue or cpm/600 mg tissue [20]. To calculate the percentage distribution of cpm among acylglycerols, cpm associated with phospholipids in TLC of neutral lipids was left out from calculation and the total cpm present in AGs was determined by TLC employed for separation of PA [11].

Results

In the first series of experiments time course studies were performed using 0.5 mM and 0.05% (v/v) final concentrations of CPZ and Triton X-100, respectively. CPZ was present from the onset of incubation, whereas Triton was added to control and CPZ-treated tissue at 30 min, and the total incubation time was 60 min.

Experiments were performed three times with different pools of placentae and the results of a representative experiment are shown in Figs 1 and 2. Triton inhibits the formation of [^{3}H]TG with an attending rise of [^{3}H]DAG (Fig. 1) and CPZ exhibits similar effects. It is also shown that Triton enhances somewhat the effect of CPZ, but the combined effect of the two compounds on DAG accumulation is roughly identical with that of Triton alone (Fig. 1). Comparing to control incubations, CPZ results in an about 2.5-fold increase of [^{3}H]PA level, and this increase is augmented further about 3-fold by the addition of Triton (Fig. 2). Triton, when added alone, also causes a marked, about 5-fold increase of [^{3}H]PA level (Fig. 2). CPZ has a great stimulatory effect on the accumulation of [^{3}H]PI, and this effect is only marginally enhanced further by Triton (Fig. 2). Again, when added alone, Triton stimulates the formation of [^{3}H]PI (Fig. 2). Relative to [^{3}H]PA formation, the effect of 0.5 mM CPZ on [^{3}H]PI accumulation is greater, whereas for 0.05% Triton the reverse is true: its effect on [^{3}H]PA exceeds that on [^{3}H]PI (Fig. 2). Under identical conditions, labelling of [^{3}H]PC was enhanced about 2-fold by CPZ and about 1.5-fold by Triton (results are not shown).

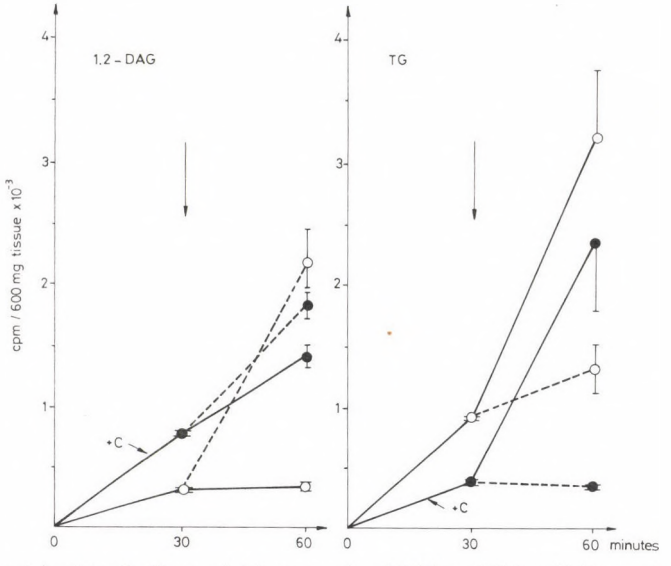

Fig. 1. Separate and combined effects of chlorpromazine (CPZ) and Triton X-100 on the conversion of [^{3}H]glucose into 1,2-diacylglycerol (1,2-DAG) and triacylglycerol (TG).

Mince of primordial placenta (600 mg) was incubated in 2.0 ml Hanks medium (prepared without glucose) with 10 μCi [^{3}H]glucose, in open vials shaken continuously at 37 °C for the time intervals indicated. Controls are denoted by open circles, solid circles refer to incubations with 0.5 mM (final conc.) CPZ. Triton X-100 (0.05%, v/v, final conc.) was added at 30 min (indicated by arrow). The effect of Triton in both control and CPZ-treated tissue is represented by dashed lines. Mean value from triplicate incubations ± SD (shown by error bars) are presented. The experiment has been repeated twice with similar results

In the second series of experiments the effect of increasing concentrations of CPZ in the presence and absence of 0.05% Triton was studied. Again, three sets of experiments were done using different pools of placenta tissue and results of a representative experiment are demonstrated by Figs 3–6. CPZ in 100–200 μM concentration exerts slight stimulatory effect on the labelling of TG, 500 μM is inhibitory and 1 mM causes an almost complete inhibition (Fig. 3). It is also shown that inhibition of TG-formation is accompanied by an accumulation of DAG. It is noteworthy that elevation of [^{3}H]DAG is observed at concentrations of CPZ where the drug-effect on TG formation is stimulatory (for example at 200 μM in Fig. 3). In agreement with our previous findings [11, 20, 21] 0.05% (v/v) Triton increases the labelling of DAG at the expense of TG labelling (Fig. 3).

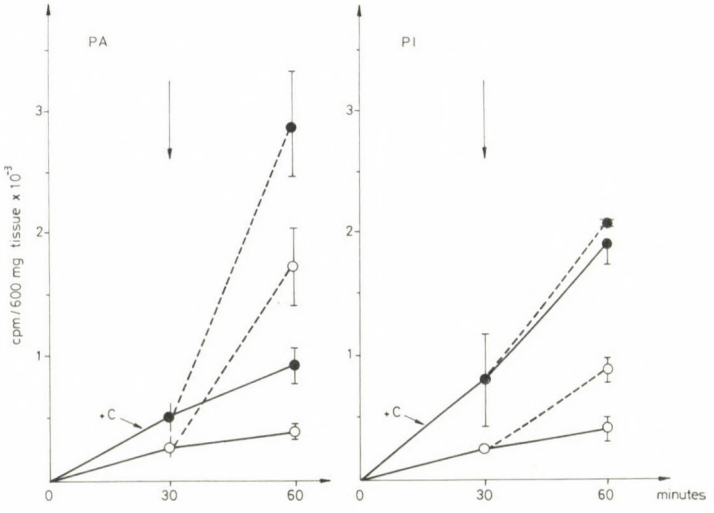

Fig. 2. Separate and combined effects of CPZ and Triton X-100 on the conversion of [^{3}H]glucose into phosphatidic acid (PA) and phosphatidylinositol (PI).
For experimental conditions and explanation of symbols and lines, see the legend to Fig. 1. Two additional experiments have given similar results

In response to 0.1–1.0 mM CPZ concentrations, there is an increase in [^{3}H]PA levels (Fig. 4). This increase is attended by a gradual rise in [^{3}H]PI, reaching the maximum at 0.5 mM CPZ, where it is 15-fold greater than the control value. It is noteworthy that elevation of [^{3}H]PI is detectable at those concentrations of CPZ which stimulates TG formation too. Triton again exerts a major, 5–6-fold effect on [^{3}H]PA accumulation, but causes only a 3-fold rise in [^{3}H]PI formation. Furthermore, Triton stimulates only slightly the synthesis of [^{3}H]PI when measured in CPZ-treated tissue. It is evident that increasing concentrations of CPZ (up to 0.5 mM) leads to gradual increase of the [^{3}H]PI/[^{3}H]PA ratio irrespective of the presence of Triton. Characteristically, between 0.5 and 1.0 mM CPZ concentrations, accumulation of [^{3}H]PA still continues, whereas the formation of [^{3}H]PI turns down.

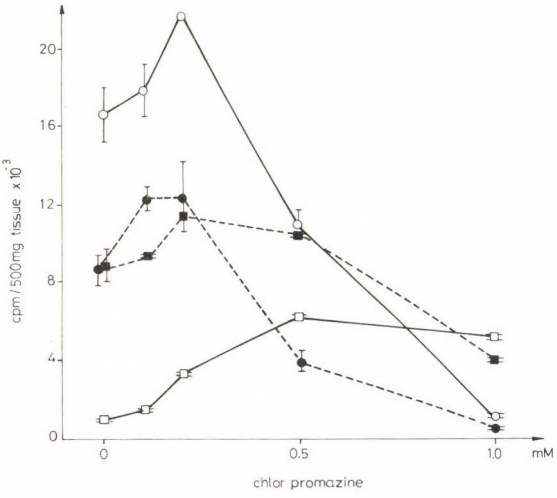

Fig. 3. Effects of increasing concentrations of CPZ on the conversion of [^{3}H]glucose into TG and DAG in the absence and presence of Triton X-100.

Mince of primordial placenta (500 mg) was incubated using the standard conditions (see the legend to Fig. 1) for 60 min. CPZ was present from zero time, whereas Triton (0.05% v/v, final conc.) was added after 30 min incubation. Dose-response curves for CPZ added alone are represented by open symbols and solid lines, whereas those for CPZ added in combination with Triton are shown by filled symbols and dashed lines. Circles: TG, squares: DAG. Each point is the average ±SD (error bars) of triplicate incubations. Results shown are a representative set out of 3 experiments

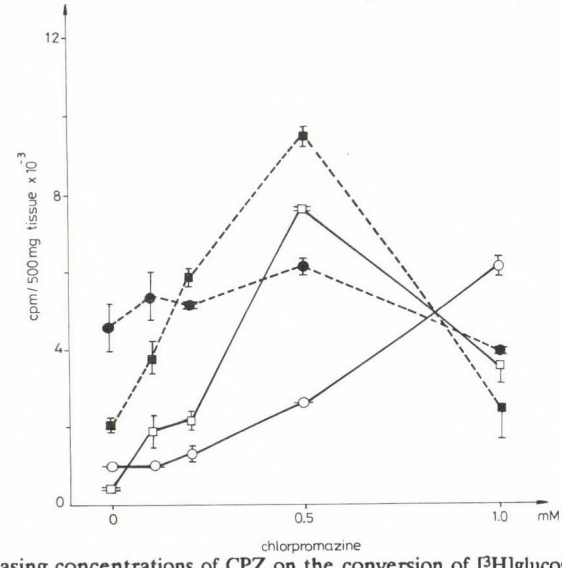

Fig. 4. Effects of increasing concentrations of CPZ on the conversion of [^{3}H]glucose into PA and PI in the absence and presence of Triton X-100.

For experimental details, see the legend to Fig. 3. Dose-response curves for CPZ added alone are represented by open symbols and solid lines, whereas those for CPZ added in combination with Triton are shown by filled symbols and dashed lines. Circles: PA, squares: PI. Each point is the average ±SD (error bars) of triplicate incubations. Two additional experiments have given similar results

In the range of zero up to 500 μM CPZ concentration the labelling of PC increases continuously (Fig. 5). Triton when given alone, increases the labelling of PC too, but reduces it when added to tissue incubated with 0.5 mM CPZ. Furthermore, 1 mM CPZ is strongly inhibitory for PC synthesis from [^{3}H]glucose (Fig. 5). Considering that in the *de novo* pathway, activity of PPH determines the rate of formation of acylglycerols (AC) and PC, the sum of radioactivities that accumulate in AC and PC can be used as an index of this activity. CPZ exhibits biphasic effect on this index: in 100–500 μM concentration range it is stimulatory, whereas 1 mM of the drug is inhibitory (Fig. 6). Triton causes a shift to the left of this dose-response curve, with the biphasic nature of the effect being sustained. In the presence of Triton, stimulation by CPZ is observed at 100 and 200 μM whereas inhibition is evident at 500 μM and 1 mM concentrations (Fig. 6).

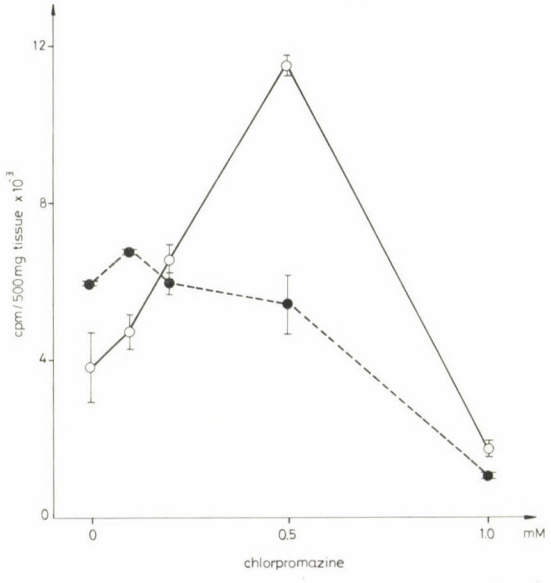

Fig. 5. Effects of increasing concentrations of CPZ on the conversion of [^{3}H]glucose into PC in the absence and presence of Triton X-100.
For experimental details, see the legend to Fig. 3. Data obtained with CPZ added alone are represented by open circles, whereas those for CPZ added in combination with Triton are shown by filled circles. Each point is the average ±SD (error bars) of triplicate incubations. Results of a representative experiment are presented; two others have given similar results

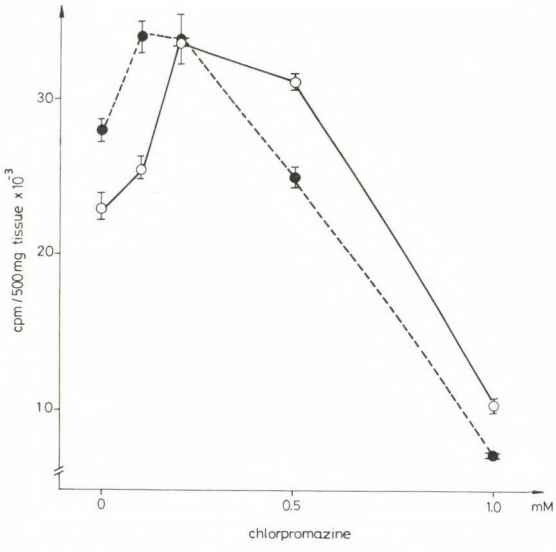

Fig. 6. Effects of increasing concentrations of CPZ on the conversion of [^{3}H]glucose into AG+PC in the absence and presence of Triton X-100.
For experimental details, see the legend to Fig. 3. Data obtained with CPZ added alone are represented by open circles, whereas those for CPZ added in combination with Triton are shown by filled circles. Each point is the average ±SD (error bars) of triplicate incubations. Results of a representative experiment are presented: two others have given similar results

Discussion

Considering the amphipathic structure of CPZ, its various effects on the activity of a number of enzymes involved in lipid metabolism [3, 5, 12, 14, 16, 18, 23] as well as on a variety of membrane enzymes [8, 10, 13, 19, 24] are not surprising. The present findings indicate two characteristic responses of the primordial placenta to treatment by CPZ: (I) the inhibition of acylglycerol and PC accumulation and (II) the enhanced rate of formation of PI and PA. These responses can be accounted for by the inhibitory effect of the drug on the activity of PPH, an effect that has been documented widely in the literature [1, 3, 5–7, 18, 23]. In our experiments, CPZ provoked such response in the primordial placenta tissue when it was applied in 1 mM final concentration, whereas in the range of 0.1–0.5 mM, the observed effects could not be explained by this simple inhibition theory.

In 0.1–0.2 mM concentrations, CPZ stimulates the synthesis of TG and PC (Figs 3 and 5). Furthermore, at 0.5 mM CPZ, the formation of PC is stimulated about 3-fold (Fig. 5), whereas that of TG is already inhibited (Fig. 3). The labelling of PC+TG, an indicator of Mg^{2+}-dependent PPH activity, exceeds the control activity

about 35% even at 0.5 mM CPZ concentration (Fig 6). These effects are attended by the increased labelling of 1,2-DAG, the immediate precursor of TG (Figs 1 and 3), and the accumulation of PI and PA (Figs 2 and 4). Collectively, these results suggest that in the 0.1–0.5 mM concentration range CPZ stimulates the *de novo* formation of PA from glucose with the consequent accumulation of some typical end products such as TG, PC and PI of lipid biosynthesis *de novo*. Apparently, CPZ exhibits some inhibitory action on DAG-acyltransferase (catalyzing the conversion of DAG to TG) which may explain the rise in the DAG level (Fig. 3). Expectedly, our data reflect the sum of the stimulatory effect of CPZ on the conversion of glucose into PA and the inhibitory effect on PPH activity. Probably, at lower CPZ concentrations the former, whereas at higher concentrations the latter effect predominates.

Our present (Fig. 5) and previous results [11, 21] also demonstrate that 0.05% (about 0.08 mM) Triton X-100 stimulates the labelling of PC from [^{3}H]glucose. This effect is attended by an increased labelling of PA and PI, while the labelling of acylglycerols (Fig. 3) remains unchanged. Therefore, low concentrations of both Triton and CPZ promote the biosynthesis of various neutral and phospholipids from glucose in the primordial placenta. Although the mechanism of this effect is still not clear, one may suppose that some membranolytic actions of these amphiphiles lead to enhanced glucose permeability of placenta cells. However, our previous results showing a major effect of Triton on [^{32}P]PA accumulation in placenta fragments dispersed in a medium containing [^{32}P]phosphate but no glucose disagree with this explanation [20].

Triton X-100 (applied in 1% concentration) has been reported to inhibit Mg^{2+}-dependent PPH activity [23]. The present finding that inhibitory effect of CPZ on the labelling of AGs+PC is potentiated by 0.05% Triton X–100 (Fig. 6), appears to be consistent with such an effect. Considering our previous findings [11, 20, 21], it seems more plausible, however, that inhibition of CDP-choline production (vide infra) by Triton with a consequent decrease in CPZ-stimulated PC formation (Fig. 5) is the major cause of the Triton-induced shift to the left of the dose-response curve depicted by Fig. 5.

The nonionic Triton and the cationic CPZ cause similar DAG accumulation (Fig. 3) which is the reflection of the inhibited activity of DAG-acyltransferase. Our present (Figs 1 and 3) and previous results [11, 20, 21] indicate that 0.05% Triton or 1 mM CPZ inhibits the activity of this enzyme almost completely. Therefore, inhibition of DAG acyltransferase activity by CPZ is due to the amphipathic nature of the drug, and the cationic property is not essential for this effect to occur.

It has been reported that both CPZ [16] and Triton X-100 [9] inhibit the production of CDP-choline by CTP: phosphorylcholine cytidylyl transferase, the putative regulatory enzyme of PC biosynthesis. Indeed, we have reported the marked inhibition by 0.05% Triton of the labelling of PC with [^{32}P]phosphate [20]. Similar

effects have been found using 0.5–2.0 mM CPZ (Tóth, unpublished results). It is important to observe that [^{32}P]phosphate incorporates into PC via the formation of labelled CDP-choline, whereas in the present experiments [^{3}H]DAG, formed from [^{3}H]glucose during incubation, supplied the label to PC. Hence, we can conclude that CPZ and Triton lead to increased labelling of PC from [^{3}H]glucose by promoting the formation of [^{3}H]DAG which then reacts with CDP-choline synthesized predominantly prior to the addition of these amphiphiles to the tissue. This explanation is consistent with previous findings in this laboratory, characterising PC formation as a reaction that is suitable for efficient attenuation of DAG-signals generated in the primordial human placenta [22].

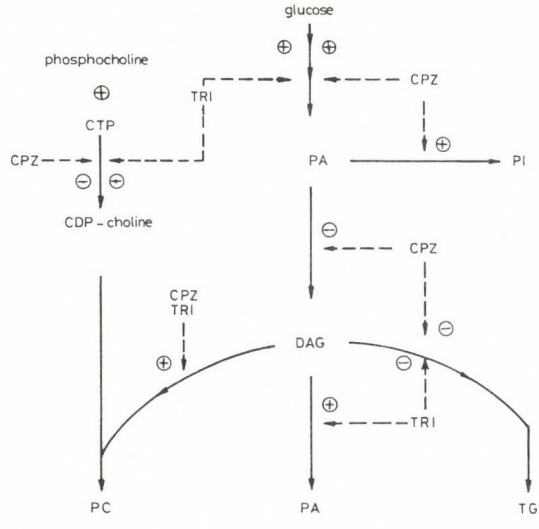

Fig. 7. Summary of the effects of chlorpromazine (CPZ) and Triton X-100 (TRI) on the rate of conversion of [^{3}H] glucose into various lipids in the primordial placenta. Stimulation is shown by + and inhibition by − signs. Abbreviations of the names of lipids: PA = phosphatidic acid; PI = phosphatidylinositol; PC = phosphatidylcholine; DAG = 1,2-diacylglycerol; TG = triacylglycerol.

Previously, we have reported that Triton in 0.01% (approximately 0.15 mM) subcellular concentration is unable to stimulate the incorporation of [^{32}P]phosphate into PA and PI [20]. Here we report that 0.1–1.0 mM CPZ stimulates efficiently the formation of PI from [^{3}H]glucose in the presence or absence of 0.05% Triton (Fig. 4). It seems suggestive to believe that the positive electric charge of CPZ plays some roles in the stimulated conversion of PA into PI. We speculate that neutralization of the negative charge of phosphatidate by CPZ may favor transphosphatidylation leading to CDP–DAG and then to PI, whereas it is not favorable for the phosphohydrolase-catalysed reaction. Finally, it is of interest that CPZ does not promote the conversion of PA, accumulated in the presence of Triton, to PI (Fig. 4).

One may think of a separate pool of PA that is not available readily for the formation of CDP – DAG. Such a pool might be generated by DAG-kinase acting on DAG that accumulates in the presence of Triton [11, 20, 21].

The proposed effects of chlorpromazine and Triton X – 100 on the formation of various lipids from glucose are illustrated by the flow-sheet shown by Fig. 7.

Acknowledgements

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REFERENCES

1. Allan, D., Michell, R. H.: Enhanced synthesis de novo of phosphatidylinositol in lymphocytes treated with cationic amphiphilic drugs. Biochem. J., **148**, 471 – 478 (1975).
2. Abramson, D., Blecher, M.: Quantitative two dimensional thin layer chromatography of naturally occurring phospholipids. J. Lipid Res., **5**, 628 – 631 (1964).
3. Bazan, N. G.: Metabolism of phosphatidic acid. In: Handbook of Neurochemistry (Ed.) Lajtha, A. Vol. 3. pp. 17 – 39 (1983). Plenum Press, New York, London.
4. Bligh, E. G., Dyer, W. J.: A rapid method of total lipid extraction and purification. Canad. J. Biochem. Physiol., **37**, 911 – 917 (1959).
5. Bowley, M., Cooling, J., Burditt, S. L., Brindley, D. N.: The effects of amphiphilic cationic drugs and inorganic cations on the activity of phosphatidate phosphohydrolase. Biochem. J., **165**, 447 – 454 (1977).
6. Brindley, D. N., Allan, D., Michell, R. H.: The redirection of glyceride and phospholipid synthesis by drugs including chlorpromazine, fenfluramine, imipramine, mepyramine, and local anaesthetics. J. Pharmachol. Pharmaceut., **27**, 462 – 464 (1975).
7. Brindley, D. N., Bowley, M.: Drugs affecting synthesis of glycerides and phospholipids in rat liver. The effects of clofibrate, halofenate, fenfluramine, amphetamine, cinchocaine, chlorpromazine, demethylimipramine, mepyramine and some of their derivatives. Biochem. J., **148**, 461 – 469 (1975).
8. Carfagna, M. A., Muhoberac, B. B.: Interaction of tricyclic drug analogs with synaptic plasma membranes: Structure-mechanism relationships in inhibition of neutral Na^+/K^+-ATPase activity. Mol. Pharmacol., **44**, 129 – 141 (1993).
9. Cornell, R., Vance, D. E.: Translocation of CTP: phosphocholine cytidylyltransferase from cytosol to membranes in HeLa cells: stimulation by fatty acid, fatty alcohol, mono- and diacylglycerol. Biochim. Biophys. Acta, **919**, 26 – 36 (1987).
10. Frölich, K. W., Aarbakke, G. M., Holmsen, H.: Chlorpromazine increases the turnover of metabolically active phosphoinotitides and elevates the steady-state level of phosphatidylinositol-4-phosphate in human platelets. Biochem. Pharmacol., **44**, 2013 – 2020 (1992).
11. Gimes, G., Tóth, M.: Low concentration of Triton X-100 inhibits diacylglycerol acyltransferase without measurable effect on phosphatidate phosphohydrolase in the human primordial placenta. Acta Physiol. Hung., **81**, 101 – 108 (1993).

12. Hedeskov, C. J., Thams, P., Gembal, M., Malik, T., Capito, K.: Characteristics of phosphoinositide-specific phospholipase C activity from mouse pancreatic islets. Mol. Cell. Endocrinol., **78**, 187–195 (1991).
13. Kanfer, J. N., Mc Cartney, D. G.: Modulation of the serine base exchange activity of rat brain membranes by amphiphilic cations and amphiphilic anions. J. Neurochem., **60**, 1228–1235 (1993).
14. Leli, U., Hauser, G.: Mechanism of modification of rat brain lysophospholipase A activity by cationic amphiphilic drugs. Biochim. Biophys. Acta, **918**, 126–135 (1987).
15. Mori, T., Takai, Y., Minakuchi, R., Yu. B., Nishizuka, Y.: Inhibitory action of chlorpromazine, dibucaine and other phospholipid-interacting drugs on calcium-activated, phospholipid-dependent protein kinase. J. Biol. Chem., **255**, 8378–8380 (1980).
16. Pelech, S. L., Vance, D. E.: Trifluoperazine and chlorpromazine inhibit phosphatidylcholine biosynthesis and CTP: phosphocholine cytidylyltransferase in HeLa Cells. Biochim. Biophys. Acta, **795**, 441–446 (1984).
17. Perez, L., Irurzun, A., Carrasco, L.: Activation of phospholipase activity during Semiliki Forest virus infection. Virology, **194**, 28–36 (1993).
18. Sturton, G., Brindley, D. N.: Factors controlling the activities of phosphatidate phosphohydrolase and phosphatidate cytidylyltransferase. The effects of chlorpromazine, demethylimipramine, cinchocaine, norfenfluramine, mepyramine and magnesium ions. Biochem. J., **162**, 25–32 (1977).
19. Takahashi, K., Kako, K. J.: Ischemia-induced changes in sarcolemmal (Na^+K^+)-ATPase, K^+-pNPPase, sialic acid and phospholipid in the dog and effects of the nisoldipine and chlorpromazine treatment. Biochem. Med., **31**, 271–286 (1984).
20. Tóth, M., Gimes, G., Hertelendy, F.: Triton X-100 promotes the accumulation of phosphatidic acid and inhibits the synthesis of phosphatidylcholine in human decidua and chorion frondosum tissues in vitro. Biochim. Biophys. Acta, **921**, 417–425 (1987).
21. Tóth, M., Gimes, G.: Phosphocholine transferase is more efficient than diacylglycerol kinase as possible attenuator of diacylglycerol signals in primordial human placenta. Acta Physiol. Hung., **81**, 341–354 (1993).
22. Tóth, M.: Attenuation of diacylglycerol signal in the primordial human placenta: role of phosphatidylcholine formation. Biochim. Biophys. Acta, **1210**, 105–112 (1993).
23. Walton, P. A., Possmayer, F.: The effects of Triton X–100 and chlorpromazine on the Mg^{2+}-dependent and Mg^{2+}-independent phosphatidate phosphohydrolase activities of rat lung. Biochem. J., **261**, 673–678 (1989).
24. Zborowski, J., Brindley, D. N.: The metabolism of CDP-diacylglycerol and phosphatidylinositol in the microsomal fraction of rat liver. Effects of chlorpromazine, magnesium and manganese. Biochim. Biophys. Acta, **751**, 81–89 (1983).

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Influence of the beta-blocker therapy on neutrophil superoxide generation and platelet aggregation in experimental myocardial ischemia and reflow

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The beneficial effect of beta-blockade has been reported in acute myocardial ischemia as well as in the postinfarction period. Recent interest focused on the special effect of beta-blocking agents regarding the changes of lipid metabolism, free radical mediated reactions and arachidonic acid cascade. In previous experiments on dogs we have shown that ultrashort-acting beta-blocker (Brevibloc) could modify production of prostacyclin and thromboxane in ischemic heart tissue. The purpose of this study was to investigate the effect of Brevibloc on the function of isolated neutrophils and platelets during myocardial ischemia and reperfusion. In mongrel dogs the left descending coronary artery (LAD) was ligated for 1 or 2 hours followed by one hour reperfusion. Animals were divided into two groups: Group I control dogs (n = 21) no drugs were given; in Group II. (n = 20) short half-life beta-blocker esmolol HCl (Brevibloc) was administered intravenously. Polymorphonuclear leukocytes (PMN) were isolated from venous blood before and after LAD ligature and following reperfusion. Spontaneous and phorbol myrystate acetate (PMA) stimulated superoxide radical generation of isolated PMN was measured. Platelets were separated at the same periods and maximal aggregation was determined in platelet rich plasma (PRP) after stimulation with collagen, adrenaline and ADP. There was no spontaneous radical production of PMN neither in the control, nor in the Brevibloc treated animals. Neutrophil superoxide production after activation in Group I was 9.54 ± 0.3 O_2^-/min/1.5×10^6 before LAD ligature, and significant elevation was present following one hour reperfusion (14.8 ± 0.8 O_2^-/min/1.5×10^6). Increased production of neutrophils was inhibited by beta-blocker therapy (9.32 ± 1.05, 8.25 ± 0.82

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respectively). Collagen and ADP stimulated platelet aggregation increased more than 20% during ischemia in Group I, which elevated further after reperfusion. Administration of Brevibloc diminished maximal aggregation in both cases, after 1–2 hours of LAD ligature and after reperfusion, compared to the initial value. Our findings suggested that ultrashort-acting beta-blocker has in vivo inhibitory action on neutrophil superoxide generation and platelet aggregation influencing the pathological cellular interactions.

Keywords: beta-blocker, isolated neutrophils, free radicals, platelets

Experimental studies demonstrate that neutrophil leukocytes play an important role in the pathophysiology of myocardial infarction [7, 8, 22, 23]. The infiltration of neutrophils is the next step when the migrating cells discharge oxygen-free radicals, proteolytic enzymes and arachidonic acid metabolites to damaged heart tissue [2, 15, 20, 21]. Platelets are able to accumulate in the ischemic myocardium as well enhancing/amplifying the development of vascular clumping [1, 6, 19, 24]. Recent investigations suggest that beneficial action of different cardioprotective drugs such as beta-blockers and Ca^{2+}-antagonist [4, 9, 13, 15, 31, 32] can be explained by their effect on the lipid metabolism and generation of free oxygen radicals [20, 27]. In previous experiments we have demonstrated that selective, ultrashort-acting beta-blocker Brevibloc could modify the arachidonic acid metabolic pathway and free radical mediated membrane damages during heart ischemia and reflow [28, 29]. The aim of our present study was to investigate the influence of Brevibloc on neutrophil superoxide production and platelet aggregation during experimental myocardial ischemia and reflow.

Materials and Methods

Surgical preparations

Forty-one mongrel dogs of both sexes were used in the study: with the average weight of 17 kg. Anesthesia was induced with sodium hexobarbital (20 mg/kg) and maintained throughout the surgery with narcotan and oxygen nitrous oxide in a ratio of 1:3. Positive pressure ventilation was used via endotracheal intubation. A branch of left femoral artery and vein were exposed and cannulated for monitoring of arterial pressure and for collection of venous blood samples. After thoracotomy the left anterior descending artery (LAD) was dissected free just distal to the first major diagonal branch. Temporary ligature was placed around the LAD, which was followed by reperfusion. ECG and systemic blood pressure were recorded in every 15 minutes with Biomedica six channels polygraph.

Experimental protocol

The dogs were devided into two groups as follows: Group I (n = 21) LAD occlusion lasted 1 or 2 hours, followed by one hour reperfusion; no drug was given. Group II (n = 20) occlusion and reperfusion as in Group I; animals were treated with ultrashort-acting cardio-selective beta-blocker

esmolol HCl (Brevibloc), which was kindly supplied by Du Pont-Pharma GmbH (Bad Homburg, Germany).

Protocol for the drug studies

After the registration of baseline hemodynamic parameters, esmolol was administered in a two-stage infusion [5]. There was an initial loading phase of 500 μg/kg/min for 1 min, followed by a maintenance infusion of 50 – 100 – 150 μg/kg/min for 4 min. This protocol was repeated until the blood pressure and heart rate decreased about twenty-five percent. After the titratable period Brevibloc was administered in a dosage of 100 – 150 μg/kg/min. The administration of esmolol had to be stopped in only a few cases for a short time, because of seriously lowered blood pressure.

Preparation of neutrophils

Peripheral blood polymorphonuclear leukocytes (PMN) were prepared by Guarnieri's [10] method from venous blood treated with EDTA. Following dextran sedimentation and hypotonic lysis (to remove contaminating erythrocytes), Ficoll Hipaque gradient centrifugation was performed. Resulting cells (95% PMN) were washed twice in phosphate buffered saline consisted of 5 mM glucose at pH 7.4 the PMN (1.5×10^6 cells/ml ≅ 40 μg protein) were incubated at 37 °C in the presence of 0.1 mM ferricytochrome c in phosphate buffer. After an initial 3 min incubation period the spontaneous radical production was measured. Also the phorbol myrystate acetate (PMA 0.1 μg/ml) stimulated activity was tested. Reaction was recorded with double blind spectrophotometer (Specord M40). Release of O_2^- was calculated from the linear portion of the cytochrome c reduction plot using a molar absorption coefficient of $21.1 \times 10^3\ M^{-1}cm^{-1}$.

The protein quantity was determined by Bradford's [3] method using bovine serum albumin as a standard.

Preparation of platelets

For the isolation of platelets venous blood was drawn without stasis into 0.13 M sodium citrate. Platelet-rich plasma (PRP) for the platelet aggregation test was prepared within 30 minutes. Platelet activity was measured in PRP with aggregometer (Mikron M 304 Diatron Kft. Hungary). The extent of aggregation to a single dose of one of the three reagents was measured in PRP and adjusted to 200 000 ± 50 000 platelets/μl then compared to autologous platelet-poor plasma (PPP). The reagents included collagen (cc. 2.5 μg/ml), adrenaline (cc. 7 μg/ml), and adenosine diphosphate (cc. 30 μg/ml) [19]. We also performed measurements involving the determination of the spontaneous aggregation of PRP without stimulants. In each of the tests the platelet aggregation a maximal optical density was estimated and compared to the PPP value and finally expressed in percentage.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). All data were compared by Student's *t*-test, taking $p < 0.05$ as the limit of significance.

Results

No spontaneous O_2^- production was detected in any experiment (non-treated and Brevibloc treated dogs). The PMN radical production can be measured only after stimulation with PMA. Table I shows the generation of superoxide radicals in non-treated and Brevibloc treated animals in different stages of experiments. These data show that without infusion of beta-blocker reperfusion can significantly increase the superoxide production following the activation of isolated neutrophils. On the other hand PMN, prepared following Brevibloc treatment show decreased radical generation after one hour of LAD ligature, and the reperfusion does not raise the O_2^- production.

Table I

PMA-stimulated O_2^- production (nmol O_2^-/min/1.5×10⁶ PMN) during myocardial ischemia and reflow

	Group I	Group II
Before LAD ligature	9.54 (0.9)	9.32 (1.05)
60'LAD ligature	11.35 (1.1)	6.88 (0.9)*
120'LAD ligature	10.2 (0.92)	7.98 (1.0)
60'LAD ligature + 60' reperfusion	14.85 (1.3)*	8.25 (0.82)

Values represent means (SD) of experiments in non-treated (Group I) and beta-blocker treated (Group II) animals.
Asterisk denotes $p < 0.05$ when compared to control values. (PMA = phorbol myrystate acetate; PMN = polymorphonuclear granulocytes).

Measuring stimulated aggregation of platelets in PRP proves that both ischemic and reperfusion state can lead to increased platelet aggregation. Table II shows that in non-treated animals the first stimulus can be detected in ischemic period, and reperfusion further aggravates the collagen and adrenaline initiated aggregation of platelets. In contrast, after the administration of Brevibloc (Table III) the level of initial aggregation decreases even after two hours of LAD ligature and reperfusion can cause only moderate elevation after ADP and collagen stimulation. The strongest effect of beta-blocker can be demonstrated after adrenaline stimulation following reperfusion.

Table II

Stimulated aggregation of platelets in non-treated group during myocardial ischemia and reflow

	Collagen	Adrenaline	ADP
Before LAD ligature	82% (12)	37% (2.5)	69% (5)
60'LAD ligature	92% (15)	42% (5.1)	102% (13)*
120'LAD ligature	95% (14)	48% (9.2)	101% (12)*
60'LAD ligature + 60' reperfusion	102% (11)*	59% (8.5)*	102% (11)*

Values represent mean (SD) of experiments in control non-treated animals (Group I). Asterisk denotes $p < 0.05$ when compared to control values.

Table III

Stimulated aggregation of platelets in Brevibloc treated group during myocardial ischemia and reflow

	Collagen	Adrenaline	ADP
Before LAD ligature	82% (10.3)	36% (2.8)	59% (8.9)
60'LAD ligature	58% (5.2)	22% (1.8)	32% (5.4)*
120'LAD ligature	51% (5.9)*	18% (1.2)*	30% (2.8)*
60'LAD ligature + 60' reperfusion	62% (6.8)	19% (0.8)	48% (8.6)

Values represent mean (SD) of experiments in beta-blocker treated animals (Group II). Asterisk denotes $p < 0.05$ when compared to control values.

Discussion

The short half-life cardio-selective beta-blocker esmolol HCl (Brevibloc) has been safely used in patients with acute myocardial infarction or in unstable angina pectoris [14, 16]. We have previously reported that Brevibloc during myocardial ischemia and reperfusion can modify the ratio of prostacyclin and thromboxane in the injured area and can significantly diminish the thromboxane release during reperfusion [29]. Our data suggest that after administration of this drug, endogenous scavengers are better preserved and peroxidation of membrane system is reduced in ischemic heart tissue [28].

In our present experiments we have studied the influence of Brevibloc on isolated neutrophil superoxide radical generation and stimulated platelet aggregation during experimental myocardial ischemia followed by reperfusion. These cells are responsible for the progressive increase of coronary vascular resistance and edema

formation during ischemia, producing oxygen-radicals and different metabolites which are directly toxic to myocytes [7, 17, 18, 30].

Our present results showed that in non-treated animals stimulated superoxide production of PMN increased at the time of coronary ligature and the generation of radicals almost doubled after reperfusion. Guarnieri [10] demonstrated that different concentrations of catecholamines cause increased radical production of human PMN, which can be decreased by beta-blocker (propanolol). Our results are in agreement with Guarnieri's data. We also demonstrated the reducing effect of Brevibloc on superoxide radical production of isolated dog PMN during ischemia and reflow. Decrease of symphathoadrenal activity together with depletion of catecholamine level after beta-blocker therapy may partly explain this beneficial effect of Brevibloc.

The interaction of platelets with leukocytes and with the blood vessel wall plays significant role in the pathogenesis of thrombus formation [12, 20, 21]. Experimental data indicates that platelet activating factor (PAF) released from ischemic myocardium can also trigger the platelet-neutrophil interaction [1, 6, 32]. Platelet-endothelial interactions [19, 25, 26] are controlled by prostacyclin and thromboxane and different mediators (PAF, leukotrienes, complement, free radicals).

Our previous experimental data showed that Brevibloc modified the prostacyclin-thromboxane ratio in ischemic heart tissue with predominance of prostacyclin [29]. Present findings confirm that decrease of stimulated platelet aggregation after administration of beta-blocker may be in close correlation with this alteration of prostacyclin content. Intraplatelet cAMP level which is the most powerful inhibitor of platelet aggregation can also be raised after elevation of prostacyclin. Such a way it can be supposed that Brevibloc which stimulates endogenous prostacyclin synthesis can elevate cAMP level inhibiting aggregation of platelet response to stimulated agents.

Based on our results, it is pointed out that Brevibloc has a beneficial effect on the pathological leukocyte-platelet-endothelial interaction during early ischemic and reperfusion state. We can conclude, as it was previously confirmed by others as well, that beta-blockers may protect the myocardium from deleterious consequences of ischemia and reperfusion.

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REFERENCES

1. Alloatti, G., Montrucchio, G., Emanuelli, G., Camussi, G.: Platelet-activating factor (PAF) induces platelet/neutrophil co-operation during myocardial reperfusion. J. Mol. Cell. Cardiol., **24**, 163–171 (1992).
2. Bolli, R.: Oxygen-derived free radicals and myocardial reperfusion injury: An overview. Cardiovascular Drugs and Therapy, **5**, 249–268 (1991).
3. Bradford, M. A.: A rapid and sensitive method of the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., **72**, 248–254 (1976).
4. Campbell, W. B., Johnson, A. R., Callahan, K. S., Graham, R. M.: Antiplatelet activity of beta-adrenergic antagonists: Inhibition of thromboxane synthesis and platelet aggregation in patients receiving long-term propranolol treatment. The Lancet, **19/26**, 1382–1384 (1981).
5. Covinsky, J. O.: Esmolol: A novel cardioselective, titratable intravenous beta-blocker with ultrashort half-life. Drug Intell. Clin. Pharm., **21**, 316–321 (1987).
6. Dinerman, J. L., Mehta, J. L.: Endothelial, platelet and leukocyte interactions in ischemic heart disease: insights into potential mechanisms and their clinical relevance. J. Am. Coll. Cardiol., **16**, 207–222 (1990).
7. Engler, R.: Consequences of activation and adenosine mediated inhibition of granulocytes during myocardial ischemia. Federation Proc., **46**, 2407–2412 (1987).
8. Engler, R.: Free radical and granulocyte-mediated injury during myocardial ischemia and reperfusion. Am. J. Cardiol., **63**, 19E–23E (1989).
9. Frishman, W. H., Skolnick, A. E., Lazar, E. J., Fein, S.: Beta-adrenergic blockade and America, **73**, 409–436 (1989).
10. Guarnieri, C., Melandri, G., Caldarera, I., Scheda, M., Ligabue, A., Guizzardi, S., Branzi, A.: Reduced oxidative activity of circulating neutrophils in patients after myocardial infarction. Cell. Biochemistry and Function, **8**, 157–162 (1990).
11. Guarnieri, C., Scheda, M., Calderera, I., Vaona, I., Melandri, G.: Higher concentrations of adrenaline and noradrenaline enhance basal and stimulated O_2^- production by isolated neutrophils. J. Mol. Cell. Cardiol., **22** (suppl. III): S49 (1990).
12. Harlan, J. M.: Leukocyte-endothelial interactions. J. Am. Soc. Hematology, **65**, 513–526 (1985).
13. Hjalmarson, A., Olsson, G.: Myocardial infarction. Effect of beta-blockade. Circulation, **84**, (suppl. VI), 101–107 (1991).
14. Hohnloser, S. H., Meinertz, T., Klingenheben, T., Sydow, B., Just, H.: Usefulness of esmolol in unstable angina pectoris. Am. J. Cardiol., **67**, 1319–1323 (1991).
15. Karmazyn, M.: The 1990 Merck Frosst Award. Ischemic and reperfusion injury in the heart. Cellular mechanisms and pharmacological interventions. Can. J. Physiol. Pharmacol., **69**, 719–730 (1991).
16. Kloner, R. A., Kirschenbaum, J., Lange, R., Antman, E. M., Braunwald: Experimental and clinical observations on the efficacy of esmolol in myocardial ischemia. Am. J. Cardiol., **56**, 40F–48F (1985).
17. Kuzuya, T., Hoshida, S., Suzuki, K., Sasaki, T., Kitabatake, A., Kada T., Minamino, T., Tada, M.: Polymorphonuclear leukocyte activity and ventricular arrhythmia in acute myocardial infarction. Am. J. Cardiol., **62**, 868–872 (1988).
18. Lucchesi, B. R., Werns, S. W., Fantone, J. C.: The role of the neutrophils and free radicals in ischemic myocardial injury. J. Mol. Cell. Cardiol., **21**, 1241–1251 (1989).
19. Mackie, I. M., Neal, C. R.: The platelet. In: Pittilo, R. M., Machin, S. J. (eds) Platelet-vessel Wall Interactions. Springer-Verlag, Berlin–Heidelberg. 1985, pp. 1–24.

20. Maza, S. R., Frishman, W. H.: Therapeutic options to minimize free radical damage and thrombogenicity in ischemic/reperfused myocardium. Medical Clinics of North America, **72**, 227–230 (1988).
21. Mehta, P., Mehta, J. L., Lawson, D., Krop, I., Letts, L. G.: Leukotrienes potentiate the effects of epinephrine and thrombin on human platelet aggregation. Thromb. Res., **41**, 731–738 (1986).
22. Mehta, J. L., Nichols, W. W., Mehta, P.: Neutrophils as potential participants in acute myocardial ischemia: relevance to reperfusion. J. Am. Coll. Cardiol., **11**, 1309–1316 (1988).
23. Mehta, J., Dinerman, J., Mehta, P., Saldeen, T. G. P., Lawson, D., Donnelly, W. H., Wallin, R.: Neutrophil function in ischemic heart disease. Circulation, **79**, 549–556 (1989).
24. Merritt, J. E., Hallam, T. J., Brown, A. M., Boyfield, I., Cooper, D. G., Hickey, D. M.B., Jaxa-Chamiec, A. A., Kaumann, A. J., Keen, M., Kelly, E., Kozlowsky, U., Lynham, J. A., Moores, K. E., Murray, K. J., MacDermot, J., Rink, T. J.: Octimibate, a potent non-prostanoid inhibitor of platelet aggregation, acts via the prostacyclin receptor. Br. J. Pharmacol., **102**, 251–259 (1991).
25. Mullane, K. M., Salanon, J. A., Kraemer, R.: Leukocyte-derived metabolites of arachidonic acid in ischemia-induced myocardial injury. Federation Proc., **46**, 2422–2433 (1987).
26. Pettersson, K., Hansson, F., Björkman, J. A., Ablad, B.: Prostacyclin synthesis in relation to sympathoadrenal activation. Circulation, **84** (suppl. VI), 38–43 (1991).
27. Reilly, P. M., Schiller, H. J., Bulkey, G. B.: Pharmacologic approach to tissue injury mediated by free radicals and other reactive oxygen metabolites. Am. J. Surg., **161**, 488–503 (1991).
28. Rőth, E.: Effect of Ca-antagonists, beta-blockers and antioxidant during reperfusion period. Bratisl. Lek. Listy, **92**, 195–200 (1991).
29. Rőth, E., Török, B.: Effect of the ultrashort-acting beta-blocker Brevibloc on free radical-mediated injuries during the early reperfusion state. Basic. Res. Cardiol., **86**, 422–433 (1991).
30. Simpson, P. J., Mickelson, J. K., Lucchesi, B. R.: Free radical scavengers in myocardial ischemia. Federation Proc., **46**, 2413–2421 (1987).
31. Torr, S., Holland, A. J. D., Main, M., Hynd, J., Isted, K., Noble, M. I. M.: Effects on infarct size of reperfusion and pretreatment with beta-blockade and calcium antagonists. Basic. Res. Cardiol., **84**, 564–582 (1989).
32. Yardumian, A., Machin, S. J.: Newer pharmacological agents. In: Pittilo, R. M., Machin, S. J., eds. Platelet-vessel Wall Interactions. Springer-Verlag, Berlin–Heidelberg. 1985, pp. 167–191.

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Histophysiological evidence for parathyroid mediation of estrogen- and prolactin-induced hypercalcemia in the toad *Bufo melanostictus* (Schneider)

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Administration of estrogen as well as prolactin to *B. melanostictus* resulted in hypercalcemia. Parathyroids and ultimobranchials of the toad were hypertrophied and the secretory epithelium reflected hyperplasia. Histophysiological manifestation of the parathyroids of treated toads suggests that estrogen and prolactin bring about hypercalcemia by stimulating the parathyroids. Changes in the ultimobranchials suggest hyperactivity of the gland to combat estrogen- and prolactin-induced hypercalcemia.

Keywords: estrogen, prolactin, hypercalcemia, parathyroid glands, toad hypercalcemia

In amphibia, parathyroids and ultimobranchials are the principal glands which regulate the plasma levels of calcium, the former secreting parathormone, the hypercalcemic principle, and the latter secreting calcitonin, the hypocalcemic hormone [7]. However, in their evolutionary predecessors, viz., the fish, which lack parathyroids, prolactin has been shown to be the hypercalcemic hormone [7, 28], and it has also been shown that prolactin retains this role in amphibia as well [10, 12, 23, 25] but it is not definitely known whether prolactin acts directly on the principal sites of calcium metabolism viz., the bone and the paravertebral lime sacs, or through modulating the secretory activity of parathyroids and ultimobranchials.

Further, it has been shown that the egg laying vertebrates, including amphibia, maintain high levels of plasma calcium during the period of sexual activity; higher

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titres of estrogen appears to be the basis of this hypercalcemia [2, 3, 13–15, 17–19, 26, 27, 33, 41, 43].

There have been attempts to find whether such an estrogen-induced hypercalcemia is mediated through the parathyroids or if comes about independently of the latter; vertebrates appear to differ in this regard, in the bullfrogs, Japanese quail [6], chicken [34] and the garden lizard [2, 3] the former, and in several other cases is the latter mechanism takes place [13, 33, 38, 30].

Thus, the objective of the present study is to find the response of the parathyroids and ultimobranchials to exogenous prolactin and estrogen in the toad *Bufo melanostictus* (Schneider) so as to infer if the hypercalcemic action of these hormones in this toad is mediated through these glands. Gross morphology, histology, seasonal changes and role in calcium homeostasis of parathyroids and ultimobranchials of this toad have already been worked out and conform to the general anuran pattern [1, 4, 20–22, 24, 25].

Materials and Methods

Adult female toads, of body weight 30.00 ± 1.25 g, were collected locally and maintained in the laboratory for one week with free access to food and water. To minimise the endogenous hormonal effects, the experiments were conducted during the non-breeding season (Dec.–March). The toads were divided into four groups of ten each and treated as follows:

Group I: Received 0.2 ml of olive oil and served as control for estrogen treatment.

Group II: Received 0.2 ml of stilbesterol dipropionate (0.2 mg) (Vetosterol, May & Baker, India) every day, for 15 days.

Group III: Received 0.5 ml of 0.9% saline and served as control for prolactin treatment.

Group IV: Received 50 μg of ovine prolactin (Sigma, USA) in 0.5 ml of 0.9% saline, every day, for 15 days.

The administration was made through intramuscular route

Three hours after the last dose, under mild ether anaesthesia blood was drawn by cardiac puncture in heparinized syringe and centrifuged for 15 minutes at 1500 × g. The plasma was separated and used for the determination of calcium spectrophotometrically [42]. Parathyroids and ultimobranchials were excised, fixed in aqueous Bouin's fixative and processed for routine histological preparations. Serial sections at 6 μm thickness were stained in haematoxylin and eosin. Diameter of the nuclei of parathyroids and ultimobranchials were measured using a calibrated ocular micrometer; cell population of the former was determined using an ocular grid. Student's *t*-test was employed for statistical comparison.

Results

Treatment of estrogen and prolactin for 15 days elevated plasma calcium to significant levels (Table I).

Table I

Plasma levels of calcium and data on the histometric parameters of parathyroids and ultimobranchials of B. melanostictus following treatment with estrogen and prolactin (values are mean ± SE of 10 animals each)

Group	Plasma calcium (mg %)	Parathyroid		Ultimobranchial
		Nuclear diameter (μm)	Cell population (/mm^2)	Nuclear diameter (μm)
Control	6.98 ± 0.03	4.52 ± 0.03	110 ± 2.16	3.89 ± 0.08
Estrogen	9.47 ± 0.05*	6.23 ± 0.01*	90 ± 1.24*	5.20 ± 0.12*
Prolactin	8.26 ± 0.06*	5.27 ± 0.01*	92 ± 1.09*	5.61 ± 0.07*

* $p < 0.01$

Changes in the parathyroid gland

Parathyroid gland of control toad was formed of the chief cells, arranged as scattered peripheral cells and dense central cells. Peripheral chief cells had spherical or oval nuclei where as central chief cells had elongated nuclei. In both cases, the cell membrane was obscure. The gland was surrounded by a thin capsule of connective tissue. The characteristic whorl-like arrangement of cells as seen in frogs was not observed. Mean diameter of nuclei was 4.52 μm and cell population was 110/mm^2 (Figs 1, 2; Table I).

Treatment of toads with estrogen as well as prolactin resulted in hyperactivity of the parathyroids as reflected in the histophysiological picture (Figs 3–6). However, such a hyperactivity was more pronounced in estrogen-treated toads than in the prolactin-treated ones; in the former, mean nuclear diameter increased to 6.23 μm, and cell population decreased to 90/mm^2, where as in the latter the values were 5.27 μm and 92/mm^2, respectively. In estrogen-treated toads, cells corresponding to central chief cells were absent and the entire gland consisted of the peripheral type (Figs 3, 4); while such a cellular transition was evident in the gland of prolactin-treated toads also, it was not as prominent following estrogen treatment (Figs 5, 6).

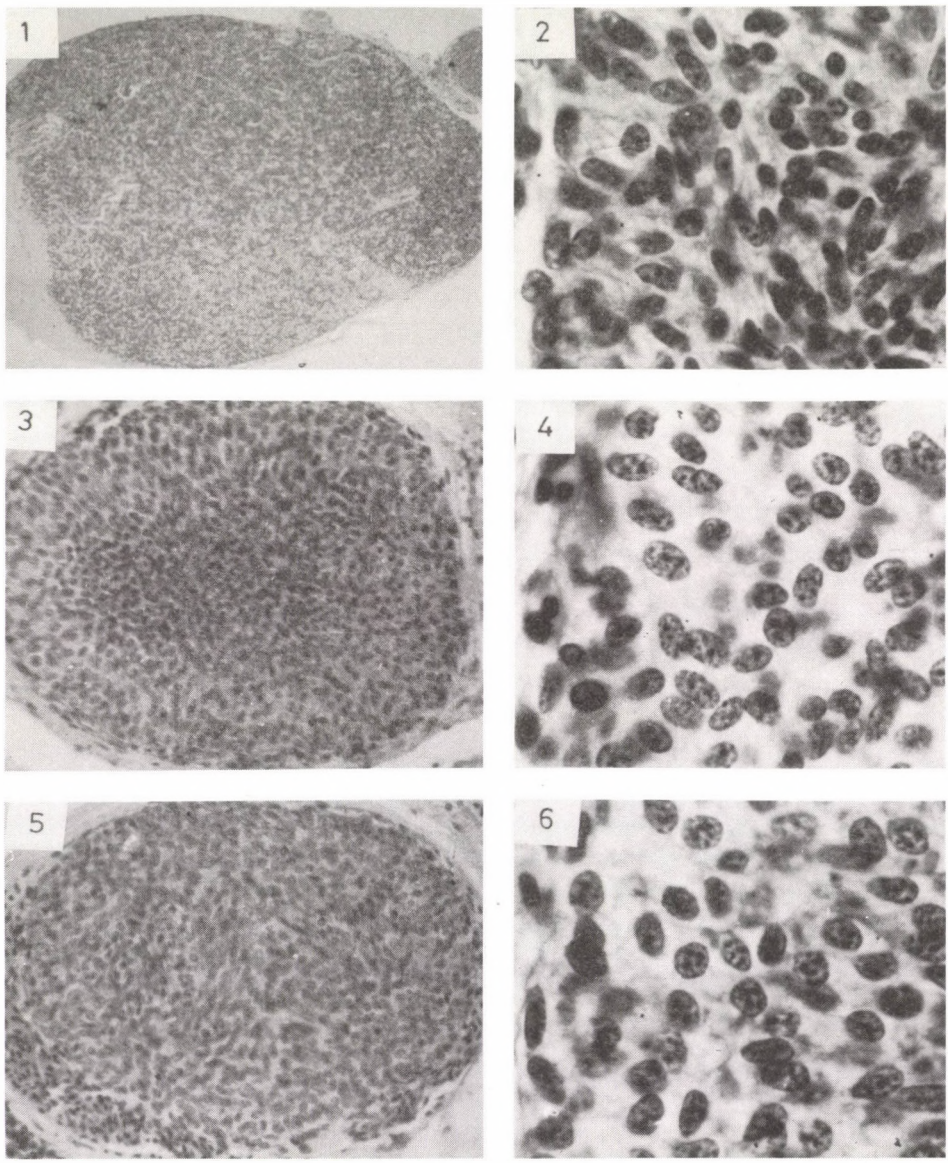

Fig. 1. T. S. of parathyroid of control toad
Fig. 2. Part of Fig. 1 enlarged showing peripheral chief cells and central chief cells
Fig. 3. T. S. of parathyroid of estrogen-treated toad
Fig. 4. Part of Fig. 3 enlarged, showing enlargement of nuclei
Fig. 5. T. S. of parathyroid of prolactin-treated toad
Fig. 6. Part of Fig. 6 enlarged, showing enlargement of nuclei

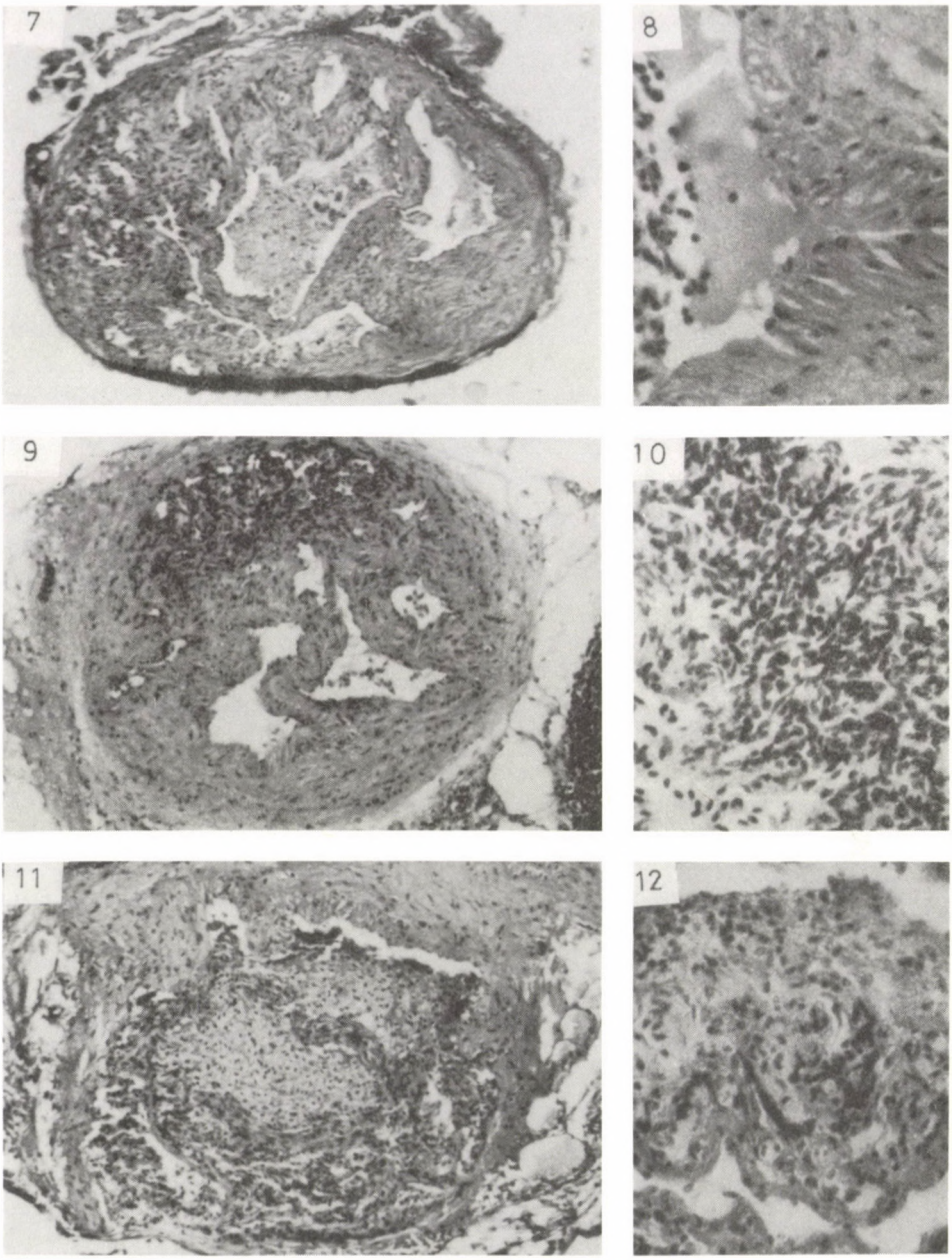

Fig. 7. T. S. of ultimobranchial of control toad
Fig. 8. Part of Fig. 7 enlarged, showing epithelium lining the follicle
Fig. 9. T. S. of ultimobranchial of estrogen-treated toad
Fig. 10. Part of Fig. 9 enlarged, showing proliferation of epithelium and formation of secondary follicles
Fig. 11. T. S. of ultimobranchial of prolactin-treated toad
Fig. 12. Part of Fig. 11 enlarged, showing a trend as in Fig. 10

Changes in the ultimobranchial gland

The ultimobranchials of the control toads consisted of one or two large follicles, with a distinct lumen filled with a colloidal material, ladened with degenerating cells. The follicle was lined with pseudostratified epithelium and showed three types of cells, viz., the basal cells, secretory cells and degenerating cells. The mean nuclear diameter was 3.89 μm (Figs 7, 8 and Table I).

In the toads treated with estrogen as well as prolactin, the ultimobranchials displayed heightened activity, as evidenced by proliferation of the secretory cells, leading to the formation of cord-like cellular arrangement, and several secondary follicles; degenerating cellular elements were hardly encountered in the colloid (Figs 9–12). Mean nuclear diameter of the epithelium was increased to 5.20 μm and 5.61 μm in estrogen- and prolactin-treated toads, respectively (Table I).

Discussion

Parathyroids of anura consist of two kinds of cells, peripheral chief cells and central chief cells; several studies have shown that the peripheral type is the active component, and any physiological demand for more calcium would result in the transformation of central resting chief cells into the peripheral type; it has also been reported that the parathyroids of anura would respond to experimental hypo- and hypercalcemia with alterations in their histophysiological status [1, 4, 9, 16, 39, 40]. On the other hand, the ultimobranchials of anura contain one or two large follicles, lined by a pseudostratified epithelium, formed of three types of cells of which only one, forming about 60% of the total population, is classified as the secretory type [31, 32]. As in the case of parathyroids, ultimobranchials also have been known to respond to experimental hypo- and hypercalcemia with alterations in their histophysiology [21, 29, 31, 32, 37].

Considering the results of the present study in this background, it is evident that estrogen as well as prolactin bring about hypercalcemia in *B. melanostictus* through activation of parathyroids, which is inferred from the increased diameter of the peripheral chief cell nuclei and decreased cell population reflecting increased cellular diameter. This inference supports the view of Baksi et al. [2, 3, 6] that estrogen-induced hypercalcemia is mediated through the parathyroid. It also suggests that prolactin, in retaining its hypercalcemic action in anurans, bring about this action through stimulating the parathyroids. The study further suggests that compared to prolactin, estrogen is more potent in stimulating the parathyroids.

It has been reported that exogenous 1.25 dihydroxycholecalciferol (1.25 $(OH)_2$ D_3, the hormonal form of vitamin D_3) is hypercalcemic in anura through increased intestinal absorption of calcium [7] and that anuran kidney contains the enzyme 1α-hydroxylase which mediates the final step in the activation of vitamin D_3 to its hormonal form [5] Baksi et al. [6] in bullfrogs and Japanese quail, and Spanos

et al. [34] in chicken, have attributed the hypercalcemic action of estrogen and prolactin to increased renal production of 1.25 $(OH)_2 D_3$. Therefore, it could be suggested that estrogen and prolactin bring about hypercalcemia by stimulating the parathyroid, to increase its output of parathormone, which would in turn enhance the formation of 1.25 $(OH)_2 D_3$ mediated by the appropriate enzyme systems, leading to increased intestinal absorption of calcium and hypercalcemia (Fig. 13).

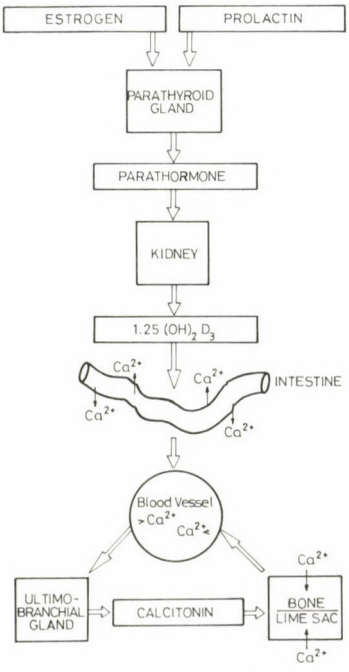

Fig. 13. Model depicting the probable mechanism of action of estrogen and prolactin in bringing about hypercalcemia

The present study also shows that experimental hypercalcemia induced by estrogen and prolactin is accompanied by hypertrophy of the ultimobranchials and hyperplasia of its epithelium, resulting in the obliteration of the primary follicles, generation of several small secondary follicles and the organization of the gland to contain a cord-type arrangement of the epithelium. This could be explained on the basis of the fact that exogenous estrogen and prolactin, in acting through the stimulation of the parathyroids, cause hypercalcemia, which is not the consequence of any physiological demand for higher level of calcium; therefore, it could be suggested that the ultimobranchials are stimulated into hyperactivity to combat the resultant hypercalcemia, as has been suggested in several other instances of experimental hypercalcemia [11, 21, 29, 32, 36].

REFERENCES

1. Akbarsha, M. A.: Parathyroid gland of the South Indian toad *Bufo melanostictus*. J. Reprod. Biol. Comp. Endocrinol., **1**, 59–68 (1981).
2. Akbarsha, M. A.: Seasonal and sexual differences in the effects of parathyroidectomy in the garden lizard *Calotes versicolor*, with a note on the follicle formation in reptilian parathyroids. Amphibia – Reptilia, **4**, 185–194 (1983).
3. Akbarsha, M. A.: Seasonal morpho- and histometric changes in the parathyroids of the lizard *Calotes versicolor* (Daudin). Z. Mikrosk. -anat. Forch., **99**, 204–208 (1985).
4. Akbarsha, M. A., Gulam Mohideen, B. M.: Studies on the functional significance of the cell types in the parathyroids of the South Indian toad *Bufo melanostictus*. J. Reprod. Biol. Comp. Endocrinol., **2**, 18–26 (1982).
5. Baksi, S. N., Galli-Gaillardo, S. M., Pang, P. K. T.: Vitamin D metabolism in amphibia and fish. Fedn. Am. Soc. exp. Biol., **36**, 1097 (1977).
6. Baksi, S. N., Kenny, A. D., Galli-Gaillardo, S. M., Pang, P. K. T.: Vitamin D metabolism in bullfrogs and Japanese quail: Effects of estradiol and prolactin. Gen. Comp. Endocrinol., **35**, 258–262 (1978).
7. Bentley, P. J.: Calcium metabolism in the amphibia. Comp. Biochem. Physiol., **79A**, 1–5 (1984).
8. Clark, N. B.: Evolution of calcium regulation in lower vertebrates. Am. Zool., **23**, 719–727 (1983).
9. Cortelyou, J. R., McWhinnie, D. J.: Parathyroid glands in amphibians. I. Parathyroid structure and function in the amphibians, with emphasis on regulation of mineral ions in body fluids. Am. Zool., **7**, 843–855 (1987).
10. Cortelyou, J. R., Hibner-Owerko, A., Mulroy, J.: Blood and urine calcium changes in totally parathyroidectomized *Rana pipiens*. Endocrinology, **66**, 441–460 (1960).
11. Das, V. K., Swarup, K.: Effect of a calcium rich environment on the ultimobranchial gland *Rana tigrina*. Arch. d'Anat. Microsc., **64**, 261–272 (1975).
12. Dougherty, W. J.: Ultrastructural changes in the secretory cycle of parathyroid cells of winter frogs (*Rana pipiens*) after pituitary homo-implantation. Z. Zellforsch., **146**, 167–174 (1973).
13. Dubewar, D. M., Suryawanshi, S. M.: Role of parathyroid gland in estrogen-induced hypercalcemia of the frog *Rana tigrina*. Rev. Roum. Biol.-Biol. Anim., **23**, 59–61 (1978).
14. Emura, S., Shoumura, S., Isono, H.: Ultrastructural changes of the parathyroid gland of the estrogen treated golden hamster. J. Clin. Electron. Microsc., **15**, 113–126 (1982).
15. Kenny, A. D.: Vitamin D metabolism: Physiological regulation in egg-laying Japanese quail. Am. J. Physiol., **230**, 1609–1615 (1976).
16. Lange, R., Von Brehm, H.: On the fine structure of the parathyroid gland in the toad and the frog. In: The Parathyroid Glands, Gailard, P. J., Talmage, R. V. and Budy, A. M., eds, University of Chicago Press, Chicago, pp. 19–26 (1965).
17. Manivannan, B.: Some histophysiological studies on parathyroid and ultimobranchial glands in vertebrates with special reference to that for birds. Ph. D. Thesis, Karnatak University, Dharwad (1992).
18. Munday, K. A., Ansari, A. Q., Oldroid, D., Akhtar, M.: Oestrogen-induced calcium binding protein in *Xenopus laevis*. Biochem. Biophys. Acta, **166**, 748–751 (1968).
19. Oguri, M., Takada, N.: Serum calcium and magnesium levels of goldfish with special reference to gonadal maturation. Bull. Jpn. Soc. Sci. Fish., **33**, 161–166 (1967).
20. Pandey, A. K.: Occurrence of accessory parathyroid and connective tissue space in the parathyroid gland of *Bufo melanostictus* (Schneider). J. Adv. Zool., **6**, 109–111 (1985).
21. Pandey, A. K.: The effect of bovine parathyroid hormone on ultimobranchial body, parathyroid gland and paravertebral lime sacs as correlated with serum calcium and inorganic phosphorus levels in *Bufo melanostictus* Schneider. Folia. Biol., **36**, 275–280 (1988).
22. Pandey, A. K.: Calcium metabolism in the female toad *Bufo melanostictus*: Effect of aging. Bull. Inst. Zool. Acad. Sin., **27**, 145–150 (1988).

23. Pandey, A. K.: Structure and behaviour of the prolactin cell in relation to parathyroid hormone induced hypercalcemia in male *Bufo andersoni*. Biol. Struct. Morphogen., **2**, 81–83 (1989).
24. Pandey, A. K.: Changes in the prolactin cells of *Bufo melanostictus* in response to experimental hypercalcemia. Bolm. Fisiol. Anim. S. Paulo, **13**, 77–83 (1989).
25. Pandey, A. K.: Seasonal changes in the ultimobranchial body, parathyroid gland, serum calcium and inorganic phosphate levels of *Bufo melanostictus*. Biol. Struct. Morphogen., **3**, 101–106 (1990).
26. Pandey, A. K.: Endocrinology of calcium regulation in reptiles: A comparative aspect in lower vertebrates. Biol. Struct. Morphogen., **3**, 159–176 (1991).
27. Pandey, A. K., Swarup, K.: Seasonal changes in the ultimobranchial body and parathyroid of *Bufo andersoni* in relation to serum calcium and inorganic phosphate level. Bolm. Fisiol. Anim. S. Paulo, **13**, 55–65 (1989).
28. Pang, P. K. T., Kenny, A. D., Oguro, C.: Evolution of endocrine control of calcium regulation, in: Evolution of Vertebrate Endocrine Systems, pp. 323–356, Pang, P. K. T., and Epple, E. eds, Texas Tech. Univ. Press, Lubbock (1980).
29. Pathan, K. M., Nadkarni, V. B.: Effect of calcium rich environment on the ultimobranchial bodies of *Rana cyanophlyctic* treated with calciferol. Proc. Indian natn. Sci. Acad., **B52**, 375–379 (1986).
30. Polin, D., Sturkie, P. D.: Pituitary and gonad relationship in regulating blood calcium fractions in chickens. Endocrinology, **63**, 177–182 (1958).
31. Robertson, D. R.: The ultimobranchial body in *Rana pipiens*. IV. Hypercalcemia and glandular hypertrophy. Z. Zellforsch., **88**, 441–452 (1968).
32. Robertson, D. R.: Cytological and physiological activity of the ultimobranchial glands in the premetamorphic anura *Rana catesbeiana*. Gen. Comp. Endocrinol., **16**, 292–341 (1971).
33. Simkiss, K.: Calcium in Reproductive Physiology, Chapman and Hall, London (1967).
34. Spanos, E., Pike, W., Hanssler, M. R., Colston, K. W., Ewans, I. M. A., Goldner, A. M., McCain, T. A., Mac Intyre, I.: Circulating 1, 25 dihydroxyvitamin D in the chicken. Enhancement by injection of prolactin and during egg laying. Life. Sci., **19**, 1751–1756 (1976).
35. Srivastav, S. D., Swarup, K., Srivastava, S.: Seasonal changes in the serum calcium level and prolactin cells of *Clarias batrachus* in relation to reproductive cycle. Ann. Endocrinol., **46**, 426–429 (1985).
36. Swarup, K., Krishna, L.: Response of ultimobranchial body, parathyroid gland and paravertebral lime sacs to parathormone administration in *Rana cyanophlyctis* (Anura, Amphibia). Arch. Biol., **90**, 355–364 (1979).
37. Swarup, K., Krishna, L.: Studies of ultimobranchial body of *Rana cyanophlyctis* in response to experimental hypercalcemia. Z. Mikrosk.-anat. Forsch., **94**, 818–824 (1980).
38. Urist, M. R.: Avian parathyroid physiology, including a special comment on calcitonin. Am. Zool., **7**, 883–895 (1967).
39. Von Brehm, H.: Morphologische Untersuchungen an Epithelkörperchen (Glandulae parathyroideae) von Anuren. Z. Zellforsch. mikrosk. Anat., **61**, 376–400 (1963).
40. Waggener, R. A.: A histological study of the parathyroids of anura. J. Morphol., **48**, 1–49 (1929).
41. Wallace, R. A., Bergnik, E. W.: Amphibian vitellogenin: Properties, hormonal regulation of hepatic synthesis and ovarian uptake and conversion of yolk proteins. Am. Zool., **14**, 1159–1176 (1974).
42. Webster, W. W.: A simple microspectrophotometrie method for the determination of serum calcium. Am. J. Clin. Pathol., **37**, 330–333 (1962).
43. Woodhead, P. M. J., Woodhead, A. D.: Seasonal changes in the physiology of the cod in relation to its environment. I. Seasonal changes in the physiological reactions of Baventa sea cod *Gadus mouhera* L., particularly affecting migration and maturation. ICNAF Environ. Symp. Ser. No. 1255A, Contrib. No F–69, p. 25 (1964).

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The effect of Ambroxol on bleomycin-induced changes in phospholipid composition of rat lung surfactant

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The influence of Ambroxol on bleomycin-induced changes in lung surfactant phospholipid composition in rats was examined. Bleomycin alone (intratracheally 10 mg/kg, single dose) produces a significant fall in phosphatidylcholine (especially in disaturated species) and phosphatidylglicerol content in both lavage and postlavaged lung tissues surfactants. Ambroxol (8 days, 2 mg/kg s.c. per day) normalizes these changes. Thus, Ambroxol has an salutary effect on bleomycin-induced changes in lung surfactant phospholipids.

Keywords: lung injury, surfactant phospholipids, bleomycin, Ambroxol

Bleomycin, a polypeptide antibiotic from *Streptomyces vesticillus* is widely used as an antineoplastic agent [9]. Unfortunately the drug provokes a cumulative dose-dependent interstitial lung fibrosis [6, 15, 28]. Intratracheal administration of bleomycin is a widespread model, suitable to investigations on the pathogenesis of lung fibrosis [28, 31] since the histopathologic and biochemical features are similar to interstitial pulmonary fibrosis seen in humans [1]. Biochemical mechanism responsible for bleomycin-induced lung fibrosis are not yet fully understood.

It is known, that bleomycin is capable of generating reactive oxygen species in the presence of molecular oxygen [23, 29, 32] causing DNA strand scissions [7, 33] and cell membrane lipid peroxidation [18, 35]. Microscopic evaluation of the lung from mice treated with bleomycin revealed that the primary lesions were endothelial

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swelling, interstitial oedema, thickening of the alveolar wall, and proliferation of type II pneumocytes containing giant lamellar bodies [8]. These bodies are considered to be the main storage granules of surfactant in type II cells [11].

There are conflicting reports with respect to changes in the level of surfactant in the lung of bleomycin-treated animals. As Burkhardt [4] and Gebbers [10] have shown, the pathogenic basis for bleomycin-induced lung damage is a reduction in lamellar bodies and in consequence, a surfactant deficiency. This is diametrically opposed to the findings of Fasske and Morgenroth [8], who reported proliferation of the type II pneumocytes with overproduction of surfactant. In previous publication [17] we have shown that endotracheal bleomycin instillation of 10 mg/kg bleomycin cause after 7 days a great fall in phosphatidylcholine, desaturated ones and phosphatidylglycerol, both in the extra- and intracellular pool of the surfactant.

Ambroxol, trans-4[(2-amine-3,5-dibrombenzyl)amine]cyclohexanole hydrochloride has a bronchosecretolytic activity [5, 27] and is used in the treatment of respiratory distress [19, 26]. Winsel et al. [36] have shown, that Ambroxol diminishes oxygen-derived free radical production in yeast glucan-stimulated alveolar macrophages. In previous publication [16] we have also shown that Ambroxol lowered the Fe^{3+}/ADP/dihydroxyfumarate-induced malondialdehyde production in dog lung mitochondria.

The aim of this study was to investigate the effect of Ambroxol on phospholipid composition of bronchoalveolar lavage surfactant and post-lavaged lung tissue surfactant in rats on the 8th day after intratracheal administration of 10 mg/kg bleomycin.

Materials and Methods

The investigations were carried out on Sprague-Dawley male rats (CD strain, 250 – 300 g body wt.) from Laboratory Animal Breeding Centre, Agriculture-Technical Academy, Olsztyn. Rats were provided with water and food (LSM mix), housed in wire cages without bedding (maximal 3 animals in a cage) and maintained on a 12 h light/dark cycle. All animals were free from pulmonary pathogens. Bleomycin sulfate (Bleocina, Nippon Kayaku Co., Ltd., Tokyo, Japan) and Ambroxol (Mucosolvan, Dr. Thomae GmbH, Biberach Deutschland) were used throughout the study. After an adaptative period of 2 weeks intratracheal instillation of bleomycin was accomplished via a stainless cannula inserted into the trachea under general anaesthesia (Ketamine 100 mg/kg + Diazepam 5 mg/kg).

One group of rats (A, n = 20) remained untreated. A second group (B, n = 20) was instilled intratracheally with bleomycin (10 mg/kg). A third group (C, n = 20) was instilled with bleomycin as above and simultaneously injections of Ambroxol (2 mg/kg, i.m.) were repeated every day for the 8 days of experiment. Two hours after the last Ambroxol injection, bleomycin group (B) and bleomycin + Ambroxol group (C) were decapitated and lungs removed for biochemical assays.

Preweighed lungs were lavaged via trachea and bronchi with 6 portions of 0.154 M NaCl, 5 ml each. Pooled lavages were centrifuged (5 min, 200 g) to sediment desquamated cells and supernatant was centrifuged again in sucrose density gradient according to Oulton et al. [24]. After a new weighing of the

lung (to quantify lavage solution retained), 0.01 M Tris-HCl buffer containing 0.145 M NaCl and 1 mM EDTA was added, to obtain a 10% homogenate and tissues were homogenized in all-glass homogenizer. The homogenate was centrifuged (5 min, 200 g) and the supernatant was centrifuged again (30 min at 10 000 g). The sediment thus obtained was washed further as described above for bronchoalveolar lavage.

Protein content was measured as described by Lowry et al. [28] with bovine serum albumin as a standard.

Lipids were extracted with chloroform-methanol 2:1 mixture, phospholipids were separated by thin layer chromatography on precoated silica gel G plates (E. Merck, Darmstadt, Germany) as described previously [35]. In individual phospholipid fractions phosphorus was estimated according to Bartlett [3]. Disaturated phosphatidylcholines were estimated according to Mason et al. [22] with osmium tetroxide.

Statistical significance was determined using Student's *t*-test for unpaired data.

Results

The chemical composition of both pools of the surfactant is shown in Table I. Post-lavaged lung tissue surfactant shows a slight minor value of fraction weight to phosphorus content ratio, higher phosphorus, phospholipids and protein content, as compared to bronchoalveolar lavage surfactant.

Table I

Rat lung surfactant isolated from bronchoalveolar lavage (BAL) and post-lavaged lung tissue (PLLT) composition

	BAL	PLLT
Weight/phosphorus, μg/nM	1.20 ± 0.15	1.16 ± 0.13
Phosphorus, % of dry wt	2.92 ± 0.30	2.98 ± 0.35
Phospholipids, % of dry wt	72.5 ± 3.4	75.1 ± 4.3
Protein, % of dry wt	6.1 ± 0.8	7.5 ± 1.1

mean ± SD

Table II shows phospholipid composition of bronchoalveolar lavage surfactant. Bleomycin causes a significant increase in phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and lysophosphoglycerides with concomitant decrease in phosphatidylcholine, especially in its content of disaturated fractions.

Table II

Phospholipid composition of lung surfactant in bronchoalveolar lavage of rats (%)

	A	B	C
Phosphatidylethanolamine	0.5±0.1	1.3±0.2*	0.8±0.1
Phosphatidylserine	2.2±0.3	5.0±0.3*	2.6±0.4*
Phosphatidylinositol	3.4±0.5	5.8±0.6*	4.0±0.4
Phosphatidylcholine	75.1±2.3	63.2±2.5*	72.0±2.6
Disaturated phosphatidylcholine	52.2±1.9	44.1±1.9*	50.0±2.0
Phosphatidylglycerol	10.7±0.7	8.6±0.6*	10.9±0.6
Sphingomyelin	8.1±0.7	14.2±0.9*	9.5±0.8
Lysophosphoglycerides	0	1.9±0.2*	0.2±0.1*

Mean ± S.D.
Group designation as in Materials and Methods.
* – $p < 0.01$ B vs A and C vs A

Similar changes were observed in post-lavaged lung tissue surfactant (Table III). In both cases, Ambroxol administration strongly diminishes these changes.

Table III

Phospholipid composition of lung tissue rat lung surfactant following lavage (%)

	A	B	C
Phosphatidylethanolamine	0.8±0.1	2.0±0.2*	1.0±0.2
Phosphatidylserine	2.2±0.3	4.8±0.5*	2.5±0.3
Phosphatidylinositol	3.6±0.4	6.5±0.9*	3.8±0.5
Phosphatidylcholine	73.2±2.3	51.4±1.9*	70.0±2.5
Disaturated phosphatidylcholine	52.6±2.6	41.2±2.5*	49.7±2.7
Phosphatidylglycerol	12.1±0.3	7.5±0.8*	11.4±0.4
Sphingomyelin	8.1±0.2	20.3±1.4*	10.7±0.6*
Lysophosphoglycerides	0	7.5±0.7*	0.6±0.1*

Mean ± S.D.
Group designation as in Materials and Methods.
* – $p < 0.01$ B vs A and C vs A

Discussion

Lavage of lungs via the trachea an bronchi to obtain an extracellular alveolar lining gave preparation contaminated with lung surfactant [12, 30]. First Macklin [21] has shown that two pools of surfactant exist, one pool as a silver-stainable

mucopolysaccharide alveolar lining and another one as pneumocyte II lamellar bodies. The introduction of the methods for selective isolation of highly defined fraction containing lung surfactant made if possible to isolate an extra- and intracellular surfactant pool [24, 25].

None of the pools of rat lung surfactants obtained in our work have shown differences in their composition, as compared to surfactants of another animal species [14, 34].

In this work, as well as previously [17] we have observed a great decrease in phosphatidylcholine, disaturated phosphatidylcholine and phosphatidylglycerol content in both bronchoalveolar lavage surfactant and post-lavaged lung tissue surfactant of rats after endotracheal bleomycin instillation. Similar changes in pulmonary surfactant composition in human adult respiratory distress syndrome were observed by Ashbaugh et al. [2] and Hallman et al. [13].

Giri [12] has examined the uptake of [^{14}C]acetate by lung slices and its incorporation into lipids at various times following intratracheal administration of a fibrogenic dose of bleomycin in hamsters. Separation of lipids into various fractions revealed that bleomycin specifically inhibits the synthesis of phosphatidylcholine and neutral lipids. It is concluded that one of the reasons why synthesis of these lipids is depressed, may be the inhibition of fatty acid synthetase complex, a cytosolic enzyme which is responsible for conversion of acetate to fatty acid. It has been shown, that 80–90% of bleomycin present in the lung is covalently linked with the cytosolic proteins [12].

The results of our work strongly suggest that Ambroxol greatly diminishes the deteriorating changes in lung surfactant caused by endotracheal instillation of bleomycin in rats.

REFERENCES

1. Adamson, I. Y. R., Bowden, O. H.: The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. Am. J. Pathol., **77**, 185–196 (1971).
2. Ashbaugh, D. G., Bigelow, D. B., Petty, T. L., Levine, B. E.: Acute respiratory distress in adults. Lancet II, 319–321 (1967).
3. Bartlett, G. R.: Phosphorus assay in column chromatography. J. Biol. Chem., **234**, 466–468 (1959).
4. Burkhardt, A., Gebbers, J. O., Holtje, W. J.: Die Bleomycin-Lunge systematische pathologisch-anatomische Untersuchungen an 15 Fallen. Deutsch. Med. Wochenschr., **102**, 281–287 (1977).
5. Curti, P. C., Renovanz, H. D.: Therapeutische Studie mit Ambroxol bei chronisch bronchopulmonalen Erkrankungen. Arzneim. Forsch., **28**, 922–926 (1978).
6. Delena, M., Guzzon, A., Monfardini, S., Bonadonna, G.: Clinical, radiologic and histopathologic studies on pulmonary toxicity induced by treatment with bleomycin. Cancer Chemother. Rep., **56**, 343–348 (1982).
7. Ekimoto, H., Kuramochi, H., Tadahashi, K., Matsuda, A., Umezawa, H.: Kinetics of the reaction of bleomycin-Fe(II)O_2 complex with DNA. J. Antibiotics, **33**, 426–431 (1980).

8. Fasske, E., Morgenroth, K.: Experimental bleomycin lung in mice: a contribution to the pathogenesis of pulmonary fibrosis. Lung, **161**, 133–138 (1983).
9. Friedman, M. A.: A review of the bleomycin experience in the United States, recent results. Cancer Res., **63**, 152–156 (1978).
10. Gebbers, J. O., Burkhardt, A., Holtje, W. J.: Morphologische Lungenveränderungen nach Bleomycin-Therapie. Verh. Dtsch. Ges. Pathol., **59**, 561–567 (1975).
11. Gil, J., Reiss, D. K.: Isolation and characterization of lamellar bodies and tubular myelin from rat lung homogenates. J. Cell. Biol., **68**, 152–157 (1973).
12. Giri, S. N.: Pharmacokinetic, subcellular distribution and covalent binding of [^{3}H]-bleomycin in hamsters after intratracheal administration. Exp. Mol. Pathol., **45**, 327–336 (1986).
13. Hallman, M., Spragg, R., Harrell, J. H., Moser, K. M., Gluck, L.: Evidence of lung surfactant abnormality in respiratory failure. J. Clin. Invest., **70**, 673–679 (1982).
14. Keough, K. M. W., Farrell, E., Cox, M., Harrell, G., Taeusch, H. W.: Physical, chemical and physiological characteristics of isolates of pulmonary surfactant from adult rabbits. Can. J. Physiol. Pharmacol., **63**, 1043–1048 (1985).
15. Krous, H. F., Hamlin, W. B.: Pulmonary toxicity due to bleomycin. Arch. Pathol., **95**, 407–412 (1973).
16. Ledwożyw, A., Jabłonka, S., Tusińska, E. : The effect of Ambroxol on peroxidative processes in dog lung mitochondria. Arch. Wet. Polon., **31**, 106–113 (1991).
17. Ledwożyw, A., Ruciński, T., Synowiec, R.: The influence of endotracheal bleomycin administration on phospholipid composition of rat lung surfactant. Arch. Vet. Polon., **32**, 41–48 (1992).
18. Ledwożyw, A., Ruciński, T., Synowiec, R.: The influence of intratracheal bleomycin instillation on peroxidative processes in rat lung tissue. Arch. Vet. Polon., **31**, 116–123 (1991).
19. Lorenz, U., Rüttgers, H., Fux, G., Kubli, F.: Fetal pulmonary surfactant induction by bromhexine metabolite VIII. Am. J. Obstet. Gynecol., **119**, 1126–1130 (1974).
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193**, 265–365 (1951).
21. Macklin, C. C.: The pulmonary alveolar mucoid film and the pneumocytes. Lancet I, 1099–1093 (1954).
22. Mason, R. J., Nellenbogen, J., Clements, J. A.: Isolation of disaturated phosphatidylcholine with osmium tetroxide. J. Lipid Res., **17**, 281–288 (1976).
23. Oberley, L. W., Buettner, G. R.: The production of hydroxyl radical by bleomycin and iron(II). FEBS Lett., **97**, 47–50 (1979).
24. Oulton, M., Fraser, M., Dolphin, M., Yoon, R., Faulkner, G.: Quantification of surfactant pool sizes in rabbit lung during perinatal development. J. Lipid Res., **27**, 602–608 (1986).
25. Pawlowski, R., Frosolono, M. F., Charms, B. L., Przybylski, R.: Intra- and extracellular compartmentalization of the surface-active fraction from dog lung. J. Lipid Res., **12**, 538–542 (1971).
26. Prevost, M. C., Soula, G., Douste-Blazy, L.: Biochemical modifications of pulmonary surfactant after bromhexine derivative injection. Respiration, **37**, 215–220 (1979).
27. Püschmann, S., Engelhorn, R.: Pharmakologische Untersuchungen des Bromhexin-Metaboliten Ambroxol. Arzneim. Forsch., **28**, 889–892 (1978).
28. Raisfeld, I. H.: Pulmonary toxicity of bleomycin analogues. Toxicol. Appl. Pharmacol., **56**, 326–331 (1980).
29. Reidbord, H. E.: An electron microscopic study of the alveolar-capillary wall following intratracheal administration of saline and water. Am. J. Pathol., **51**, 275–286 (1967).

30 Shelley, S., A., Paciga, J. E., Ballis, J. U.: Lung surfactant phospholipids in different animal species. Lipids, **19**, 857–861 (1984).

31. Snider, G. L., Celli, B. R., Goldstein, R. H., O'Brien, J. J., Lucy, E. C.: Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin. Am. Rev. Resp. Dis., **177**, 289–295 (1978).

32. Sugiura, Y, Suzuki, T., Kuwahara, J., Tanaka, H.: On the mechanism of hydrogen peroxide, superoxide and ultraviolet light-induced DNA cleavage of inactive bleomycin-iron(II) complex. Biochem. Biophys. Res. Commun., **105**, 1511–1515 (1982).

33. Tanaka, Y., Takei, T.: Lung surfactants. I. Comparison of surfactants prepared from lungs of calf, ox, dog and rabbit. Chem. Pharm. Bull., **31**, 4091–4096 (1983).

34. Thrall, R. S., McCormack, J. R., McReynolds, R. M., Ward, P. A.: Bleomycin-induced pulmonary fibrosis in the rat. Am. J. Pathol., **95**, 117–125 (1979).

35. Wawrzkiewicz, K., Ledwożyw, A., Ziółkowska, G.: Lipid composition of the mycelial and spore forms of *Trichophyton verrucosum*. Zentralbl. Mikrobiol., **142**, 549–555 (1987).

36. Winsel, K., Grollmuss, H., Unger, U., Eckert, H.: Modulation der Alveolarmakrophagenaktivität durch Ambroxol, Bromhexin und exogene Arachidonsäure. Z. Erkrank. Atm. Organe, **165**, 149–155 (1985).

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Protective effect of the thiol agent WR–2721 against cyclophosphamide-induced cytotoxicity in the mouse erythropoietic system

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The effect of WR–2721 against cyclophosphamide-induced cytotoxicity was studied using the mouse micronucleus test. Adult male Swiss mice were treated intraperitoneally with a dose of 200 mg/kg or 400 mg/kg b.w. WR–2721, 15 or 30 minutes prior to cyclophosphamide administration, with a dose of 200 mg/kg b.w. The frequency of micronuclei in polychromatic erythrocytes and the number of polychromatic erythrocytes in the peripheral blood were determined 24-hours after CP treatment. In comparison with the controls, in mice injected with cyclophosphamide, the number of micronucleated polychromatic erythrocytes was increased, and the number of polychromatic erythrocytes was decreased. In mice treated with WR–2721 and CP, in relation to those injected with cyclophosphamide alone, the frequency of micronuclei in polychromatic erythrocytes was lower, but the number of polychromatic erythrocytes was found to be greater. The protective effect of WR–2721 against cyclophosphamide-induced clastogenicity and suppressing mitotic activity of the erythropoietic system caused by the alkylating drug was shown. The effect was dependent on the dose of the thiol compound applied and it was more expressive when WR–2721 was given in the higher dose, 400 mg/kg b.w. However, the protection by aminothiol appeared not to depend on the time intervals between WR–2721 and CP administration into mouse organism. The result may be useful for therapeutic application of WR–2721 with cyclophosphamide therapy.

Keywords: WR–2721, cyclophosphamide, mouse micronucleus test, peripheral blood

During the last decade, the mouse micronucleus test has become increasingly popular as a primary assay for detecting genotoxic agents [7, 9, 10, 12, 20]. The

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micronucleus test is regarded as a sensitive and rapid in vivo method for measuring certain types of chromosomal aberrations induced by ionizing radiation or chemical clastogens/aneugens [8, 10–12, 23, 29].

Cyclophosphamide, one of the biological alkylating agents is widely used to treat a variety of malignant and nonmalignant disorders. Although CP has a wide spectrum of clinical uses, it also possesses a wide spectrum of toxicity [1, 2, 4, 14, 17, 22]. The acute toxicity of this compound is related to its cytotoxicity. Like other alkylating drugs, cyclophosphamide is most toxic to rapidly proliferating tissues such as the haematopoietic system [1, 2, 4, 10].

The aminothiol compound WR–2721 (S–2–/3-aminopropylamino/ethyl-phosphorothioic acid) is known to protect cells from the cytotoxic effects of ionizing radiation and chemotherapeutic cytostatic agents [3, 6, 13, 15, 18, 21, 24, 26–28]. However, little information is available on the influence of WR–2721 on the effects of the chemotherapeutic drugs and especially cyclophosphamide [6, 18, 24, 25, 27, 28].

The purpose of the present experiments was to study possible protective effect of WR–2721 against cyclophosphamide-induced cytotoxicity in the mouse erythropoietic system. In the investigations the micronucleus test was used.

Materials and Methods

The experiments were carried out on adult male Swiss mice. All were kept under constant environmental conditions with a 12/12-h light/dark cycle (light: 08.00–20.00, dark: 20.00–08.00). They were fed standard granulated chow and given drinking water ad libitum.

WR–2721 (S–2-/3-aminopropylamino/ethyl-phosphorothioic acid, Institute of General Chemistry, Warsaw Agricultural University, Poland) was administered intraperitoneally, in a single dose of 200 mg/kg or 400 mg/kg body weight, 14 or 30 minutes prior to cyclophosphamide (Endoxan 200, CP, Asta Pharma AG, Germany) treatment. Cyclophosphamide was injected intraperitoneally, in a single dose of 200 mg/kg body weight, always at about 10.00 a.m. The doses of WR–2721 and CP applied in the present experiments, and the time intervals between the thiol administration and cyclophosphamide injection into mouse organism coincide with those used in experimental biology [1, 5, 13, 30]. WR–2721 was dissolved in aqua pro injectione (Polia, Poland) and CP was diluted in physiological saline (Polfa, Poland). The injected volume of each agent analysed was 300 μl per mouse. All solutions were freshly prepared immediately before treatment of the animals. Untreated mice served as controls. Each experimental group of mice consisted of 3–5 animals. The control group counted 7 mice.

Peripheral blood samples were collected 24 hours after CP treatment of mice. Peripheral blood smears were prepared (two slides per mouse) using blood obtained by snipping off the end of the tail. After air-drying, the smears were stained with May-Grunwald and Giemsa combination [16, 19]. With this method polychromatic erythrocytes (PCEs) stain reddish-blue and normochromatic erythrocytes (NCEs) stain orangey while nuclear material shows a dark purple color.

The number of micronucleated polychromatic erythrocytes (MNPCEs) among 2000 PCEs per mouse (1000 PCEs per slide) and the number of PCEs among 2000 erythrocytes per animal

(1000/PCEs+NCEs/ per slide) were determined twice by two persons. The slides were examined under 1250× magnification using a light Zeiss microscope (Germany).

Differences in the incidence per animal of MNPCEs per 1000 PCEs and of PCEs per 1000 erythrocytes /PCEs+NCEs/ were compared using an analysis of variance and the Duncan's new multiple range test.

Results

The experimental groups of mice showed statistically significant differences ($P < 0.05$) in the number of micronucleated polychromatic erythrocytes and the total number of polychromatic erythrocytes in the peripheral blood (Table I).

Table I

Number of MNPCEs per 1000 PCEs and PCEs per 1000 (PCEs+NCEs) in the peripheral blood of the particular groups of mice

Group characteristic	Number of MNPCEs per 1000 PCEs (mean ± SD)	PCEs per 1000 (PCEs+NCEs) (mean ± SD)
I $CP_{200\ mg}$	56.80 ± 10.75	18.10 ± 3.51
II $WR-2721_{200\ mg,\ -15\ min}$ + $CP_{200\ mg}$	35.25 ± 11.18	22.55 ± 3.05
III $WR-2721_{200\ mg,\ -30\ min}$ + $CP_{200\ mg}$	33.57 ± 9.16	25.64 ± 6.10
IV $WR-2721_{400\ mg,\ -15\ min}$ + $CP_{200\ mg}$	25.00 ± 2.53	28.14 ± 6.82
V $WR-2721_{400\ mg,\ -30\ min}$ + $CP_{200\ mg}$	22.71 ± 4.07	30.14 ± 7.24
VI control	3.67 ± 1.23	36.70 ± 2.50
	VI V IV III II I	I II III IV V VI
	—	—
	——	——
	———	———
	—	—

Significant differences at $P < 0.05$

Number of MNPCEs per 1000 PCEs

The number of micronucleated polychromatic erythrocytes among 1000 polychromatic erythrocytes was the greatest in Group I, intermediate in Groups II,

III, IV, and V, and the smallest one in Group VI. In this respect, there were no differences between Groups II and III, and Groups IV and V, respectively.

Number of PCEs per 1000 /PCEs+NCEs/

The number of polychromatic erythrocytes among 1000 polychromatic and normochromatic erythrocytes was the smallest in Group I, intermediate in Groups II, III, IV and V, and the greatest in Group VI, however no differences between Groups I and II, Groups II and III, and Groups III, IV and V, respectively, were found.

Discussion

The results of the present study have shown the protective effect of the aminothiol compound WR-2721 against cyclophosphamide-induced clastogenicity and suppressing mitotic activity caused by the alkylating drug in the mouse erythropoietic system. The effects of WR-2721 administration prior to cyclophosphamide injection were observed in the peripheral blood at 24 hours after CP treatment of mice. In relation to the controls, in mice treated with cyclophosphamide, in a dose of 200 mg/kg b.w., the number of micronucleated polychromatic erythrocytes was increased, and the number of polychromatic erythrocytes was decreased. In mice injected with WR-2721 and CP, in comparison with those treated with cyclophosphamide alone, the frequency of micronuclei in polychromatic erythrocytes was lower, and the number of polychromatic erythrocytes was found to be greater. The protection by WR-2721 against cyclophosphamide-induced cytotoxicity was dependent on the dose of the thiol agent given, and it was more expressive when WR-2721 was applied in the dose of 400 mg/kg than in the dose of 200 mg/kg b.w. However, the effect caused by the aminothiol appeared not to be dependent on the time intervals between WR-2721 and CP administration into mouse organism.

The cytotoxicity induced by cyclophosphamide is directly connected with its metabolism and toxic reactions of its metabolites. It is accepted that the alkylating agent can bind to a variety of molecules including water, amino acids, proteins, and peptides, but the most important site of binding is DNA where cross-linkages occur. This can lead to DNA strand breaks, inability to synthesise DNA, disruption of cell growth, mitotic activity, differentiation and function of cell, and ultimately to cell death [1, 2, 4, 10, 14, 17].

The proposed mechanisms for protection by WR-2721 are considered at the molecular and biochemical-physiological level as well as at the organ level. WR-2721's dephosphorylated metabolite, WR-1065, is thought to be the active

protective agent. However, the exact mechanisms by which the aminothiol produces protection against toxicity by the alkylating agents are still unclear [14, 21].

Considering the results obtained in the present investigations, it can be concluded, that WR-2721 offers protection for the normal tissue against the cytotoxic effects of the alkylating drug. So, a therapeutic application of WR-2721 with CP administration may provide a remarkable decrease of the adverse effects on normal cells, and especially cells of the rapidly proliferating tissues. Clinical significance of these findings with regard to the utilization for patients undergoing cyclophosphamide treatment has to be considered.

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REFERENCES

1. ASTA-Oncologica: Endoxan (Cyclophosphamide), Cytostatic. Basic Scientific Information, pp. 4-60 (1982).
2. Brookes, P.: The early history of the biological alkylating agents, 1918-1968. Mutat. Res., **233**, 3-14 (1990).
3. Brown, J. M.: Sensitizers and protectors in radiotherapy. Cancer, **55**, 2222-2228 (1985).
4. Fraiser, L. H., Kanekal, S., Kehrer, J. P.: Cyclophosphamide toxicity. Characterizing and avoiding the problem. Drugs, **42**, 781-795 (1991).
5. Gamba-Vitalo, Ch., DiGiovanna, M. P., Sartorelli, A. C.: Effects of recombinant murine granulocyte-macrophage colony-stimulating factor in cyclophosphamide-treated mice. Blood Cells, **17**, 193-205 (1991).
6. Glower, D., Grabelsky, S., Fox, K., Weiler, C., Cannon, L., Glick, J.: Clinical trials of WR-2721 and cis-platinum. Int. J. Radiat. Oncol. Biol. Phys., **16**, 1201-1204 (1989).
7. Grawe, J., Zetterberg, G., Amneus, H.: Flow-cytometric enumeration of micronucleated polychromatic erythrocytes in mouse peripheral blood. Cytometry, **13**, 750-758 (1992).
8. Heddle, J. A., Salamone, M. F.: Chromosomal aberrations and bone marrow toxicity. Environ. Health Perspectives, **39**, 23-27 (1981).
9. Heddle, J. A., Hite, M., Kirkhart, B., Mavourin, K., Mac Gregor, J. T., Newell, G. W., Salamone M. F.: The induction of micronuclei as a measure of genotoxocity. A report of the U.S. Environmental Protection Agency Gene-Tox Program, Mutat, Res., **123**, 61-118 (1983).
10. Krishna, G., Fiedler, R., Theiss, J. C.: Simultaneous evaluation of clastogenicity, aneugenicity and toxicity in the mouse micronucleus assay using immunofluorescence. Mutat. Res., **282**, 159-167 (1992).
11. Ludewig, E., Koch, F., Kamprad, F., Melzer, R.: The micronucleus test in pigs: induction of micronuclei in polychromatic erythrocytes by various doses of X-rays. Mutat. Res., **249**, 1-6 (1991).
12. Mavournin, K. H., Blakey, D. H., Cimino, M. C., Salamone, M. F., Heddle, J. A.: The in vivo micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox program. Mutat. Res., **239**, 29-80 (1990).

13. Milas, L., Hunter, N., Reid, B. O., Thames, H. D.: Protective effects of S-2-/3-aminopropylamino/ethyl-phosphorothioic acid against radiation damage of normal tissues and fibrosarcoma in mice. Cancer Res., **42**, 1838-1897 (1982).
14. Moore, M. J.: Clinical pharmacokinetics of cyclophosphamide. Clin. Pharmacokinet., **20**, 194-208 (1991).
15. Nori, D., Kim, J. H., Hilaris, B., Chu, F. C.: Radiosensitizers and protectors. Radiat. Ther. Gynecol. Cancer, Alan, R. Liss Inc., p. 319-329 (1987).
16. Pascoe, S., Gatehouse, D.: The use of a simple haemotoxylin and eosin staining procedure to demonstrate micronuclei within rodent bone marrow. Mutat. Res., **164**, 237-243 (1986).
17. Patel, J. M.: Metabolism and pulmonary toxicity of cyclophosphamide. Pharmac. Ther., **47**, 137-146 (1990).
18. Peters, G. J., van der Wilt, C. L., Gyergyay, F., van Laar J. A. M., Treskes, M., van der Vijgh, W. J. F., Pinedo, H. M.: Protection by WR-2721 of the toxicity induced by the combination of cisplatin and 5-fluorouracil. Int. J. Radiat. Oncol. Biol. Phys., **22**, 785-787 (1992).
19. Schmid, W.: The micronucleus test. Mutat. Res., **31**, 9-15 (1975).
20. Selig, Chr., Schlegelmilch, R., Wolf, H. U.: Development of a method to increase the proportion of polychromatic erythrocytes from mouse bone marrow for more rapid evaluation of the micronucleus assay. Mutat. Res., **234**, 23-30 (1990).
21. Shaw, L. M., Glover, D., Turrisi, A., Brown, D. Q., Bonner, H. S., Norfleet, A. L., Weiler, C., Glick, J. H., Kligerman, M. M.: Pharmacokinetics of WR-2721. Pharmac. Ther., **39**, 195-201 (1988).
22. Thompson, Ch. A.: Safe handling of cyclophosphamide. Am. J. Hosp. Pharmac., **47**, 75-76 (1990).
23. Tice, R. R., Erexson, G. L., Hilliard, C. J., Huston, J. L., Boehm, R. M., Gulati, D., Shelby, M. D.: Effect of treatment protocol and sample time on the frequencies of micronucleated polychromatic erythrocytes in mouse bone marrow and peripheral blood. Mutagenesis, **5**, 313-321 (1990).
24. Treskes, M., Holwerde, U., Klein, I., Pinedo, H. M., van der Vijgh, W. J. F.: The chemical reactivity of the modulating agent WR-2721 (ethiofos) and its main metabolites with the antitumor agents cisplatin and carboplatin. Biochem. Pharmacol., **42**, 2125-2130 (1991).
25. Twentyman, P. R.: Modification of tumor and host response to cyclophosphamide by misonidazole and by WR-2721. Br. J. Cancer, **43**, 745-755 (1981).
26. Weiss, J. F., Kumar, K. S., Walden, T. L., Neta, R., Landauer, M. R., Clark, E. P.: Advances in radioprotection through the use of combined agent regimens. Int. J. Radiat. Biol., **57**, 709-722 (1990).
27. Van der Wilt, C. L., van Laar, J. A. M., Gyergyay, F., Smid, K., Peters, G. J.: Biochemical modification of the toxicity of the anti-tumour effect of 5-fluorouracil and cisplatinum by WR-2721 in mice. Eur. J. Cancer, **28A**, 2017-2024 (1992).
28. Van Laar, J. A. M., can der Wilt, C. L., Treskes, M., van der Vijgh, W. J. F., Peters, G. J.: Effects of WR-2721 on the toxicity and antitumor activity of the combination of carboplatin and 5-fluorouracil. Cancer Chemother. Pharmacol., **31**, 97-102 (1992).
29. Vanparys, P., Deknudt, G., Vermeiren, F., Sysmans, M., Marsboom,R.: Sampling times in micronucleus testing. Mutat. Res., **282**, 191-192 (1992).
30. Yuhas, J. M.: Biological factors affecting the radioprotective efficiency of S-2(3-aminopropylamino)ethylphosphorothioic acid (WR-2721). $LD_{50/30}$ doses. Radiat. Res., **44**, 621-628 (1970).

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The influence of proline analogs on Bleomycin-induced lung injury in rats

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The influence of 3,4-dehydro-DL-proline and 3,4-dehydro-L-proline on lysyl oxidase, prolyl hydroxylase activities, collagen cross-linking and types of collagen in bleomycin-induced lung injury was investigated.

Both proline analogs cause a great fall in prolyl hydroxylase activity without effect on lysyl oxidase, diminish the number of cross-links and normalize the type I/type III collagen ratio.

It is suggested that proline analogs may be valuable agents in lung fibrosis treatment.

Keywords: proline analogs, lung fibrosis

At the present time, there is only poor understanding of the series of events which in some cases of acute lung injury lead to a rapidly developing pulmonary fibrosis [39], with an irreversible loss of respiratory function and death [25, 29]. In these instances, acute lung injury, regardless of the cause, sets in motion a series of metabolic events which ultimately result in an abnormal healing process with excessive or inappropriate deposition of collagen fibers.

Several classes of pharmacologic agents have been employed in efforts to limit the deposition of excessive amounts of collagen. Among these are proline analogs. These chemicals, such as cis-4-hydroxy-L-proline, azetidine-2-carboxylic acid, 3,4-dehydro-L-proline, cis-4-bromproline and cis-4-fluoro-L-proline have been studied extensively over the past decade as inhibitors of collagen synthesis both in vitro [12, 37] and in vivo [14, 28]. These analogs are incorporated in place of proline during the biosynthesis of proteins. The first experiments demonstrated clearly that collagen synthesized in the presence of proline analogs was secreted from connective tissue at

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a reduced rate [33, 34]. The inhibitory effects of the proline analogs on collagen synthesis were greater than those on protein synthesis in general [15, 32].

It was demonstrated, that proline analogs either directly, or indirectly affect the stability of the collagen triple helix [12].

The proline analogs incorporated into proteins contain either a modified pyrrolidine ring or substituents on C−4 of the pyrrolidine ring and thus cannot serve as substrates for prolylhydroxylase. Thus the molecules synthesized in the presence of these analogs are secreted at a reduced rate [13] and are more easily degraded [4, 5]. Of the proline analogs so far studied, 3,4-dehydro-L-proline is the most potent. This may be partially related to the fact, that of the proline analogs, 3,4-dehydro-L-proline is the most potent competitive inhibitor of prolyl-t-RNA synthetase [16]. Treatment with 3,4-dehydro-L-proline, more than with any of the other proline analogs, decreases the activity of prolyl hydroxylase [14, 17] and reduces the extent to which the normal proline residues in newly synthesized procollagen were hydroxylated [8].

The prevalent model for bleomycin-induced lung injury exposes animals via intratracheal instillation [3, 36]. This is an accepted general model for fibrogenic pulmonary toxicity widely used, because fibrosis readily occurs.

Some of the morphological and biochemical events associated with the fibrotic phase of the injury have been examined [19, 20]. There is an early inflammatory phase with a marked influx of neutrophils. Fibrosis develops with maximal collagen deposition 2−4 weeks after administration. Collagen synthesis rates are also raised, and there may be a reduced rate of collagen degradation [21]. It is also known that the relative ratios of the two major collagen types of the lung, types I and III are altered in patients that died of idiopathic pulmonary fibrosis or of the adult or infant respiratory distress syndrome. In animal models of acute lung fibrosis it has been established that comparable shifts in collagen type ratios are the result of excessive deposition of newly synthesized type I collagen in affected lungs [26].

The mode of investigation of the reducible cross-links has been the use of tritiated borohydride, but it should be remembered, that cross-links exist as the unreduced form in collagen [23]. Dehydrohydroxylysinonorleucine is an aldimine bifunctional cross-link which is formed by condensation of an allysine residue with a residue of hydroxylysine. Dehydrodihydroxylyxsinonorleucine results from the condensation of a hydroxyallysine residue with a residue of hydroxylysine and undergoes an Amadori rearrangement to the ketoimine form [27].

In previous work [22] it was demonstrated, that β-aminopropionitrile reduces the number of cross-links in collagen molecules and normalizes the collagen type I/type III ratio in bleomycin-injured rat lungs.

In this experiment, the influence of 3,4-dehydro-DL-proline and 3,4-dehydro-L-proline on collagen metabolism in bleomycin-induced lung fibrosis in rats is investigated.

Materials and Methods

3,4-Dehydro-DL-proline and 3,4-dehydro-L-proline were purchased from Sigma, St. Louis, USA.

Studies were carried out on 320–350 g male Sprague Dawley rats, certified free of specific pathogens. Rats were provided with food and water ad libitum, housed in laminar flow cages with bedding, and maintained on a 12 h light/dark cycle. Bleomycin sulfate (Bleocina, Nippon Kayaku Co., Tokyo, Japan) was used throughout this study. After an adaptative period of 4 weeks, intratracheal instillation of bleomycin was accomplished via a stainless steel cannula inserted into the trachea under general anaesthesia (Ketamine 100 mg/kg + Diazepam 5 mg/kg).

One group of rats (n = 10) remained completely untreated. A second group (n = 10) was instilled intratracheally with saline (0.2 ml), whereas another group (n = 30) received one single dose of bleomycin (10 mg/kg) in a volume of 0.2 ml. On this day an intraperitoneal injections of proline analogs in the doses of 100 mg/kg were started. These injections were repeated every 24 h thereafter during the 14-day experiment. Two hours after the last proline analogue injection (day 14 after bleomycin instillation) the rats of all groups were decapitated, the lungs were perfused through the pulmonary artery with 0.154 M NaCl to remove blood for the biochemical measurements.

A 10% homogenate of the left lung was prepared in 50 mM Tris-HCl buffer, pH = 7.2 containing 1×10^{-4} M dithiothreitol. 1×10^{-5} M EDTA and 0.1% Triton X–100 using a Waring Blendor homogenizer. The homogenate was centrifuged at 30 000 g for 30 min and the clear supernatant was assayed for prolyl hydroxylase activity by the method of Hutton et al. [11] as modified by Cutroneo et al. [7]. Lysyl oxidase activity was measured according to Harris and Garcia de Quevedo [10].

Fine pieces of the right lung (about 40 mg) were washed with 5 mM phosphate buffer containing 0.9% NaCl, pH = 7.4 and incubated in 3 ml of 0.1 M sodium phosphate, pH = 7.4 for 4 h at room temperature. $NaB[^3H]_4$ (142 Ci/mole, Amersham, England) was then added at a ratio of 1:30 (based on dry weight). After 1 h the reduction was stopped by the addition of 1 ml of glacial acetic acid. The tissue was then thoroughly rinsed with water, hydrolyzed in 6 M HCl for 18 h at 110 °C, evaporated to remove HCl, filtered and in the filtrate hydroxyproline content was estimated according to Kivirikko et al. [18].

The remained portions of right lungs were homogenized in a meat grinder and treated by a three-step pepsin digestion (0.5%). After centrifugation at 10 000 g, the supernatants were pooled and a crude mixture of collagens was precipitated from the 0.5 M acetic acid by addition of NaCl up to 1.2 M. The precipitate was dialyzed against distilled water and lyophilized. Lyophilized samples of purified collagen were digested by CNBr according to Bellon and Borel [2].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of collagens was performed in cylindrical tubes with a stacking gel of 3.5% and a separation gel of 5% acrylamide in 50 mM phosphate buffer, pH = 7.2, containing 0.1% sodium dodecyl sulfate. A current of 1 mA per tube (i.d. 7 mm) was used for 1.5 hour. Electrophoresis of CNBr-peptides was performed in gels of 12.5% acryl-amide in 0.37 M Tris-HCl buffer, pH = 7.8 containing 0.1% of sodium-dodecyl-sulfate. After electrophoresis, the gels were stained with Coomassie brilliant blue G 250 for 30 min and destained in 10% acetic acid for two periods of 24 h each. Then, the gels were sliced with a razor blade and every stained zone was transfered to a test tube to which 1 ml of 6 M HCl was added. The tubes were sealed and the content was hydrolyzed for 24 h at 110 °C. The hydrolyzates were evaporated to dryness under a nitrogen stream. The residues were dissolved in hot distilled water and then passed through a 6.5 cm × 1.0 cm (i.d.) column of

Dowex 50W – X2 in the H^+ form. Bound amino acids were then eluted with 15 ml of 2 M NH_4OH, which was then evaporated under a nitrogen stream. The residue was treated by a 7-chloro-4-nitrobenzo-2-oxo-1,3-diazole (Aldrich, Milwaukee, USA) solution and chromatographed on silica gel G precoated plates (E. Merck, Darmstadt, Germany). The plates were developed with the solvent system acetone-toluene-methanol-triethylamine 40:40:15:5 v/v/v/v. Fluorescent spots were scanned in a Farrand spectrofluorimeter equipped with a thin-layer attachment. As collagen standards Type I from bovine Achilles tendon, Type III from calf skin, Type IV from human placenta and Type V from bovine Achilles tendon (all from Sigma, St. Louis, USA) were used.

Statistical evaluation was accomplished by the Student's *t*-test for unpaired data.

Results

As shown in Table I lung lysyl oxidase and prolyl hydroxylase activities increase significantly in lung tissue of bleomycin-treated rats. Both 3,4-dehydro-DL-proline and 3,4-dehydro-L-proline cause a great fall in activity of prolyl hydroxylase, without effect on lysyl oxidase.

Table I

Effect of proline analogues on lysyl oxidase and prolyl hydroxylase activities in bleomycin-injured rat lungs (cpm/mg protein × 10^{-3}).

	Lysyl oxidase	Prolyl hydroxylase
Control	10.6 ± 0.8	8.7 ± 0.5
Bleomycin	122.7 ± 5.1*	65.3 ± 4.1*
Bleomycin + 3,4-dehydro-DL-proline	119.4 ± 4.8*	33.4 ± 1.6*
Bleomycin + 3,4-dehydro-L-proline	125.2 ± 5.3*	20.6 ± 1.1*

Mean values ± S.D. * – $p < 0.002$ treated vs control

Table II shows collagen cross-links in bleomycin- and proline analogs-treated rats. Cross-link numbers measured as dihydroxylysinenorleucine and hydroxylysinenorleucine contents were greatly increased in bleomycin-treated rats. Both proline analogues diminish, the number of cross-links, although its level remains elevated as compared to the control.

Table III shows collagen types in rat lungs examined this way. In fibrotic lung an increase in type I collagen with decrease in type III collagen were observed. Supplementation with proline analogues normalizes the type I/type III collagen ratio.

Table II

Collagen cross-links in bleomycin-injured rat lungs after the treatment with proline analogues (cpm/μg hydroxyproline)

	DHLNL	HLNL	DHLNL:HLNL
Control	108 ± 8	54 ± 7	2.0 ± 0.3
Bleomycin	882 ± 56*	178 ± 21*	5.0 ± 0.6*
Bleomycin + 3,4-dehydro-DL-proline	541 ± 32*	132 ± 9*	4.1 ± 0.5*
Bleomycin + 3,4-dehydro-L-proline	422 ± 27*	117 ± 10*	3.6 ± 0.5*

Mean values ± S.D. DHLNL – dihydroxylysinenorleucine. HLNL – hydroxylysinenorleucine. * – $p < 0.001$ treated vs control

Table III

Collagen types in bleomycin-injured rat lungs after proline analogues treatment

	I	III	IV	V	I/III
Control	40.2±3.1	37.5±1.7	14.8±0.8	7.5±0.9	1.07±0.08
Bleomycin	62.3±3.4	20.6±1.4	13.2±1.0	3.8±0.5	3.03±0.24
Bleomycin +3,4-dehydro--DL-proline	51.6±4.1	30.3±1.8	12.6±0.9	5.5±0.7	1.70±0.22
Bleomycin +3,4-dehydro--L-proline	45.2±2.8	34.0±2.0	13.8±1.1	7.0±0.8	1.33±0.16

Mean values ± S.D. Total collagen in each gel is assumed to be 100% and the different collagen types are calculated as relative percentages

Discussion

Attempts to prevent or to reverse pathophysiology of fibrotic diseases by altering the metabolism of fibrous tissue represent a relatively new area of pharmacologic interest. Because the physical characteristics of all fibrous lesions seem to depend on the physical properties of collagen fibers, investigators have concentrated on developing methods of affecting collagen metabolism. In order to be useful clinically, anticollagenous agents must specifically affect collagen without influence on the metabolism of other biologically important molecules and selectively affect collagen in pathologic lesions without influencing the structural integrity of normal mesenchymal tissues.

The most promising method of inhibiting collagen synthesis involve attempts to block specifically the hydroxylation of proline and lysine. Because collagen is the only protein containing significant amounts of hydroxyproline and hydroxylysine, a specific inhibition of hydroxylation should produce few, if any, effects on other biologically important proteins. It was shown, that with prolonged inhibition of hydroxylation, synthesis of collagen slows significantly [9], and underhydroxylated collagen seems to be degraded more rapidly than normal collagen [6].

Theoretically, the efficiency of proline analogs in inhibiting collagen hydroxylation depends on the magnitude of incorporation into collagen peptides. Under these assumption that both proline and its analog are incorporated randomly into protein, analog: free proline ratio should determine relative incorporation rates, although Thompson et al. [35] showed, that fasting animals reduce the free proline pool in the liver more than the level of other free amino acids. Madden et al. [24] demonstrate that eliminating dietary proline has no effect on free proline pool size. The endogenous production of proline from glutamate, ornithine and arginine seems sufficient to replace the endogenous source [1].

The assumption that incorporation of proline or its analogues into protein is random may not be valid. Rosenbloom and Prockop [30] using chick embryo cartilage, have shown that 3,4-dehydroproline is incorporated into protein at a rate one-fifth that of proline. This observation was confirmed by Kerwar and Felix [15] using L929 mouse fibroblasts, 3T3 mouse fibroblasts and WI-38 human diploid lung fibroblast cell cultures.

In addition to their effects on collagen biosynthesis, proline analogues may also have an effect on the proliferation of the fibroblasts which synthesize collagen. In vitro experiments with cis-4-hydroxyproline and azetidine-2-carboxylic acid have demonstrated that these analogues inhibit the proliferation of fibroblasts in culture [38].

In rat studies, the D-isomer of 3,4-dehydroproline had only a small inhibitory effect upon collagen synthesis which possibly was due to its conversion to the L-isomer *in vivo* [32].

Toxicity of 3,4-dehydroproline is relatively low. In the rat, the acute intravenous LD_{50} dose was significantly higher with the D-(800 mg/kg) than with L-isomer (400 mg/kg). In 2-month-old rats of both sexes, the administration of 25 and 50 mg/kg/day of 3,4-dehydro-L-proline did not cause microscopic or clinical chemistry changes of concern. Beginning signs of toxicity were observed in rats administered 100 mg/kg/day and these become more severe at 200 mg/kg/day [31].

As far as we know there is the first time to give evidence that both 3,4-dehydro-DL-proline and 3,4-dehydro-L-proline reduce the number of collagen cross-links and normalize the type I/type III collagen ratio in bleomycin-induced lung fibrosis in rats.

REFERENCES

1. Adams, E.: Metabolism of proline and of hydroxyproline. Int. Rev. Connect. Tis. Res., **5**, 2–91 (1970).
2. Bellon, G., Borel, J. P.: Quantitative evaluation of collagen fractions separated by acrylamide gel electrophoresis: its application for collagen typing in normal and pathological tissues. J. Chromatogr., **433**, 81–94 (1988).
3. Berend, N.: The effect of bleomycin and oxygen on rat lung. Pathology, **16**, 136–142 (1984).
4. Berg, R. A., Schwartz, M. L., Crystal, R.: Regulation of the production of secretory proteins: intracellular degradation of newly synthesized "defective collagen". Proc. Natl. Acad. Sci. USA, **77**, 4746–4750 (1980).
5. Bienkowski, R. S., Baum, B. J., Crystal, R. G.: Fibroblasts degrade newly synthesized collagen within the cell before secretion. Nature, **276**, 413–416 (1978).
6. Chvapil, M., Hurych, J.: Control of collagen biosynthesis. Int. Rev. Connect. Tissue Res., **4**, 67–196 (1968).
7. Cutroneo, K. R., Bewman, R. A., Prichard, D. M., Guzman, N. A., Sharawy, M. M.: Localization of collagen synthesizing ribosomes on the dense segments of the endoplasmic reticulum. Int. J. Biochem., **8**, 421–427 (1977).
8. Fiedler-Nagy, C., Bruckner, P., Hayashi, T., Fietzek, R. P., Prockop, D.: Structure and helical stability of a modified procollagen synthesized in the presence of 3,4-dehydroproline. J. Biol. Chem., **257**, 9181–9188 (1982).
9. Grant, M. E., Prockop, D. J.: The biosynthesis of collagen. N. Engl. J. Med., **286**, 194–199, 242–249, 291–300 (1972).
10. Harris, E. D., Garcia de Quevedo, M. C.: Reaction of lysyl oxidase with soluble protein substrate: effect of neutral salts. Arch. Biochem. Biophys., **190**, 227–233 (1978).
11. Hutton, J. J., Tappel, A. L., Udenfriend, S.: A rapid assay for collagen proline hydroxylase. Anal. Biochem., **16**, 384–394 (1966).
12. Jimenez, S. A., Rosenbloom, J.: Decreased thermal stability of collagens containing analogs of proline or lysine. Arch. Biochem. Biophys., **163**, 239–299 (1974).
13. Kao, W. W. Y., Prockop, D. J., Berg, D. A.: Kinetics for the secretion of non-helical procollagen by freshly isolated tendon cells. J. Biol. Chem., **254**, 2234–2244 (1979).
14. Kelley, J., Newman, R. A., Evans, J. N.: Bleomycin-induced pulmonary fibrosis in the rat. Prevention with an inhibitor of collagen synthesis. J. Lab. Clin. Med., **96**, 954–963 (1980).
15. Kerwar, S. S., Felix, A. M., Marcel, R. J., Tsai, I., Salvador, R. A.: Effect of L-3,4-dehydroproline on collagen synthesis and prolyl hydroxylase activity in mammalian cell cultures. J. Biol. Chem., **251**, 503–508 (1976).
16. Kerwar, S. S., Marcel, R. J., Salvador, R. A.: Studies on the effect of L-3,4-dehydroproline on collagen synthesis by chick embryo polysomes. Arch. Biochem. Biophys., **172**, 685–688 (1976).
17. Kerwar, S., S., Oronsky, A. L., Choe, D., Alvarez, B.: Studies on the effects of 3,4-dehydroproline on collagen metabolism in carbon tetrachloride-induced hepatic fibrosis. Arch. Biochem. Biophys., **182**, 118–123 (1977).
18. Kivirikko, K. I., Laitinen, O., Prockop, D. J.: Modifications of a specific assay for hydroxyproline in urine. Anal. Biochem., **19**, 249–255 (1967).
19. Laurent, G. J.: Biochemical pathways leading to collagen deposition in pulmonary fibrosis. Ciba Found. Sump., **114**, 223–233 (1985).
20. Laurent, G. J.: Lung collagen: more than scaffolding. Thorax, **41**, 418–428 (1986).

21. Laurent, G. J., McAnulty, R. J.: Protein metabolism during bleomycin-induced pulmonary fibrosis in rabbits. In vivo evidence for collagen accumulation due to increased synthesis and decreased degradation of the newly synthesized collagen. Am. Rev. Resp. Dis., **128**, 82–88 (1983).
22. Ledwozyw, A.: The effect of β-aminopropionitrile on bleomycin-induced lung injury in rats. Submitted for publication.
23. Light, N. D., Bailey, A. J.: Covalent cross-links in collagen. Meth. Enzymol., **82**, 360–372 (1982).
24. Madden, J. W., Chvapil, M., Carlson, E. C., Ryan, J. N.: Toxicity and metabolic effects of 3,4-dehydroproline in mice. Toxicol. Appl. Pharmacol., **26**, 426–437 (1973).
25. Petty, T. L., Newman, J. H.: Adult respiratory distress syndrome. West. J. Med., **128**, 399–407 (1978).
26. Reiser, K. M., Last, J. A.: A molecular marker for fibrotic collagen in lungs of infants with respiratory distress syndrome. Biochem. Med. Metab. Biol., **37**, 16–21 (1987).
27. Ricard-Blum, S., Ville, G.: Collagen cross-linking. Cell. Mol. Biol., **34**, 581–590 (1988).
28. Riley, D. J., Berg, R. A., Edelman, N. H., Prockop, D. J.: Prevention of collagen deposition following pulmonary oxygen toxicity in the rat by cis-4-hydroxy-L-proline. J. Clin. Invest., **65**, 643–651 (1980).
29. Rinaldo, J. E., Rogers, R. M.: Adult respiratory distress syndrome. N. Engl. J. Med., **300**, 900–909 (1982).
30. Rosenbloom, J., Prockop, D. J.: Incorporation of 3,4-dehydroproline into protocollagen and collagen. J. Biol. Chem., **245**, 3361–3368 (1970).
31. Salvador, R. A., Fiedler-Nagy, C., Coffey, J. W.: Biochemical basis for drug therapy to prevent pulmonary fibrosis in ARDS. In: Acute respiratory failure, eds Zapol, W. M., Falke, K. J., Marcel Dekker Inc., New-York – Basel 1985, p. 477.
32. Salvador, R. A., Tsai, I., Marcel, R. J., Felix, A. M., Kerwar, S. S.: The in vivo inhibition of collagen synthesis and the reduction of prolyl hydroxylase activity by 3,4-dehydroproline. Arch. Biochem. Biophys., **174**, 381–392 (1976).
33. Takeuchi, T., Prockop, D. J.: Biosynthesis of abnormal collagens with amino acid analogs. I. Incorporation of L-azetidine-2-carboxylic acid and cis-4-fluoro-L-proline into protocollagen and collagen. Biochim. Biophys. Acta, **174**, 142–155 (1969).
34. Takeuchi, T., Rosenbloom, J., Prockop, D. J.: Biosynthesis of abnormal collagen with amino acid analogs. II. Inability of cartilage cells to extrude collagen polypeptides containing L-azetidine-2-carboxylic acid or cis-4-fluoro-L-proline. Biochim. Biophys. Acta, **175**, 156–164 (1969).
35. Thompson, H. T., Schurr, P. E., Henderson, L. M., Elvehjem, C. A.: The influence of fasting and nitrogen deprivation on the concentration of free amino acids in rat tissues. J. Biol. Chem., **182**, 47–53 (1950).
36. Tryka, A. F., Godleski, J. J., Brain, J. D.: Differences in effects of immediate and delayed hyperoxic exposure on bleomycin-induced pulmonary injury. Cancer Treat. Rep., **68**, 759–764 (1984).
37. Uitto, J., Prockop, D. J.: Incorporation of proline analogues into collagen polypeptides. Biochim. Biophys. Acta, **336**, 241–251 (1974).
38. Uitto, J., Tan, E. M. L., Ryhanen, L.: Inhibition of collagen accumulation in fibrotic process: review of pharmacological agents and new approaches with amino-acids and their analogues. J. Invest. Dermatol., **79**, 113s–120s (1982).
39. Zapol, W. M., Trelstad, R. L., Coffey, R. L., Tsai, J. W., Salvador, R. A.: Pulmonary fibrosis in severe acute respiratory failure. Am. Rev. Resp. Dis., **119**, 547–553 (1979).

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In Memoriam Professor **László Hársing,** M. D., D. Sc. Med. (1920–1995)

Professor László Hársing died in Budapest on April 8 1995 at the age of 75. He graduated in 1944 at the Medical Faculty of the Pázmány Péter University in Budapest, Hungary. His interest in the field of physiology commenced at the 1st Department of Internal Medicine working on the pathomechanism of tubular azotaemia, under the influence of Professors István Rusznyák, Pál Gömöri and Péter Bálint.

He then moved to the Institute of Physiology, in 1950, where he became interested in renal physiology. Indeed, he was the first scientist to describe the functional aspects of the tubuloglomerular feed-back mechanism, which was based on the morphological findings of DeMuylder and Peters. This important classical finding was the first observation of this later widely investigated phenomenon in the field of nephrology.

Later in his life, he worked in many other fields. For example, he investigated the effect of hypothermia on renal circulation and later developed a new radioactive method measuring the intrarenal distribution of blood flow in different normal and pathological states.

He obtained his Ph. D. degree in 1955, his D. Sc. degree in 1969. Then in 1974 he became Professor and Director of the Institute of Pathophysiology of the Budapest University (in 1969 renamed the Semmelweis University Medical School) and remained at this post until his retirement in 1990. In his new Institute he once more developed a flourishing school of renal physiology. In these years he turned to the problem of compensatory renal hypertrophy. Although he remained an international expert in nephrology, he also served as Scientific Counsellor of the Department of Oral Biology since 1990.

He was a memorable teacher, and an excellent lecturer, he encouraged a high standard of teaching in his Institute. He promoted a new curriculum in pathophysiology as a modern, functional scientific subject. He emphasized the value of preclinical lecturers who were best suited to convey to vocational students the clinical relevance of the preclinical subjects. In the mid-80s he was one of about dozen experts who have founded the International Society for Pathophysiology.

In 1948 he was elected ordinary member of the Hungarian Physiological Society; he served between 1966–1982 as General Secretary, and between 1982–1990 as President of the Society. His most important achievement was to continue the traditional society-life environment, unique in Hungary at that time, of free discussion and debate, and in which all members, whatever position they held at the time, might join.

Another of his important efforts was to promote the participation of young Hungarian physiologists in international congresses. He was General Secretary of the XXVIIIth International Union of Physiological Sciences Congress held in Budapest, in 1980 with more than 6000 participants. The Congress which was the first large, international scientific event organized in the Central European territory, was exceptionally well-organized and gained wide international acknowledgement.

He was respected and well-liked as a colleague. He combined a formidable intellect with deep humanity, wisdom, and optimism.

He will be well remembered by his many colleagues, students and friends.

Emil Monos

Professor of Physiology
President of the Hungarian
Physiological Society

Lajos Szollár

Professor of Pathophysiology
General Secretary of the
Hungarian Physiological Society

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The effect of long-term tilting on capillary supply in rat hindlimb muscles

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Rats were kept for three weeks tilted head-up at 45° and their capillary supply (capillary density CD, number of capillaries/mm^2, and capillary/fibre ratio C/F), fibre density FD, and muscle weights were compared with a group of animals with similarly restricted movement but at horizontal position, and with control freely moving animals. Movement restriction caused loss of muscle (but not body) weight in fast muscles in all rats, and gain of weight in slow postural soleus in the tilted group. Neither of these changes were due to changes in the content of water. Loss of weight was accompanied by increased fibre density indicative of muscle atrophy due to movement restriction. Capillary supply was not affected by movement restriction, but was significantly decreased, particularly in the oxidative part of tibialis anterior and in the soleus of tilted animals (C/F: 1.78 ± 0.11 vs 2.11 ± 0.05 and 2.06 ± 0.08 vs 2.29 ± 0.10). It is assumed that this reduction might be explained by changes in hemodynamic characteristics of the venous vascular bed due to a chronic increase in hydrostatic pressure load.

Keywords: angiogenesis, gravitational load, muscle vascularisation, oxidative muscles, vascular rarefaction

Capillary growth in skeletal muscles can be achieved by mechanical forces resulting from increased blood flow [3, 10] which include either increased shear stress or increased capillary wall tension [4]. Capillary wall tension is quite clearly dependent on capillary diameter and transcapillary pressure, but there are very few data relating the latter parameter to possible capillary growth. There is no evidence for capillary growth in hypertensive animals [5, 7] where capillary pressure may be increased [20], and there is no reference towards growth in skin capillaries in cases of increased venous pressure (e.g. in case of vein valve insufficiency). In skeletal

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muscles, capillary growth can be elicited within 2–4 days by chronic electrical stimulation [9], it starts at the venous end of capillaries which have wider diameters [15] but it is not known whether this can be due to increased venular pressure. Monos et al. [14] described an experimental model where hind-limb venous pressure was doubled when animals were kept chronically in 45° head-up tilted position. The purpose of this tilt investigation was to establish the effect of the tilt procedure on capillary supply in hind-limb skeletal muscles.

Methods

Nine male Sprague-Dawley rats (initial body weight 290.5 ± 5.1 g) were divided into three groups. The control group was kept in regular cages of horizontal position, chronically tilted animals were kept in tube-like plastic cages maintained at 45° for three weeks, similarly as described previously [14], and the last group (restricted animals) was kept for three weeks in similar tubes in a horizontal position. Animals with restricted movement were allowed one hour of free movement for grooming each day, and had normal intake of water and food.

After three weeks, all animals were anaesthetized with sodium pentobarbitone (5 mg/100 g body weight) and fast (tibialis anterior TA, and extensor digitorum longus EDL), as well as slow (soleus) muscles were taken out from both limbs (n = 54 muscles total). All muscles were weighed, the middle part of each muscle was taken for histology and the rest was minced and dried to estimate the content of water. Samples intended for histochemical examination were mounted on corks using OTC medium, frozen in isopenthane, precooled in liquid nitrogen and stored at −70°. Cryostat sections 10 μm thick were stained for alkaline phosphatase (which depicts all anatomically present capillaries as black dots) using tetrazolium method [21] and for ATPase after acid preincubation (pH 4.35) to estimate the proportion of fast glycolytic, fast oxidative, and slow muscle fibres [1]. The number of all capillaries and muscle fibres were counted using an unbiased method in two square field (each 0.2 mm^2) in EDL and in soleus, and separately in a predominantly glycolytic cortex and oxidative core (two fields in either region) in TA. Capillary supply was then expressed either as capillary density CD/mm^2, or capillary/muscle fibre ratio C/F. Fibre density FD, was expressed as number of fibres/mm^2. Care was taken to select areas without connective tissue or larger vessels.

All results are presented as means ± SEM and were analysed using Student's unpaired *t*-test.

Results

The results are presented in Tables I, II and III, and in Fig. 1. Body weights were slightly but not significantly lower in rats with restricted movement but not in tilted rats compared to controls. In contrast to body weights, fast muscles were lighter in both restricted and tilted rats while the postural slow soleus was significantly heavier in tilted and slightly but not significantly heavier in the restricted animals (Table I). The changes in muscle weights indicating artophy of fast and hypertrophy of slow muscle were not due to a change in the content of water which was not significantly different from controls in any of the muscles studied in either experimental group (Table II).

Table I

Body weights, muscle weights, and muscle/body weights
(Means ± SEM)

Body weights (g)
n = 3 in each group

Control	Restricted	Tilted
363 ± 17	324 ± 5	350 ± 4*

*P < 0.05 vs restricted

Muscle weights (mg)
n = 6 in each group

Control			Restricted			Tilted		
TA	EDL	SOL	TA	EDL	SOL	TA	EDL	SOL
691	191	160	534	168	178	600	177	188
±32	5	8	13	2	7	19	4	5
			***	***	NS	*	*	**

***P < 0.005 vs C
**P < 0.02 vs C
*P < 0.05 vs C
T vs R NS with the exception of TA P < 0.02

Muscle/body weights (mg/g)
n = 6 in each group

Control			Restricted			Tilted		
TA	EDL	SOL	TA	EDL	SOL	TA	EDL	SOL
1.900	0.524	0.442	1.651	0.520	0.549	1.719	0.506	0.540
±0.04	0.006	0.02	0.039	0.007	0.21	0.045	0.008	0.016
			***	NS	***	**	NS	***

***P < 0.005 vs C
**P < 0.02 vs C
T vs R: NS

The loss of muscle weight was linked with muscle fibre atrophy: fibre density, and thus number of fibres per area was slightly increased in fast muscles in all animals with restricted movement irrespective of position, but the difference was only significant in the glycolytic cortex of TA which has larger fibres than the oxidative core or than EDL. The small increase in FD in soleus was not significant, and it could thus be assumed that the increase in muscle weight in this muscle in the tilted animals could be due to hyperplasia rather than hypertrophy.

Table II

% water content in rat muscles

(All values are based on an average of 6 muscles from 3 rats and given as means ± SEM)

	TA	EDL	SOL
Control	74.67 ± 0.14	71.36 ± 0.79	70.75 ± 1.74
Restricted	71.98 ± 1.43	71.10 ± 0.42	73.60 ± 0.65
Tilted	74.31 ± 0.48	71.25 ± 1.24	71.95 ± 1.17

Proportion of slow fibres

(All values are based on counting fibres in 20 muscle bundles from one animal)

	TA		EDL	SOL
	Core	Cortex		
Control	6.5 ± 1.1	0	8.3 ± 1.1	78.6 ± 1.9
Restricted	10.6 ± 1.5**	13.6 ± 2.0	7.5 ± 0.6	88.7 ± 1.6**
Tilted	10.4 ± 1.7*	0	9.5 ± 1.3	86.6 ± 1.9*

*P < 0.02 vs C **P < 0.01 vs C

Table III

Capillary density, fibre density and capillary/fibre ratio

	CD			
	TA		EDL	SOL
	Core	Cortex		
Control	1073 ± 71	228 ± 23	552 ± 49	685 ± 29
Restricted	1141 ± 33	345 ± 17	508 ± 35	688 ± 26
Tilted	980 ± 68+	325 ± 35	555 ± 72	624 ± 33
	FD			
Control	495 ± 23	203 ± 11	295 ± 14	282 ± 11
Restricted	542 ± 15	293 ± 13**	309 ± 21	311 ± 22
Tilted	549 ± 19	261 ± 18**	337 ± 32	317 ± 32
	C/F			
Control	2.17 ± 0.11	1.40 ± 0.07	1.85 ± 0.11	2.45 ± 0.10
Restricted	2.11 ± 0.05	1.22 ± 0.10	1.68 ± 0.12	2.29 ± 0.10
Tilted	1.78 ± 0.11●**	1.18 ± 0.07**	1.60 ± 0.08	2.06 ± 0.08+**

+P < 0.05 vs R ●P < 0.01 vs R **P < 0.02 vs C

CD = cap/mm^2, FD = fibres/mm^2

(All values are from 6 muscles in 3 animals of each group, 2 fields per muscle – 144 fields total, each field 0.4 mm^2)

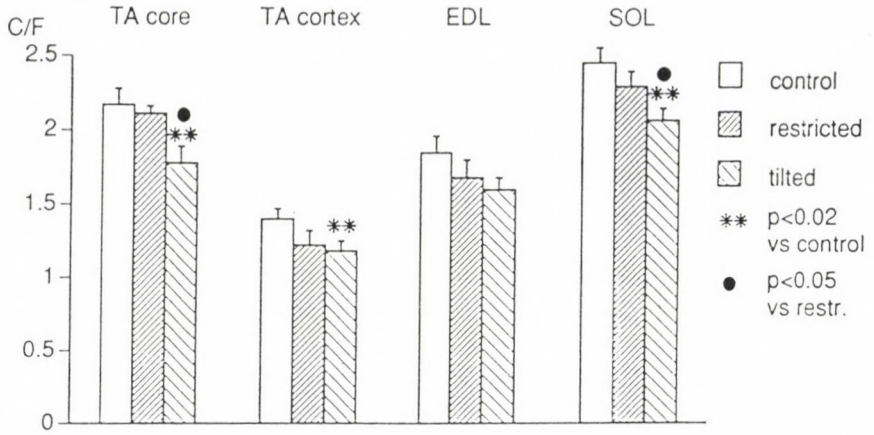

Fig. 1. Capillary/fibre ratio (C/F) in hindlimb skeletal muscles: oxidative (core) and glycolytic (cortex) part of tibialis anterior (TA), mixed extensor digitorum longus (EDL) and oxidative soleus (SOL)

Capillary supply was assessed both as CD and as C/F (Table III). CD was not changed in EDL or soleus, and was slightly but not significantly increased in the glycolytic part of TA in both groups; however, in view of fibre atrophy this increase was solely due to the fact that capillaries were closer to each other because of smaller muscle fibres, and capillary supply assessed as C/F ratio in tilted rats was actually significantly lower in comparison with controls (Fig. 1). CD in the oxidative muscles was either not significantly changed (soleus) or decreased (TA core), and C/F was significantly lower in these muscles not only in comparison with controls, but also with respect to animals with restricted movement (Fig. 1). Thus only tilting but not movement restriction resulted in a change in capillary supply with a decrease occurring preferentially in oxidative muscles.

Discussion

Restriction of movement caused a mild but not significant slowdown of physiological body weight increase which was not evident in tilted rats. Although the number of animals in each group in this series of experiments is rather small, the results are in accordance with those reported earlier [14].

Muscle weights were affected in the restricted as well as in the tilted group, with significant loss of weight in fast muscles (TA and EDL) in comparison with controls, and gain in weight in the postural slow oxidative soleus; however, the gain of weight in soleus was significant only in the tilted rats.

Muscle/body weight ratio reflected changes similar to muscle weights, i.e. loss of weight in the fast tibialis (but not EDL), and gain in soleus which was significant in restricted as well as tilted rats in comparison with controls. The difference between the tilted and restricted animals was in most cases not significant, with the exception of a smaller loss of weight in TA. This, together with a greater weight gain in soleus in tilted rats, indicates that tilted animals were using the postural muscle to a grater extent, but this was not accompanied by fibre hypertrophy.

Loss of weight in tibialis anterior and in EDL was accompanied by a higher density of muscle fibres indicative of fibre atrophy, in TA this was significant only in the glycolytic cortex and was probably due to movement restriction. There was an increase in the proportion of slow fibres both in TA and soleus which is consistent with a greater usage of these fibres in animals with restricted movement.

Capillary supply was not affected by movement restriction, but was decreased in tilted rats and this was more obvious in highly oxidative muscles. Although most changes in muscle weight and fibre composition may be attributed to the restriction of movement, decreased capillary supply – rarefaction of capillaries – was specific for tilting and is certainly contrary to the expected findings with prediction of increased capillary filtration due to increased capillary pressure and thus increased volume of interstitial fluid. This obviously did not happen since there was no evidence of oedema formation.

There are at least two possible explanations of decreased capillary supply after chronic tilting – one connected with changes in microcirculatory hemodynamics and the other one includes humoral mediators. Although the venous pressure is increased by tilting, it is possible that this might result in a veno-arterial reflex and limitation of flow through the capillary bed described earlier by Henriksen et al. [8]. Capillary pressure and wall tension would then be descreased and this could lead to lack of flow through some capillaries with their consequent closure and disappearance as it was described during development by Clark [2]. Simulation experiments in a network model applying a stress-growth principle suggest that circumferential wall stresses above an upper threshold cause vascular growth while stresses below a lower threshold cause resorption and thus microvascular rarefaction [17]. Since the difference between arterial and venous pressure in soleus is less than in EDL [19], it is possible that flow from soleus would be affected more and more capillaries would collapse. The decrease in capillary supply of certain muscles in tilted rats is similar to rarefaction of capillaries and arterioles found in various types of hypertension [6] where it was explained by a decreased perfusion possibly due to increased contraction to e.g. oxygen or decreased response to dilators such as adenosine [13]. We do not know whether tilting affects the reactivity of arterioles and/or venules to various metabolites, but it could alter the hormonal balance. In hypertension, microvascular rarefaction can be explained by increased levels of angiotensin II

which is supposed to stimulate TGF-beta [11, 12] – a factor which inhibits angiogenesis [16, 18]. Although it has been shown that tilting does not produce permanent stress and does not increase the levels of ACTH and corticosterone [14], there is so far no information about possible changes in angiotensin II or other factors which might be involved in growth or involution of capillaries in this specific model of vascular adaptation.

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REFERENCES

1. Brooke, M. H., Kaiser, K. K.: Some comments on the histochemical characterization of muscle adenosine triphosphatase. J. Histochem. Cytochem. **17**, 431 – 432 (1969).
2. Clark, E. R.: Studies on the growth of blood vessels in the tail of the frog. Am. J. Anat. **23**, 37 – 88 (1918).
3. Dawson, J. M., Hudlicka, O.: The effect of long-term administration of prazosin on the microcirculation in skeletal muscles. Cardiovasc. Res. **23**, 913 – 920 (1989).
4. Dawson, J. M., Hudlicka, O.: Can changes in microcirculation explain capillary growth in skeletal muscles? Int. J. Exp. Path. **74**, 65 – 71 (1993).
5. Gray, S. D.: Morphometic analysis of skeletal muscle capillaries in early spontaneous hypertension. Microvasc. Res. **27**, 39 – 50 (1984).
6. Greene, A. S., Lombard, J. H., Cowley, A. W. Jr., Hansen-Smith, F.: Microvessel changes in hypertension measured by Griffonia Simplicifolia I lectin. Hypertension **15**, 779 – 783 (1990).
7. Henrich, H. A., Romen, W., Heingarter, W., Hartung, E., Maumer, F.: Capillary rarefaction characteristic of the skeletal muscle of hypertensive patients. Klin. Wochenschr. **66**, 54 – 60 (1988).
8. Henriksen, O., Serjsen, P., Paaske, W. P., Eickhoff, J. H.: Effect of chronic sympathetic denervation upon transcapillary filtration rate induced by venous stasis. Acta Physiol. Scand. **117**, 171 – 176 (1983).
9. Hudlicka, O., Dodd, L., Renkin, E. M., Gray, S. D.: Early changes in fiber profiles and capillary density in long-term stimulated muscles. Am. J. Physiol. **243**, 11528 – 535 (1982).
10. Hudlicka, O.: What makes blood vessels grow? J. Physiol. Lond. **444**, 1 – 24 (1991).
11. Koibuchi, Y., Lee, W. S., Gibbons, G. H., Pratt, R. E.: Role of transforming growth factors beta-1, in the cellular growth response to angiotensin II. Hypertension **21**, 1046 – 1050 (1993).
12. Krieger, J. E., Dzau, V. J.: Molecular biology of hypertension. Hypertension **18**, 13 – 17 (1991).
13. Lombard, J. H., Hinojosa-Laborde, C., Cowley, A. W. Jr.: Hemodynamics and microcirculatory alterations in reduced renal mass hypertension. Hypertension **13**, 128 – 138 (1989).
14. Monos, E., Contney, S. F., Cowley, A. W. Jr.: Effect of long-term tilt on mechanical and electrical properties of rat saphenous vein. Am. J. Physiol. **256**, H1185 – 1191 (1989).
15. Myrhage, R., Hudlicka, O.: Capillary growth in chronically stimulated adult skeletal muscle as studied by intravital microscopy and histological methods in rabbits and rats. Microvasc. Res. **16**, 73 – 90 (1978).
16. Pepper, M. S., Vassali, J. D., Orci, L., Montesano, R.: Biphasic effect of transforming growth factor beta 1 on in vitro angiogenesis. Exp. Cell Res. **204**, 356 – 363 (1993).

17. Price, R. J., Skalak, T. C.: Circumferential wall stress as a mechanism for arteriolar rarefaction and proliferation in a network model. Microvasc. Res. **47,** 188 – 202 (1994).
18. Roberts, A. B., Sporn, M. B.: Regulation of endothelial cell growth, architecture and matrix synthesis by TGFbeta. Am. Rev. Resp. Dis. **140**, 1126 – 1128 (1989).
19. Williams, D. A., Segal, S. S.: Feed artery role in blood flow control to rat hindlimb skeletal muscles. J. Physiol. Lond. **463**, 631 – 646 (1993).
20. Williams, S. A., Boolell, M., MacGregor, G. A., Smaje, L. H., Wasserman, S. M., Tooke, J. E.: Capillary hypertension and abnormal pressure dynamics is patients with essential hypertension. Clin. Sci. **19**, 5 – 8 (1990).
21. Ziada, A., Hudlicka, O., Tyler, K. R., Wright, A.: The effect of long-term administration of apha 1 blocker prazosin on capillary density in cardiac and skeletal muscle. Cardiovasc. Res. **18**, 724 – 732 (1984).

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The role of urokinase-type plasminogen activator and its inhibitor PAI-1 in gastric cancer

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Background: It has been proposed that the serine protease urokinase-type plasminogen activator (UPA) and its inhibitor type-1 (PAI-1) may play an important part in cancer spread and metastasis.

Aims of the study: 1. To determine the UPA, PAI-1 antigen levels in gastric cancer (GC) and in normal gastric mucosa far from the tumor (NORM). 2. To evaluate changes related to the presence of metastasis, tumor differentiation (grading) and histotype.

Patients and methods: Samples of tumor and of normal mucosa were obtained from 25 GC patients undergoing surgery (17 males, 8 females, mean age 62, range 31–84). Antigen concentrations were measured using the ELISA method.

Results: Significantly higher UPA and PAI-1 antigen levels were found: 1. in GC vs. NORM tissue; 2. in patients with poorly and moderately differentiated GC vs. well-differentiated GC. UPA was significantly higher in GC with vs. without metastasis. PAI-1 was also higher, though not significantly so, in GC with metastasis than in GC without metastasis.

Conclusion: Our results confirm that the serine protease UPA and its inhibitor PAI-1 play an important role in GC progression. Considering the higher protease levels detected in GC with metastases and poorer differentiation, UPA and PAI-1 might be useful for identifying gastric cancer patients with a poor prognosis.

Keywords: gastric cancer, protease, urokinase-type plasminogen activator, plasminogen activator inhibitor type-1, prognosis

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Proteolytic enzymes, such as serine proteases, play an important part in physiological (e.g. protein turnover) and in pathological processes [8]. The proteolysis of tissue barriers is an essential step in tumor spread and has been linked to the production by cancer cells of degrading enzymes [23].

Plasminogen activators (PA) form part of the group of serine proteases and are involved in many protein-degrading processes, converting plasminogen into active plasmin, which degrades fibrin, leading to fibrinolysis. The tissue-type plasminogen activator (TPA) is a key enzyme in the fibrinolytic cascade, while the urokinase-type plasminogen activator (UPA) plays a major part in extracellular matrix degradation, tumor invasion and the metastatic spread of malignant cells [5, 11, 24, 31, 37]. PA activity is controlled by serine protease inhibitors (serpins) [42]. The plasminogen activator inhibitor type-1 (PAI-1) is produced by vascular endothelial cells and inhibits both TPA and UPA by forming a covalent inhibitor-enzyme complex [13, 42].

A large number of solid tumors such as those of the breast [1, 2, 7, 9, 16], lung [25], prostate [17], brain [21] and colon [6, 10, 26, 38] were found to correlate with raised concentrations of UPA.

An increased PAI-1 has also been found in patients with cancer of the breast [1, 9, 16], brain [21] and colorectum [4, 15, 18, 38, 45], strongly suggesting that PAI-1 may be very important in tumor invasion and metastasis, too. The best evidence to support a role for PA in metastasis comes from experimenting with malignant cell lines in vitro and with tumor cells transplanted into laboratory animals, in which increased levels of UPA correlate with the invasive and metastatic potential of malignant tumor, and anti-UPA antibodies strongly inhibited metastatic spread [32, 34].

In the case of gastric cancer, relatively few studies have been published on serine proteases: elevated UPA [12, 30, 31, 39, 44, 46] and PAI-1 [30, 45] have been described, but mostly in the references cited, activity tests were applied for UPA and only Nekarda [30] evaluated UPA and PAI-1 together in the same cancer patients and also only findings reported by Nekarda [30] and by Umehara [46] have demonstrated the clinical and possible prognostic significance of the correlations of histomorphological data with UPA and PAI-1 levels.

The aim of the present study was therefore to determine, using the ELISA method, the antigen concentrations of the serine protease UPA and its inhibitor PAI-1 in gastric cancer (GC) and in normal tissue obtained far from the tumor (NORM) and to evaluate any relationship these proteases may have with the prognostic parameters such as the presence of metastases, the differentiation (grading) and histotype of the cancer.

Patients and methods

The study involved 25 gastric cancer patients undergoing gastroresection (17 males, 8 females, mean age 62, range 31 – 84). Informed consent was obtained from all patients taking part in the study. Immediately after removal of the stomach, fresh samples of tumor and of normal tissue (taken more

than 10 cm from the macroscopic border of the tumor) were obtained. After removing fat and muscle layers, the samples were frozen at −70 °C until needed. All patients had histologically-confirmed advanced adenocarcinoma. Pathologic staging was done for the presence (n = 13) or absence (n = 12) of metastasis; differentiation-grading (well-differentiated, G1 (n = 4); moderately-differentiated, G2 (n = 14); and poorly-differentiated, G3 (n = 17); histotype (intestinal-type (n = 13) and diffuse-type (n = 12)) according to Lauren [22].

Specimens were homogenized for 2 min in melting ice using a teflon homogenizer in 1 ml Tris Tween buffer (0.1 M, 0.1% Tween 80, pH 7.5). After centrifugation for 10 min at 10.000 × g at 4 °C, the supernatants were stored at −70 °C in 200 µl before assay. Protein concentrations of the supernatants were determined by the Bradford method [3] (Bio-Rad, München, Germany). The antigen levels were measured using the ELISA method as follows:

Assay for urokinase-type plasminogen activator (UPA)

Antigen quantification was performed using the TintElize uPA ELISA (Biopool, Umea, Sweden) [40]. Briefly, a mouse monoclonal anti-urokinase-type plasminogen activator was used as a catching antibody. After incubation with the tissue homogenates, a second goat anti-human UPA, conjugated with horseradish peroxidase, was used to form a "sandwich" ELISA and ortho-phenylene diamine was added as a substrate. The amount of UPA antigen in the samples was calculated from a six-point standard curve of UPA (0−4 ng/ml). The detection limit is about 0.1 ng/ml for UPA.

Assay for plasminogen-activator inhibitor type 1 (PAI-1)

PAI-1 antigen was determined using Asserachrom PAI-1 − ELISA (Diagnostica Stago, Asnieres-sur-Seines, France) [40], with mouse monoclonal anti-human PAI-1 as a catching antibody. A second mouse monoclonal anti-PAI-1 is coupled with peroxidase and binds to another antigenic determinant at a distance from the first one, forming the "sandwich". The bound enzyme peroxidase is then revealed, in the same way as for UPA, in the presence of hydrogen peroxide. Absolute quantities of PAI-1 antigen on the samples were calculated from a five-point standard curve of PAI-1 (0−100 ng/ml). The detection limit is about 2.5 ng/ml for PAI-1.

Antigen concentrations were expressed as ng of antigen per mg of protein. Results are given as mean values ± SD. Differences between groups were statistically tested using Student's *t*-test on paired and unpaired data, or the Mann−Whitney U test where applicable. Differences were considered as significant with $p < 0.05$.

Results

For the 25 patients, the antigen concentrations for UPA and PAI-1 in the gastric cancer tissue and normal mucosa, expressed in ng/mg protein, are shown in Table I. UPA and PAI-1 were significantly higher in gastric cancer tissue than in normal mucosa.

Table I

UPA and PAI-1 in gastric cancer and in normal tissue far from tumor

	GC (n = 25)	NORM	*p
UPA	1.85 ± 1.05*	0.45 ± 0.37	$p < 0.001$
PAI-1	2.35 ± 2.69*	0.50 ± 0.30	$p < 0.001$

[ng/mg protein] [*p: GC vs. NORM]
[mean ± SD]

UPA was significantly higher in specimens from patients with lymph node (n = 11) or liver metastases (n = 2) than in specimens from patients without metastases (1.88 ± 0.82 vs. 1.36 ± 0.72 ng/mg protein) (Fig. 1). PAI-1 was also higher, though not significantly so, in GC with metastasis than in GC without metastasis (2.54 ± 3.06 vs. 2.14 ± 2.35 ng/mg protein).

Significantly higher UPA and PAI-1 antigen levels were seen in poorly- and moderately-differentiated than in well-differentiated tumors (Table II).

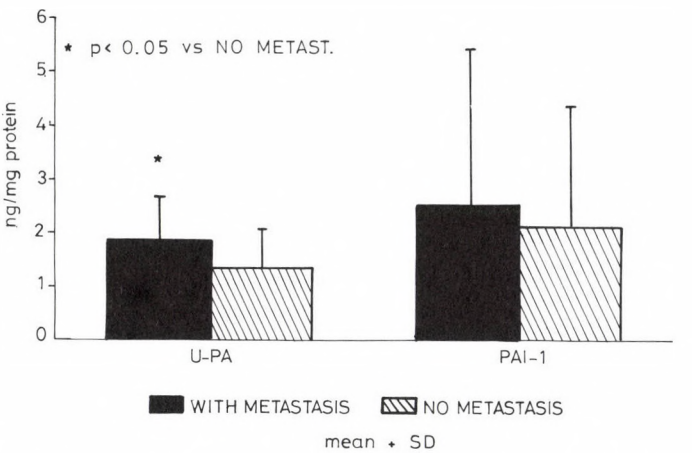

Fig. 1. UPA, PAI-1 in gastric cancer: correlation with the presence or absence of metastases

Table II

UPA, PAI-1 and differentiation (grading) of gastric tumors

	Well G1 (n = 4)	Moderately G2 (n = 14)	Poorly G3 (n = 7)
UPA	0.86 ± 0.34	2.29 ± 0.91	1.94 ± 2.03
PAI-1	0.79 ± 0.30	3.00 ± 3.21	1.53 ± 0.99

[ng/mg protein]
[mean ± SD]

UPA: G2 vs. G1: $p < 0.005$
G3 vs. G1: $p < 0.05$
PAI-1: G2 vs. G1: $p < 0.01$
G3 vs. G1: $p < 0.05$

No significant differences were observed with respect to the histotype according to Lauren (intestinal vs diffuse type GC: UPA = 1.90±1.19 vs 1.80±0.94; PAI-1 = 2.80±3.46 vs 1.87±1.49 ng/mg protein).

A significant correlation was found in GC levels of UPA and PAI-1 ($r = 0.50$; $p = 0.01$).

Discussion

Proteolytic mechanisms have been suggested to represent important factors in the process of tumor invasion and metastasis [5, 11, 23, 24, 31, 37]. A number of studies have also been published on gastric cancer, but in most of the relevant references cited here, activity tests were applied for UPA. In contrast to activity tests, in which only free, enzimatically active UPA can be determined, the most accurate UPA ELISAs may recognize various forms of UPA, including the enzymatic inactive precursor pro-UPA, free and complexed form of activated form of UPA, and (pro)UPA bound to its receptor UPA-R [16, 30]. Likewise, various inactive and active forms of PAI-1 are recognized by the PAI-1 ELISA used [16]. ELISA tests for UPA and PAI-1 have been used previously in prognosis-related breast cancer studies and were found to be superior to activity assays [7, 16].

In the present study we therefore surveyed the relationship between the antigen concentrations (determined by ELISA method) of the serine protease UPA and its inhibitor PAI-1 in human gastric cancer tissues and the antigen levels of UPA, PAI-1 were also considered in correlation with clinical and pathological parameters of prognostic relevance (the presence of metastases, GC differentiation-grading and histotype).

We demonstrated that the antigen levels of UPA and PAI-1 are significantly higher in homogenates of GC tissues than in those of non-cancerous samples obtained from the same stomach. Our results therefore confirm earlier studies reporting increased levels of UPA [12, 30, 31, 39, 44, 46] in GC tissue, also

confirming clearly that this enzyme is involved in the process of gastric cancer development and growth. In our experience, PAI-1 antigen was also found significantly increased in gastric cancer tissue, confirming findings reported by Tanaka [45] and Nekarda [30]. Under normal physiological conditions indeed, UPA forms inactive enzyme complexes with the inhibitor PAI-1, when the latter is present [13, 42]. The exact functional role of PAI-1 in tumor biology is however not well established. It has been suggested that PAI-1 may represent a specific protein of transformed malignant tissue [33, 35], or PAI-1 may serve to protect cancer tissue against the proteolytic degradation triggered by the tumor on surrounding normal tissue [4, 19]. Another possible explanation for the significance of PAI-1 is that the inhibitor might play a role in angiogenesis, thus favouring tumor spread and metastasis [23, 28]. UPA was significantly higher in specimens from patients with lymph node or liver metastases than in specimens from patients without metastases and this confirms the correlation between the protease synthesis and tumor invasion potential [40]. PAI-1 was also higher, though not significantly so, in GC with metastasis than in GC without metastasis.

Significantly higher UPA, PAI-1 antigen levels were found in poorly- and moderately-differentiated tumors than in well-differentiated cancers, thus indicating that the more aggressive tumors are characterized by a more enhanced protease synthesis. UPA, PAI-1 antigen levels were not significantly changed in the different histological subtypes of gastric cancer.

The importance of our data from the biological and clinical point of view is relevant. Gastric cancer is one of the most lethal gastrointestinal cancers in terms of cure rate and survival [29]. It would therefore be most useful for clinicians to give more accurate prognostic indications on patients in order, for instance, to identify those needing adjuvant treatment.

Our findings concerning gastric cancer appear to be supported also from experience regarding other tumors. In fact, recent studies on breast carcinoma have shown that high UPA and PAI-1 in tissue extract were associated with tumor aggressiveness and poor prognosis, and appeared to be independent prognostic factors [2, 7, 16].

Obviously, the determination of proteases is not the only approach to this problem. Intracellular mucus production, the DNA contents of the cancer cells, the presence of vascular invasion and ploidy, has been suggested to be the most significant prognostic parameters after histomorphological staging [27, 36, 43]. It is foreseeable that determining the levels of tissue UPA and PAI-1 will also facilitate the identification of high-risk gastric cancer patients who are candidates for extensive resection and/or adjuvant or neo-adjuvant therapy [14, 20, 41, 47].

In conclusion, our results confirms that the serine protease UPA and its inhibitor PAI-1 may have a crucial role in gastric cancer progression. Considering the higher antigen levels detected in gastric cancer with metastases and poorer differentiation, UPA and PAI-1 might prove of great value in identifying patients with a poor prognosis.

REFERENCES

1. Bianchi, E., Cophen, R. L., Thor, A. T., Todd, R. F., Mizukami, I. F., Lawrence, D. A.: The urokinase receptor is expressed in invasive breast cancer but not in normal breast tissue. Cancer Res. **54**, 861–866 (1994).
2. Bouchet, C., Spyratos, F., Martin, P. M., Hacène, K., Gentile, A., Oglobine, J.: Prognostic value of urokinase-type plasminogen activator (UPA) and plasminogen activator inhibitors PAI-1 and PAI-2 in breast carcinomas. Br. J. Cancer **69**, 398–405 (1994).
3. Bradford, M. A.: Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254 (1976).
4. Buo, L., Lyberg, T., Jorgensen, L., Johansen, H. T., Aasen, A. O.: Location of plasminogen activator (PA) and PA inhibitor in human colorectal adenocarcinomas. APMIS **101**, 235–241 (1993).
5. Dano, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L. S., Skriver, L.: Plasminogen activators, tissue degradation and cancer. Adv. Cancer Res. **44**, 139–266 (1985).
6. De Bruin, P. A. F., Verspaget, H. W., Griffioen, G., Nap, M., Verheijen, J. H., Lamers, C. B. H. W.: Plasminogen activator activity and composition in human colorectal carcinomas. Fibrinolysis **1**, 57–62 (1987).
7. Duffy, M. J., Reilly, D., O'Sullivan, C., O'Higgins, N., Fennelly, J. J., Andreasen, P.: Urokinase-plasminogen activator, a new and independent prognostic marker in breast cancer. Cancer Res. **50**, 6827–6829 (1990).
8. Emonard, H., Grimaud, J. A.: Matrix metalloproteins. A review. Cell. Mol. Biol. **36**, 131–153 (1990).
9. Grondahl-Hansen, J., Christensen, I. J., Rosenquist, C., Brünner, N., Mouridsen, H. T., Dano, K.: High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. Cancer Res. **53**, 2513–2521 (1993).
10. Grondahl-Hansen, J., Rolfkiaer, E., Kirkeby, L. T., Kristensen, P., Lund, L. R., Dano, K.: Localisation of urokinase-type plasminogen activator in stromal cells in adenocarcinomas of the colon in humans. Am. J. Pathol. **138**, 111–117 (1991).
11. Hart, I. R., Saini, A.: Biology of tumor metastasis. Lancet **339**, 1453–1457 (1992).
12. Harvey, S. R., Lawrence, D. D., Madeja, J. M., Abbey, S. J., Markus, G.: Secretion of plasminogen activators by human colorectal and gastric tumor explants. Clin. Exp. Metastasis **6**, 431–450 (1988).
13. Hekman, C. M., Loskutoff, D. J.: Fibrinolytic pathways and the endothelium. Semin. Thromb. Hemost. **13**, 514–527 (1987).
14. Horváth, O. P., Iványi, B., Oláh, T., Leindler, L., Karácsonyi, S.: Surgical treatment of stomach cancer with extended removal of lymph nodes. Orv. Hetil. **131**, 2307–2310 (1990).
15. Huber, K., Wojta, J., Kirchheimer, J. C., Ermler, D., Binder, B. R.: Plasminogen activators and plasminogen activator inhibitor in malignant and non-malignant ascitic fluid. Eur. J. Clin. Invest. **18**, 595–599 (1988).
16. Jänicke, F., Schmitt, M., Pache, L., Ulm, K., Harbeck, N., Hofler, H., Graeff, H.: Urokinase (uPA) and its inhibitor PAI-1 are strong and independent prognostic factors in node-negative breast cancer. Breast Cancer Res. Treat. **24**, 195–208 (1993).
17. Kirchheimer, J. C., Köller, A., Binder, B. R.: Isolation and characterisation of plasminogen activators from hyperplastic and malignant prostate tissue. Biochim. Biophys. Acta **797**, 256–265 (1984).
18. Kirchheimer, J. C., Huber, K., Wagner, O., Binder, B. R.: Pattern of fibrinolytic parameters in patients with gastrointestinal carcinomas. Br. J. Haematol. **66**, 85–89 (1987).
19. Kristensen, P., Pyke, C., Lund, L. R., Andreasen, P. A., Dano, K.: Plasminogen activator inhibitor type-1 in Lewis lung carcinoma. Histochemistry **93**, 559–566 (1990).

20. Kuklis, B., Iliás, L., Simig, M., Sebesi, J., Pattantyus, J., Fiala, E.: Experience with cytostatic therapy of stomach cancer. Orv. Hetil. **132**, 1691–1694 (1991).
21. Landau, B. J., Kwaan, H. C., Verrusio, E. N., Brem, S. S.: Elevated levels of urokinase-type plasminogen activator and plasminogen activator inhibitor type 1 in malignant human brain tumors. Cancer Res. **54**, 1105–1108 (1994).
22. Lauren, P.: The two histologic main types of gastric carcinoma: Diffuse and so-called intestinal type carcinoma. An attempt at a histo-clinical classification. Acta Pathol. Microbiol. Scand. **64**, 31–49 (1965).
23. Liotta, L. A., Steeg, P. S., Stetler-Stevenson, W. G.: Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell **64**, 327–336 (1991).
24. Markus, G.: The relevance of plasminogen activators to neoplastic growth – a review of recent literature. Enzyme 1988 **40**, 158–172 (1988).
25. Markus, G., Takita, H., Camiolo, S. M., Corasanti, J. G., Evers, J. L., Hobika, H.: Content and characterisation of plasminogen activators in human lung tumors and normal lung tissue. Cancer Res. **40**, 841–848 (1980).
26. Markus, G., Camiolo, S. M., Kohga, S., Madeja, J. M., Mittelman, A.: Plasminogen activator secretion of human tumors in short-term organ culture, including a comparison of primary and metastatic colon tumors. Cancer Res. **43**, 5517–5525 (1983).
27. Martin, I. G., Dion, M. F., Sue-Ling, H., Axon, A. T. R., Johnston, D.: Goseki histological grading of gastric cancer is an important predictor of outcome. Gut **35**, 758–763 (1994).
28. Montesano, R., Pepper, M. S., Mohlesteinlein, U., Risau, W., Wagner, E. F., Orci, L.: Increased proteolytic activity is responsable for the aberrant morphogenetic behaviour of endothelin cells expressing the middle T-oncogene. Cell **62**, 435–445 (1990).
29. Nakamura, K., Ueyama, T., Yao, T., Xin, X. Z., Ambe, K., Adachi, Y., Yakeishi, Y., Matsukuma, A., Enjoji, M.: Pathology and prognosis of gastric carcinoma. Findings in 10000 patients who underwent primary gastrectomy. Cancer **70**, 1030–1037 (1992).
30. Nekarda, H., Schmitt, M., Ulm, K., Wenninger, A., Vogelsang, H., Becker, K., Roder, J. D., Fink, U., Siewert, J. R.: Prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in completely resected gastric cancer. Cancer Res. **54**, 2900–2907 (1994).
31. Nishino, N., Aoki, K., Tokura, Y., Sakaguchi, S., Takada, Y., Takada, A.: The urokinase type of plasminogen activator in cancer of digestive tract. Thromb. Res. **50**, 527–535 (1988).
32. Ossowski, L., Reich, E.: Antibodies to plasminogen activator inhibit human tumor metastasis. Cell **35**, 611–619 (1983).
33. Paoli, B. U., Knudson, W.: Tumor invasion: A consequence of destructive and compositional matrix alterations. Hum. Pathol. **19**, 628–639 (1988).
34. Pereyra-Alfonso, S., Haedo, A., Bal de Kier, J. E.: Correlation between urokinase type plasminogen activator production and the metastasising ability of two murine mammary adenocarcinomas. Int. J. Cancer **42**, 59–63 (1988).
35. Reilly, D., Christensen, L., Duch, M., Nolan, N., Duffy, M. J., Andreasen, P. A.: Type-1 plasminogen activator inhibitor in human breast carcinomas. Int. J. Cancer **50**, 208–214 (1992).
36. Rugge, M., Sonego, F., Panozzo, M., Baffa, R., Rubio, J. Jr., Farinati, F., Nitti, D., Ninfo, V., Ming, S. C.: Pathology and ploidy in the prognosis of gastric cancer with no extranodal metastasis. Cancer **73**, 1127–1133 (1994).
37. Saksela, O., Rifkin, D. B.: Cell-associated plasminogen activation: regulation and physiological functions. Ann. Rev. Cell. Biol. **4**, 93–126 (1988).
38. Sier, C. F. M., Verspaget, H. W., Griffioen, G., Verheien, J. H., Quax, P. H. A., Dooijewaard, G., De Bruin, P. A. F., Lamers, C. B. H. W.: Imbalance of plasminogen activators and their inhibitors in human colorectal neoplasia. Implication of urokinase in colorectal carcinogenesis. Gastroenterology **101**, 1522–1528 (1991).
39. Sier, C. F. M., Verspaget, H. W., Griffioen, G., Ganesh, S., Vloedgraven, H. J. M., Lambers, C. B. H. W.: Plasminogen activators in normal tissue and carcinomas of the human oesophagus and stomach. Gut **34**, 80–85 (1993).

40. Sier, C. F. M., Vloedgraven, H. J. M., Ganesh, S., Griffioen, G., Quax, P. H. A., Verheien, J. H., Dooijewaard, G., Welvaart, K., Van den Velde, C. J. H., Lambers, C. B. H. W., Verspaget, H. W.: Inactive Urokinase and increased levels of its inhibitor type-1 in colorectal cancer liver metastasis. Gastroenterology **107**, 1449–1456 (1994).
41. Siewert, J. R., Böttcher, K., Roder, J. D., Busch, R., Hermanek, P., Meyer, H. J.: Prognostic relevance of systemic lymph node dissection in gastric carcinoma. Br. J. Surg. **80**, 1015–1018 (1993).
42. Sprengers, E. D., Kluft, C.: Plasminogen activator inhibitors. Blood **69**, 381–387 (1987).
43. Szentirmay, Z., Udvarhelyi, N., Besznyák, I.: Cellular DNA determination and its significance in the identification of tumor recurrance, metastases and second primary tumors. Orv. Hetil. **133**, 1015–1016 (1992).
44. Takai, S., Yamamura, M., Tanaka, K., Kawanashi, H., Tsuji, M., Nakane, Y., Hioki, K., Yamamoto, M.: Plasminogen activators in human gastric cancers: correlation with DNA ploidy and immunohistochemical staining. Int. J. Cancer **48**, 20–27 (1991).
45. Tanaka, N., Fukao, H., Ueshima, S., Okada, K., Yasutomi, M., Matsuo, O.: Plasminogen activator inhibitor 1 in human carcinoma tissues. Int. J. Cancer **48**, 481–484 (1991).
46. Umehara, Y., Yoshida, M., Oba, N., Harada, Y.: Relationship between plasminogen activators and stomach carcinoma stage. Acta Oncol. **30**, 815–818 (1991).
47. Wilke, H., Preusser, P., Fink, U., Achterrath, H., Meyer, H. J., Stahl, M., Lenaz, L., Meyer, J., Siewert, J. R., Geerlings, H.: New developments of gastric carcinoma. Semin. Oncol. **17**, 61–70 (1990).

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Antiarrhythmic activity of oximethers

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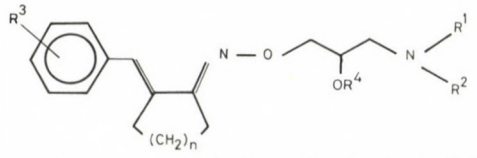
More than sixty 2-substituted-cycloalkanone-oxim-/2-hydroxy-3-dialkylamino/-propylethers were investigated on aconitine induced ventricular arrhythmia model in rats. According to the structure-activity relationships cyclohexanes containing diisopropylamine in the basic group were the most effective derivatives. Based on the highest per os activity and lowest toxicity EGIS-3966 (ED_{50} values 1.21 mg/kg iv. and 27.3 mg/kg per os) was selected for further development.

Keywords: rats, aconitine arrhythmia, structure-activity relationship

During the last few years more than sixty analogues of the 2-substituted-cycloalkanone-oxim-/2-hydroxy-3-dialkylamino/-propylethers [1] were synthetized and tested in our pharmacological screening program.

The general structure of the compounds:



The majority of the derivatives studied showed activity in different test systems, but the highest effect was found on the aconitine induced ventricular arrhythmia model in rats. Aconitine induces serious ventricular arrhythmias on rats, therefore this simple and quick test was chosen to screen molecules for antiarrhythmic activity. Based upon the results of the antiarrhythmic test we described the structure-activity relationships for the oximether derivatives. The most effective compound was selected for further development.

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Method

Aconitine-induced arrhythmia in rats

Experiments were carried out in Wistar rats of either sex weighing 160 to 300 g according to the modified method of Marmo et al. [3]. The animals were anaesthetized with 1.2 g/kg ethylurethane (Fluka) intraperitoneally. Aconitine (Fluka) was administrated as a bolus injection in a dose of 75 μg/kg into the tail vain of rats. Each test compound was administered to 6 rats in a dose of 4 mg/kg intravenously, 2 minutes before the aconitine injection. The ECG changes were monitored for 5 minutes after aconitine injection in standard lead II and scored from zero to 5 points.

Score points:

0 – absence of extrasystoles
1 – sporadical incidence of extrasystoles
2 – bigeminy
3 – groupwise extrasystoles
4 – continuous extrasystoles
5 – fibrillation, death

Mean score values in the fifth minute were calculated for each group and the effects of the test compounds were expressed as a percent inhibition compared to the control group. Those compounds exhibiting more than 50 percent inhibition in the screen dose, ED_{50} values were determined by regression analysis.

Results and discussion

The results of some compounds are presented in Table I in order to demonstrate the most characteristic examples of the structure-activity relationship.

1. Among the different cycloalkanes, cyclohexanes (n = 2) seem to be the most preferable. Ring enlargement (n = 3, 4), or contraction (n = 1) impairs the activity studied.

2. In the case of cyclohexane compounds containing different basic groups (R^1, R^2) the diisopropylamine derivative proved to be the most effective. The 4-benzyl-piperidyl substitution decreased the activity.

3. Examining the role of R^3 substitution, Cl in position 2 enhances parenteral antiarrhythmic activity, however Cl in position 4 ceases it.

4. The benzoyl group in R^4 position nearly abolishes the efficacy.

According to these results, and per os antiarrhythmic activities (EGIS-4887 in 50 mg/kg per os dose: −12%), EGIS-3966 (ED_{50} values 1.21 mg/kg iv. and 27.3 mg/kg per os [2]) was chosen for further development. In our experiments ED_{50} values for chinidine, propranolol, and pindolol, drugs used for the treatment of ventricular arrhythmias, were 9.73 mg/kg, 5.83 mg/kg, 1.81 mg/kg respectively [5].

In conclusion, the aconitine arrhythmia test seems suitable for determining structure-activity relationships.

A brief account of some of this work has been published previously [4].

Table I

Results in aconitine arrhythmia test by iv. administration in rats

Code-number EGIS-	n	-N R^1 / R^2	R^3	R^4	Antiarrhythmic activity iv. % in 4 mg/kg	Antiarrhythmic activity iv. ED_{50} mg/kg
6020	1	$-N(CH(CH_3)_2)_2$	–	H	–46%	
3966	2	$-N(CH(CH_3)_2)_2$	–	H		1.21
4334	3	$-N(CH(CH_3)_2)_2$	–	H		1.64
5972	4	$-N(CH(CH_3)_2)_2$	–	H		3.29
3966	2	$-N(CH(CH_3)_2)_2$	–	H		1.21
6424	2	$-N \quad -CH_2-C_6H_5$	–	H		3.47
6021	2	$-NH-C(CH_3)_3$	–	H	–44%	
4887	2	$-NH-C)(CH_3)_3$	2 Cl	H		0.36
4563	2	$-NH-C(CH_3)_3$	4 Cl	H	–4%	
3966	2	$-N(CH(CH_3)_2)_2$	–	H		1.21
6410	2	$-N(CH(CH_3)_2)_2$	–	$-CO-C_6H_5$	–11%	

REFERENCES

1. Budai, Z., Mezei, T., Petőcz, L., Hegedüs, M., Fekete, M., Fekete, V., Reiter, J-né, Kiszelly, E., Zsila, G., Gigler, G., Kápolnai, L.: Eljárás új ciklikus-keton-származékok és hatóanyagként e vegyületeket tartalmazó gyógyászati készítmények előállítására. Hung. Patent Application No. 4401/89, 1989.
2. Hegedüs, M., Petőcz, L., Szemerédi, K.: Egy potenciális antiaritmikum, az EGIS-3966 hatásigazoló vizsgálata. LIX. Meeting of the Hungarian Physiological Society, Budapest, Hungary, July 10–13, 1994. Poster 2 (abstract) (in Hungarian).
3. Marmo, E., DiGiacomo, S., Imperatore, A.: Metoclopramid und elektrokardiographische Veränderungen durch KCl, $BaCl_2$, $CaCl_2$, $MgCl_2$, Aconitin, k-Strophanthosid, Vasopressin und durch Asphyxie. Arzneimittel-Forschung **20**, 12–18 (1970).
4. Petőcz, L., Budai, Z., Mezei, T., Hegedüs, M.: Cyclic oximether derivatives with mainly antiarrhythmic activity. XIth International Symposium on Medicinal Chemistry, Jerusalem, Israel, September 2–7, 1990. Poster II/37 (abstract).
5. Szécseyné Hegedüs, M.: Oximether derivatives with antiarrhythmic activity: pharmacological analysis of the most potent molecule. PhD Thesis, Technical University of Budapest, Hungary (in Hungarian).

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Effect of volume expansion induced by Ringer's solution or plasma on the natriuretic response of the kidney

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Studies were carried out in anaesthetized dogs, to determine the effects of volume expansion induced by Ringer's solution, plasma taken from non-hydrated or previously volume expanded animals on the natriuretic response of the kidney.

When the animals were infused with Ringer's solution(0.25 ml/kg/min iv.) water and sodium excretion increased gradually reaching a peak value at about 70 min. after starting the infusion (3.01 ± 0.21 ml/min and 329 ± 23.9 mmol/min, respectively).

Infusing the dogs with plasma taken from non-hydrated animals (0.25 ml/kg/min iv.) water and sodium excretion showed a small increase in the first 15 min, but afterwards there was hardly any change in them, and water and sodium excretions were significantly less in the remaining part of the experiment than in with Ringer's solution expanded animals. (Peak values 1.44 ± 0.53 ml/min and 193 ± 85.3 mmol/min.)

In animals infused with plasma taken from previously hydrated dogs (0.25 ml/kg/min iv.) water and sodium excretion were already after the first 15 min significantly higher than in the two other groups, they increased further in the following 15 to 30 minutes and remained elevated during the time of the study. (Peak values for water and sodium excretion being 3.30 ± 0.58 ml/min and 344 ± 73.5 mmol/l.)

Blood pressure, C_{PAH} and glomerular filtration rate were the same and did not change in either of the experimental series.

We concluded, that besides the physical forces playing certainly a role mainly in the Ringer's solution hydrated dogs, some humoral natriuretic factor liberated in the volume expanded animals plays also a role in the diuretic-natriuretic response. This humoral factor can be released from the atria or other organs, but its production is not primarily regulated by intravasal volume/or pressure changes.

Keywords: volume expansion, sodium and water excretion, natriuretic factor, anaesthetized dog

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Fluid and electrolyte regulation is accomplished by complex interactions of physical factors, neural and endocrine systems, and intrarenal paracrine-autocrine mechanisms [5, 11].

Sodium excretion by the kidney increases significantly during acute volume expansion, this increase in renal sodium excretion is mediated by multiple regulatory mechanisms. The renin-angiotensin system, arginine vasopressin (AVP), renal sympathetic nerve activity, atrial natriuretic factor (ANF), physical factors (blood pressure, colloid osmotic pressure) and the urodilatin produced by the kidney itself are all clearly capable of importantly influencing sodium and water excretion under various conditions.

It has already long ago been demonstrated in rats, that blood from volume-expanded donor animals induced natriuresis in the cross-circulated recipient, furthermore blood and uric extracts from volume-expanded subjects caused natriuresis in assay animals [2, 28, 34].

Despite of the description of the numerous overlapping mechanisms, their relative importance in the acute regulation of sodium and water excretion is not yet really cleared up. Recently, Cowley et al. [7] demonstrated that during acute volume expansion, the increase in the urine-, and sodium excretion was minimally affected when the various neural and endocrine controllers were fixed or eliminated. They emphasized the role of the physical factors.

The aim of our studies was to reinvestigate in dogs, how the intravenous infusion of the same amount of Ringer's solution, or plasma taken from non-hydrated or previously volume expanded animals can influence the diuretic and natriuretic response of the kidneys. We also intended to follow the time course of the changes observed.

Methods

The experiments were carried out on mongrel dogs of both sexes, under Nembutal anaesthesia (30 mg/kg given intravenously).

During the last 24 h prior to the experiments the animals were given water alone.

Following the anaesthesia the femoral arteries on both sides and the femoral vein on one side were exposed for taking blood samples and recording the blood pressure, in the case of the vein for giving the infusions.

Arterial blood pressure was measured by an electronic tonometer inserted into the femoral artery, and recorded continuously on a RADELKISZ OH 814/1-type recorder.

From lower median laparatomy the bladder was exposed and the ureters were cannulated supravesically.

The urine from both kidneys was collected for 10 minutes in order to have the initial values for urine and sodium excretion.

The experiments were carried out in three series. Following the initial urine collection the animals of each series got the clearance substances (PAH and inulin at an amount to reach a plasma concentration of 2 mg/dl and 30 mg/dl respectively) in 40 ml of Ringer's solution intravenously. The maintaining dose of the clearance substances was administered in a continuous iv. infusion of 1 ml/min.

This volume infused was substracted from the calculated infusion rate of 0.25 ml/kg/min, so the rate of infusion per body weight was the same in each case.

Afterwards in the *first experimental series* an intravenous infusion of 0.25 ml/kg/min of Ringer's solution of body temperature was started an maintained through the experiment.

In the *second experimental series*, the administration of the clearance substances was followed by an intravenous infusion of 0.25 ml/kg/min of plasma taken from non-hydrated dogs (no previous intervention, no water intake 12 hours prior to the experiment).

In the *third experimental series*, the animals were volume expanded with the infusion of 0.25 ml/kg/min of plasma taken from previously hydrated dogs (before taking the blood these animals got 0.25 ml/kg/min iv. Ringer's solution for one hour).

In each series, the first 15 minutes were left to elapse, afterwards urine of both kidneys was collected in three subsequent 15 min [1, 2, 3] followed by three subsequent 10 min [4, 5, 6] periods. At the midpoint of each period arterial blood sample was taken. At the end of the experiment the animals were killed the kidneys were weighed.

PAH concentration in urine and plasma was determined by the method Smith et al. [43] that of inulin by the method of Little [32]. Urinary and plasma sodium and potassium concentrations were measured by flame photometry. Total osmolality of urine and plasma was measured by the method of freezingpoint depression in a Fiske osmometer. Hematocrit was determined by means of Hawskley microhematocrit centrifuge, plasma protein concentration by the biuret method [24].

The clearances of PAH (C_{PAH}) and of inulin (C_{inulin}) were calculated by the usual formules. The data for C_{PAH} C_{inulin}, urine flow, sodium excretion ($U_{Na} \times V$), potassium excretion ($U_K \times V$), free water clearance (C_{H_2O}) were referred to 100 g kidney tissue weight.

Statistical evaluation of the data was made by paired and unpaired analysis using Student's *t*-test. A p value of less that 0.05 was considered significant.

Results

In two of three experimental series the initial (preinfusion) values for urine output, sodium-, and potassium excretion have been determined. (Values are depicted on the ordinata on the Figure 2.) The initial urine output was 0.27 ± 0.06 ml/min in the animals volume expanded later on with plasma taken from non-hydrated dogs, and 0.22 ± 0.04 ml/min in those volume expanded later on with plasma taken from previously hydrated animals. The respective values for sodium excretion were 33.7 ± 7.12 mmol/min and 33.8 ± 5.86 mmol/min, those for potassium excretion were 28 ± 4.9 mmol/min and 29 ± 5.3 mmol/min, showing that there was no appreciable initial difference in the state of the animals of different groups.

There was no difference in the starting blood pressure, C_{PAH} and C_{inulin} values between the animals of the three experimental groups and these parameters remained unchanged throughout the experiment (Fig. 1).

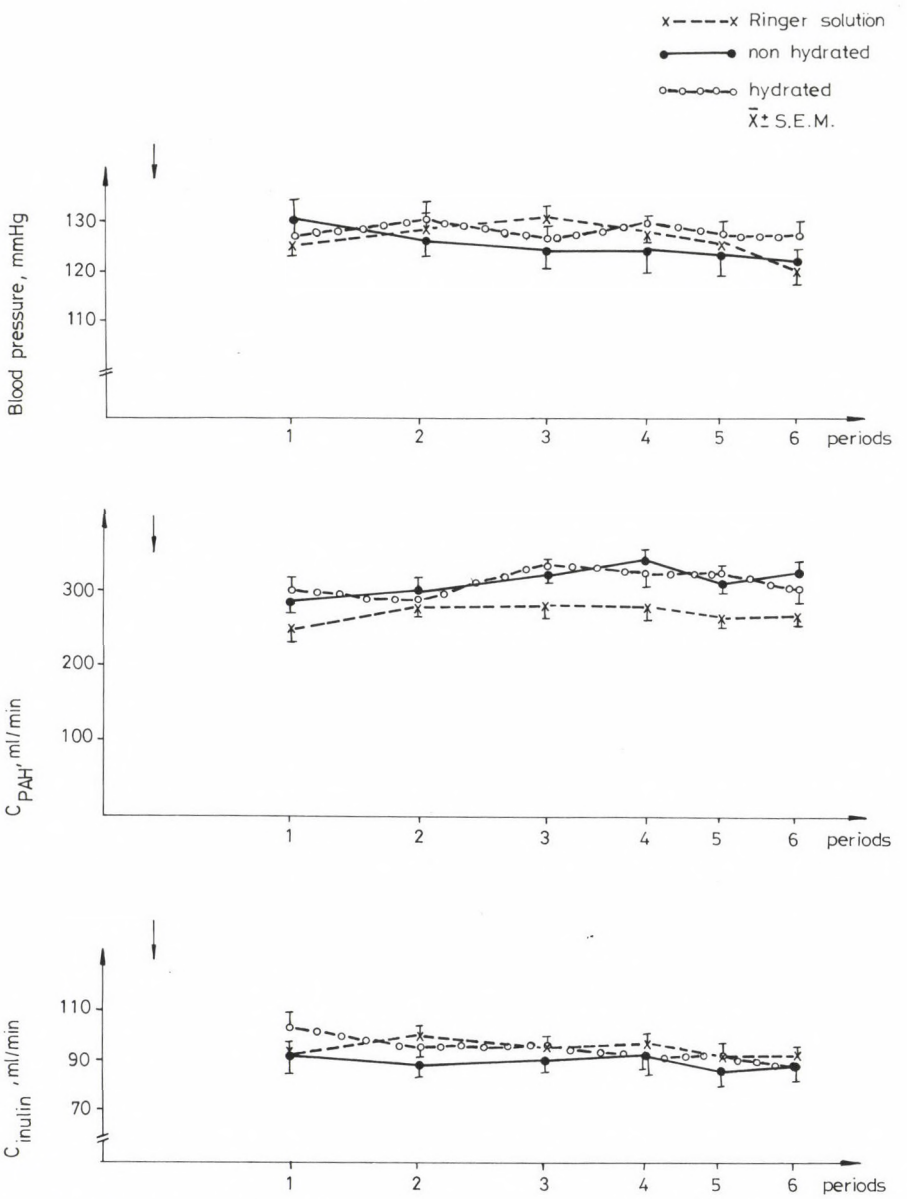

Fig. 1. Values for the blood pressure, C_{PAH} and C_{inulin} in the three series of experiments. Broken line: results obtained in animals volume expanded with Ringer's solution; Solid line: with plasma taken from non-hydrated animals; Dotted line: those with plasma taken from previously hydrated animals. Average values and S.E.M. are given

In the first experimental series (volume expansion with Ringer's solution), the urine output in the first period was 0.84 ml/min, then it increased gradually, reaching a peak value of 3.01±0.21 ml/min in the last period $p < 0.001$ (Fig. 2, Table I). The sodium excretion in the first period was 110±12.2 mmol/min, reaching the maximal value of 329±23.9 mmol/min in the 4 period ($p < 0.01$), afterwards it remained practically unchanged (Fig. 2, Table I). Potassium excretion increased from 47±3.51 mmol/min to 70±3.95 mmol/min ($p < 0.05$) while urinary osmolality decreased from 725±16.6 mosm/l to 353±14.0 mosm/l ($p < 0.001$) (Fig. 3, Table II). C_{H_2O} increased gradually, sodium and potassium rejection values changed parallelly with the sodium and potassium excretions.

In the second experimental series (volume expansion with plasma taken from non-hydrated animals), the urine excretion in the first period was practically the same as in the former group; 0.92±0.17 ml/min and did not show any significant change during the experiment, in the last period it was 1.09±0.32 ml/min (Fig. 2, Table I). The sodium and potassium excretions were in the first period 143±35.1 mmol/min and 65±15.8 mmol/min respectively, and these values again did not show any change during the experiment. Only a small, not significant drop in the urinary osmolality and increase in C_{H_2O} could be observed. The sodium and potassium rejection values changed parallelly with the sodium and potassium excretion (Fig. 4, Table I).

In the third experimental series (volume expansion with plasma taken from previously hydrated dogs), the urine excretion in the first period was 1.99±0.41 ml/min, significantly higher than the corresponding values in the other two experimental series ($p < 0.01$). It increased further on reaching the maximal value of 3.30±0.58 ml/min in the 3 period, being till significantly higher than the values of the two other series. Afterwards it showed a very small decline, the last two values being not different from those measured in the with Ringer's solution volume expanded group. The urine excretion through the whole experiment was significantly higher than the corresponding values of the second experimental serie (from the third period $p < 0.001$).

The sodium excretion in the first period was 296±61.5 mmol/min, also significantly higher, then in the other two series ($p < 0.01$). In the second period it increased to 344±73.5 mmol/min, afterwards it declined very slowly remaining till the end of the experiment significantly higher than in the second group ($p < 0.01$).

Potassium excretion was also higher than in the other two groups through the whole experiment (Fig. 2, Table I).

Urinary osmolality decreased, C_{H_2O} increased gradually during the study. Water and sodium rejections changed parallelly with the urine, and sodium excretion.

Comparing the plasma parameters (Table II) in the three series of experiments, one can conclude, that they are – within the limits of error – identical in the three series and do not show any significant change during the study with the only exception of the plasma protein concentration being significantly ($p < 0.05$) lower in the with Ringer's solution hydrated animals than is the two other groups.

Table I

Blood pressure and renal parameters in dog volume expanded with Ringer's solution, or plasma taken from previously non-hydrated or hydrated animals

(x ± S.E.M.)

		Periods					
		1	2	3	4	5	6
	R	126 ± 1.4	128 ± 1.6	130 ± 1.6	128 ± 1.6	125 ± 1.7	121 ± 2.2
Blood pressure	P	130 ± 6.7	126 ± 6.3	125 ± 6.0	124 ± 6.6	123 ± 7.4	121 ± 7.9
mmHg	HP	127 ± 5.7	129 ± 5.7	126 ± 6.0	128 ± 5.4	127 ± 5.4	127 ± 5.4
	R	250 ± 7.8	284 ± 7.7	281 ± 7.3	278 ± 6.2	270 ± 6.1	268 ± 8.1
C_{PAH}	P	284 ±18.4	294 ±20.5	321 ±13.2	321 ±13.2	310 ±17.1	323 ±17.0
ml/min/100 g	HP	291 ±22.6	285 ±23.5	328 ±22.0	325 ±18.7	326 ±18.7	305 ±16.9
	R	93.5 ± 2.8	101.1 ± 2.9	96.4 ± 2.6	93.8 ± 2.5	91.7 ± 2.5	91.4 ± 2.8
C_{Inulin}	P	91.5 ± 8.7	86.5 ± 4.1	89.6 ± 4.9	93.8 ± 6.9	85.8 ± 6.9	88.3 ± 5.9
ml/min/100 g	HP	104.0 ± 4.7	96.4 ± 5.7	96.5 ± 5.1	90.0 ± 4.6	91.8 ± 6.1	89.9 ± 4.0
	R	0.84 ± 0.07	1.33 ± 0.11	2.19 ± 0.15	2.80 ± 0.18	2.89 ± 0.19	3.01 ± 0.21
Urine output	P	0.92 ± 0.17	1.19 ± 0.27	1.44 ± 0.53	1.43 ± 0.48	1.15 ± 0.34	1.09 ± 0.32
ml/min/100 g	HP	1.99 ± 0.41	2.67 ± 0.52	3.30 ± 0.58	3.15 ± 0.50	3.04 ± 0.44	2.70 ± 0.34
	R	110 ±12.2	193 ±18.3	285 ±21.5	329 ±23.9	320 ±22.8	291 ±22.8
$U_{Na} \times V$	P	143 ±35.1	167 ±49.0	193 ±85.3	181 ±78.8	139 ±60.5	132 ±57.4
μmol/min/100 g	HP	269 ±61.5	344 ±73.5	339 ±60.6	302 ±38.7	283 ±29.4	235 ±24.2
	R	47 ± 3.5	53 ± 3.4	65 ± 3.5	69 ± 3.7	72 ± 3.5	70 ± 4.0
$U_K \times V$	P	65 ±15.8	60 ±12.4	65 ±13.6	66 ±13.3	61 ±10.6	60 ±11.0
μmol/min/100 g	HP	84 ±11.6	94 ±12.3	110 ±11.4	109 ±11.5	102 ± 9.1	95 ± 7.8

Table I (cont.)

		Periods					
		1	2	3	4	5	6
U_{osm} mosm/l	R	725 ±16.6	656 ±19.5	522 ±16.3	456 ±14.9	416 ±14.3	353 ±14.0
	P	699 ±38.9	617 ±34.5	590 ±50.7	542 ±28.0	554 ±23.5	565 ±26.5
	HP	577 ±53.3	525 ±55.4	459 ±55.2	415 ±49.7	396 ±45.2	386 ±44.0
Water rejection %	R	0.90 ± 0.02	1.32 ± 0.03	2.27 ± 0.05	2.98 ± 0.03	3.15 ± 0.06	3.29 ± 0.07
	P	1.01 ± 0.10	1.38 ± 0.11	1.61 ± 0.12	1.52 ± 0.12	1.34 ± 0.14	1.23 ± 0.16
	HP	1.91 ± 0.18	2.77 ± 0.20	3.41 ± 0.22	3.50 ± 0.26	3.31 ± 0.25	3.00 ± 0.18
Sodium rejection %	R	0.80 ± 0.03	1.30 ± 0.06	2.00 ± 0.05	2.37 ± 0.08	2.35 ± 0.10	2.14 ± 0.09
	P	1.09 ± 0.11	1.36 ± 0.12	1.56 ± 0.13	1.36 ± 0.14	1.18 ± 0.15	1.09 ± 0.12
	HP	1.82 ± 0.21	2.96 ± 0.22	2.41 ± 0.25	2.50 ± 0.19	2.33 ± 0.18	2.15 ± 0.16
C_{H_2O} ml/min/100 g	R	−0.86 ± 0.05	−1.03 ± 0.05	−0.92 ± 0.07	−0.65 ± 0.08	−0.35 ± 0.09	+0.02 ± 0.11
	P	−1.37 ± 0.26	−1.44 ± 0.30	−1.15 ± 0.30	−1.02 ± 0.20	−0.91 ± 0.19	−0.94 ± 0.21
	HP	−1.21 ± 0.50	−1.01 ± 0.60	−0.68 ± 1.21	−0.38 ± 1.35	−0.32 ± 1.41	−0.41 ± 1.26

R = volume expansion with Ringer's solution (n = 60)

P = volume expansion with plasma taken from non-hydrated dog (n = 10)

HP = volume expansion with plasma taken from hydrated dog (n = 11)

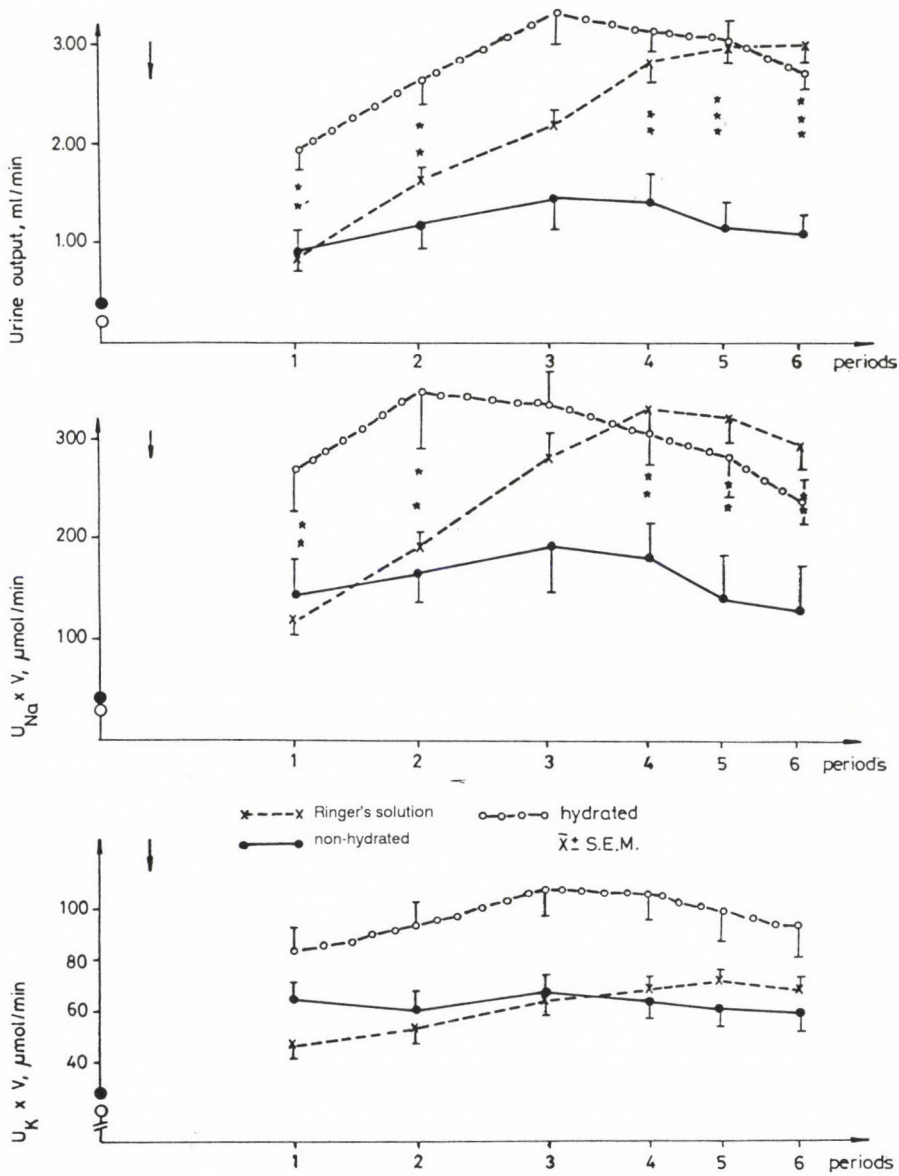

Fig. 2. Values for urine output, $U_{Na} \times V$ and $U_K \times V$. For designations see Fig. 1. The stars indicate the level of significance between the data obtained with Ringer's solution and with plasma taken from non-hydrated animal volume expanded dogs. The surrounded stars indicate the level of significance between data received in dog volume expanded with plasma from non-hydrated, and with plasma taken from previously hydrated, animals. In both cases $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. The filled and empty circles on the ordinate show the initial (preinfusion) values obtained in the second and third experimental series

Table II

Plasma parameters in dog volume expanded with Ringer's solution on plasma taken from previously non-hydrated or hydrated animals
(x ± S.E.M.)

		Periods					
		1	2	3	4	5	6
Hematocrit %	R	39 ± 0.5	38 ± 0.5	39 ± 0.5	39 ± 0.6	40 ± 0.6	39 ± 0.6
	P	38 ± 1.5	38 ± 1.6	38 ± 1.7	39 ± 1.8	39 ± 1.9	38 ± 1.8
	HP	35 ± 1.2	34 ± 0.9	35 ± 1.0	35 ± 1.0	35 ± 1.2	35 ± 1.2
Plasma protein concentration g/dl	R	5.02 ± 0.07	4.91 ± 0.08	4.87 ± 0.08	4.82 ± 0.07	4.81 ± 0.07	4.81 ± 0.11
	P	5.50 ± 0.25	5.45 ± 0.23	5.76 ± 0.28	5.48 ± 0.22	5.57 ± 0.20	5.33 ± 0.19
	HP	5.44 ± 0.23	5.41 ± 0.20	5.48 ± 0.19	5.53 ± 0.19	5.43 ± 0.16	5.67 ± 0.16
Plasma Na^+ concentration mmol/l	R	147 ± 1.21	146 ± 1.76	148 ± 1.18	148 ± 1.31	149 ± 1.24	149 ± 0.81
	P	143 ± 2.00	142 ± 1.90	139 ± 2.10	142 ± 1.51	137 ± 1.49	137 ± 1.58
	HP	142 ± 1.52	141 ± 1.60	142 ± 1.61	140 ± 1.50	142 ± 1.58	140 ± 1.49
Plasma K^+ concentration mmol/l	R	3.9 ± 0.08	3.8 ± 0.07	3.6 ± 0.07	3.5 ± 0.06	3.5 ± 0.06	3.6 ± 0.05
	P	3.8 ± 0.12	3.5 ± 0.12	3.5 ± 0.11	3.6 ± 0.11	3.3 ± 0.07	3.3 ± 0.10
	HP	4.2 ± 0.30	3.8 ± 0.20	3.9 ± 0.25	3.6 ± 0.18	3.4 ± 0.10	3.5 ± 0.13
Plasma osmolality mosm/l	R	319 ± 2.6	318 ± 2.7	317 ± 2.8	319 ± 2.5	319 ± 2.5	309 ± 1.8
	P	295 ± 5.4	291 ± 5.6	294 ± 5.3	299 ± 2.4	300 ± 2.7	300 ± 2.7
	HP	295 ± 3.1	299 ± 2.5	300 ± 2.2	303 ± 1.4	303 ± 1.4	303 ± 1.8

R = volume expansion with Ringer's solution (n = 60)

P = volume expansion with plasma taken from non-hydrated dogs (n = 10)

HP = volume expansion with plasma taken from hydrated dogs (n = 11)

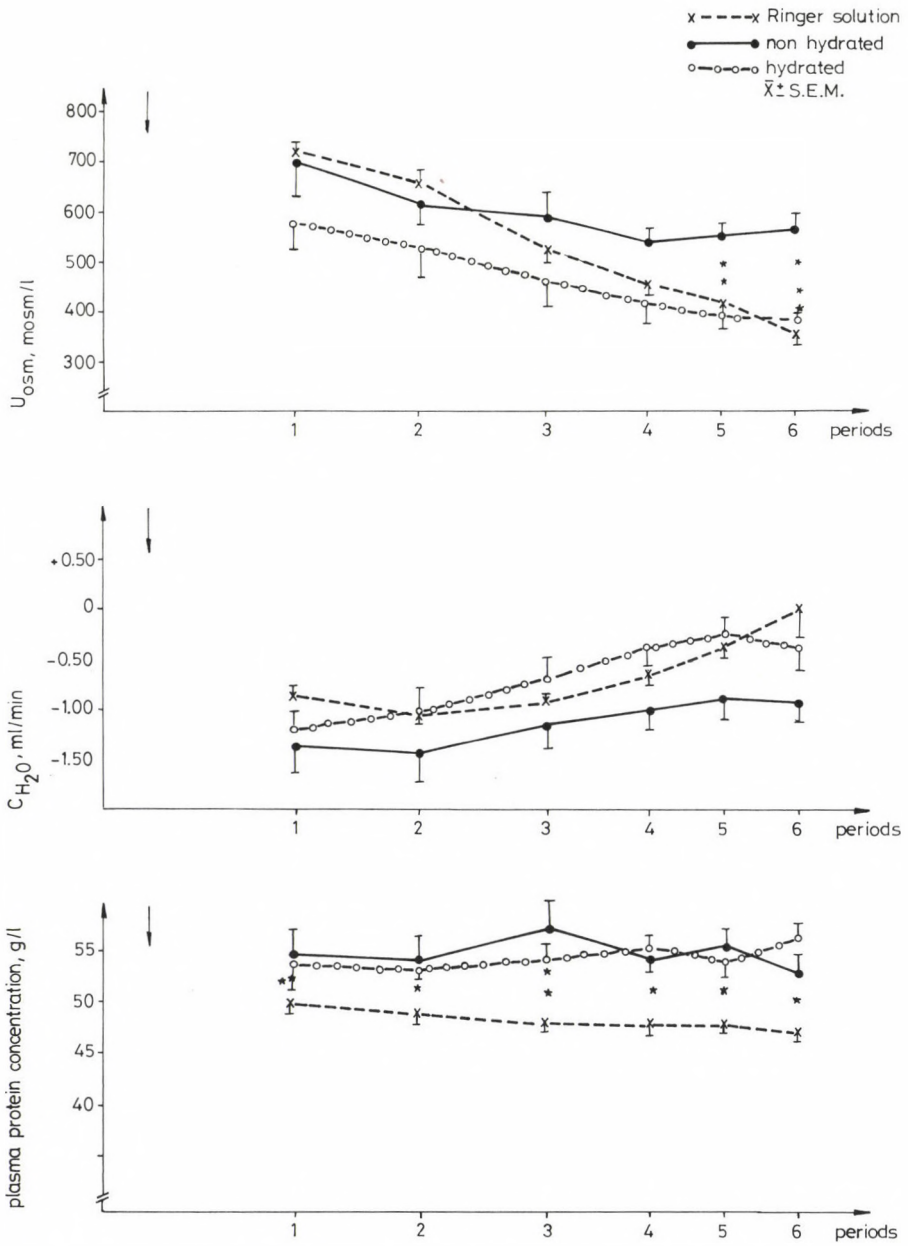

Fig. 3. The figure shows the urinary osmotic activity, the C_{H_2O} and the plasma protein concentrations. Designations are the same as in Figs 1 and 2

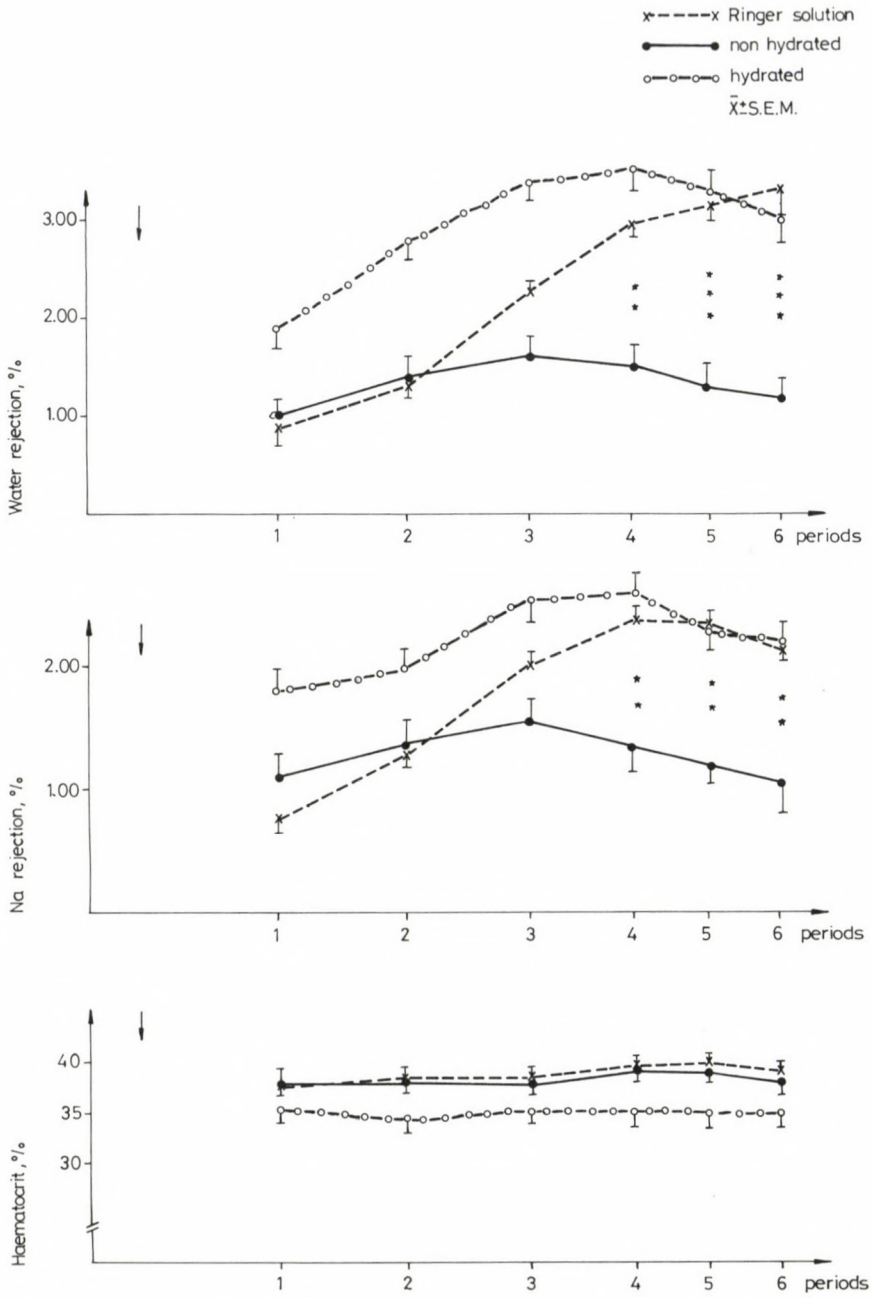

Fig. 4. Values for water rejection, Na-rejection and hematocrit in the three series of experiments. Designations are the same as in Figs 1 and 2

Discussion

The effect of isotonic volume expansion on the kidney function has widely been investigated and it has been reported to produce diuresis and natriuresis. The diuretic and natriuretic response can be iniciated by a lot of overlapping mechanisms. The decrease in plasma renin activity, drop in circulating levels of aldosterone and vasopressin, changes in physical factors influencing glomerular and tubular function, changes in the intrarenal distribution of blood flow, circulating and local natriuretic factors may all be determinants of the natriuretic response.

The atrial natriuretic factor was described by De Bold et al. [8]. The working hypothesis supported by circumstantial evidences [20, 38, 40] was, that the atrial factor is released into the blood in greater amount, when atrial pressure is elevated. Its primary function – it was postulated – was to cause natriuresis thereby reducing blood volume (and atrial pressure) closing a negative feedback loop involved in the regulation of body fluid balance. Later on the chemical structure, the binding sites, the second messenger mechanism for the ANP were discovered [9, 10, 14, 26, 47]. A lot of evidences accumulated proving the role of ANP in inducing natriuretic response [9, 30, 31, 41, 42, 44, 47]. A little bit later it was demonstrated, that "the" natriuretic peptide can also be produced in several other organs [17, 18, 33, 36, 45, 46]. More recently numerous evidences have shown, that the atriopeptin is not the primary physiologic regulator of sodium excretion [1, 3, 12, 19, 22, 23, 27, 35, 37]. Consequently, even after more than a decade of intensive investigation there is no consensus concerning neither, the factors regulating ANP release, nor its primary physiological effects, but it is postulated, that atriopeptin is primarily a regulator of the cardiovascular system with relatively minimal direct effects on the kidneys under normal physiological conditions.

Extracellar volume expansion with saline has been reported to produce a diuretic and natriuretic response in renal denervated dogs in the absence of changes in plasma renin activity and circulating levels of aldosterone, vasopressin and atrial natriuretic peptide (ANP) [4, 6, 7].

The urodilatin, a member of the natriuretic peptide family produced by the kidney has also been postulated to be the intrarenal mediator of the regulation of Na^+ and Cl^- transport in the kidneys within various conditions. It was discorved by Schultz-Knappe et al. in 1988 [39]. In contrast to atriopeptin it is resistant to the action of peptidases of cortical membranes obtained from dog kidney [25]. Uridilatin has an affinity for the same binding sites as ANP in several tissues, and activates guanylate cyclase generating cGMP [25, 48]. It accounts largely for the ANP immunreactivity in human urine but cannot be detected in the plasma [13]. The regulation of the urodilatin release is not sufficiently cleared up, but it has already been demonstrated that left atrial distension through cardiac neural pathway, volume expansion independently of the cardiac neural pathways, and elevated plasma sodium concentration stimulating cephalic receptors and using neural or humoral efferent pathways can increase its excretion and induce natriuretic response [13, 21].

In our experiments extracellular volume expansion with Ringer's solution induced a gradual increase in urinary water and sodium excretion, and water and sodium rejection. When the same amount of plasma taken from non-hydrated animals was infused only a small increase in the first 15 min was observed, afterwards urine and sodium excretions remained unchanged in spite of the fact, that in this case much more of the infused fluid remained within the vascular bed than in the case of Ringer's solution infusion [7]. The blood pressure, C_{PAH}, GFR, and plasma parameters (plasma osmolality, sodium and potassium concentrations) were – within limits of error – identical in the two series and did not change during the experiment, with the only exception of the plasma protein concentration being significantly lower in the animals infused with Ringer's solution. These findings are in good agreement with the data in the literature pointing out, that changes in physical factors, like decrease in plasma colloid osmotic pressure (COP), a consequent increase in the renal interstitial pressure (RIP) and perhaps intrarenal redistribution of blood flow [7, 14, 29] may play an important role in the development of natriuresis following isotonic volume expansion.

In the third experimental series the same amount of plasma taken from previously hydrated animals was infused intravenously. In this case, again without any significant difference in blood pressure, C_{PAH}, GFR and without any significant difference in the measured plasma parameters, the water and sodium excretions, were already in the first 15 min significantly higher than the corresponding values in the animals infused with plasma taken from non-hydrated dogs, and remained significantly higher throughout the whole experiment. These results point to the probability of the presence of a circulating factor in the plasma of the hydrated animals, being at least partially responsible for the development of the diuretic/natriuretic response. This factor cannot be the urodilatin because it does not circulate in the plasma [12]. On the base of our experiments we cannot tell what this factor can be, it can be a natriuretic factor liberated from the atria or from other organs, or it can be some humoral factor increasing the release of urodilatin in the kidneys. Whatever this factor can be, the stimulus for its release cannot be the increased intravascular volume/or pressure, because the intravascular volume must have been about in the same magnitude in the two groups of plasma infusion, and certainly much higher in the plasma infusion series than in the case of Ringer's solution infusion.

We can conclude from our experiments, that besides the physical forces playing certainly a role mainly in the Ringer's solution hydrated dogs, same humoral factor liberated in the volume expanded animals plays also a role in the diuretic-natriuretic response. This humoral factor can be released from the atria of other organs, can be natriuretic itself or can increase the release of the urodilatin, but its production is not primarily regulated by intravasal volume/or pressure changes.

REFERENCES

1. Brenner, B. N., Ballermann, B. J., Gunning, M. E., Zeidel, M. L.: Diverse biological actions of atrial natriuretic peptide. Physiol. Rev. **70**, 665 – 699 (1990).
2. Buchaler, W. M., Gruber, K. A. Jr.: Natriuretic hormone. In the Kidney in Liver Disease, ed. M. Epstein, New York: Elsevile 2nd ed., 479 – 499 (1983).
3. Cogan, M. G.: Nephrology forum: atrial natriuretic peptide. Kidney Int. **37**, 1148 – 1160 (1990).
4. Cowley, A. W. Jr., Andreas, P. R., Shelton, M. M.: Acute saline loading in normal and bilaterally atrial-resected conscious dogs. Am. J. Physiol. **255**, H144 – H152 (1988).
5. Cowley, A. W., Roman, R. J. : The pressure-diuresis-natriuresis mechanism in normal and hypertensive states. In: Handbook of Hypertension-Pathophysiology of Hypertension-Regulatory Mechanisms, edited by A. Zanchetti and R. C. Tarazi. Amsterdam, Elsevier **8**, 295 – 314 (1986).
6. Cowley, A. W. Jr., Roman, R. J.: Control of blood and extracellular volume. In: Bailliere's Clinical Endocrinology and Metabolism, ed. by P. H. Baylis Tindall, p. 331 – 369 (1989).
7. Cowley, A. W. Jr., Shelton, M. M.: Dominance of colloid osmotic pressure in renal excretion after isotonic volume expansion. Am. J. Physiol. **261**, H1214 – H1225 (1991).
8. De Bold, A. J., Borenstein, H. B., Veress, A. T., Sonnenberg, H.: A rapid and potent natriuretic response to intravenous injection of atrial myocardial extracts in rats. Life Sci. **28**, 89 – 94 (1981).
9. De Lean, A., Vinay, P., Cantin, M.: Distribution of atrial natriuretic factor receptors in dog kidney fractions. FEBS Lett. **193**, 239 – 242 (1985).
10. De Wardener, H. E., McGregor, G. A., Clarkson, E. M., Alaghband-Zadeh, J., Birtensky, L., Chayen, J.: Effect of sodium intake on ability of human plasma to inhibit renal $Na^+ - K^+$ ATPase in vitro. Lancet **1**, 411 – 412 (1981).
11. Di Bona, G. F.: The functions of the renal nerves. Rev. Physiol. Biochem. Pharmacol. **94**, 75 – 181 (1982).
12. Drummer, C., Fiedler, F., König, A., Gerzer, R.: Urodilatin, a kidney-derived natriuretic factor, is excreted with circadian rhythm and is stimulated by saline infusion in man. J. Am. Soc. Nephrol. **1**, 1109 – 1113 (1991).
13. Emmeluth, C., Schütten, H. J., Knigge, V., Warberg, J., Bie, P.: Increase in plasma sodium entrances natriuresis in response to sodium load unable to change plasma atrial peptide concentration. Acta Physiol. Scand. **140**, 119 – 127 (1990).
14. Fenoy, F. J., Roman, R. J.: Effect of volume expansion on papillary blood flow and sodium excretion. Am. J. Physiol. **260**, F813 – F822 (1991).
15. Flynn, T. G., De Bold, M. L., De Bold, A. J.: The amino acid sequence of on atrial peptide with potent diuretic and natriuretic properties. Biochem. Biophys. Res. Commun. **117**, 859 – 865 (1983).
16. Gagelmann, M., Hock, D., Forsmann, W. G.: Urodilatin (CDD – ANP – 95 – 126) is not biologically inactived by a peptidase from dog kidney cortex membrane in contrast to atrial natriuretic peptide (cardiodilatin – H.NAP/CDD – 99 – 126). FEBS Lett. **283**, 249 – 254 (1988).
17. Gardner, D. G., Deschepper, C. F., Ganong, W. F., Hane, S., Fiddes, J., Baxter, J. D., Lewicki, J.: Extra atrial expression of the gene for atrial natriuretic factor. Proc. Natl. Acad. Sci. USA **83**, 6697 – 6701 (1986).
18. Gardner, D. G., Deschepper, C. F., Baxter, J. D.: The gene for the atrial natriuretic factor is expressed in the aortic arc. Hypertension Dallas **9**, 103 – 106 (1987).
19. Goetz, K. L.: Evidence, that atriopeptin is not a physiologic regulator of sodium excretion. Hypertension Dallas **15**, 9 – 19 (1990).
20. Goetz, K. L., Bond, G. C., Bloxham, D. D.: Atrial receptors and renal function. Physiol. Rev. **55**, 157 – 205 (1975).
21. Goetz, K. L., Drummer, C., Zhu, J. L., Leadley, R. Jr., Fiedler, F., Gerzer, R.: Evidence that urodilatin rather then ANP, regulates renal sodium excretion. J. Am. Soc. Nephrol. **1**, 867 – 874 (1990).

22. Goetz, K. L., Wang, B. C., Bie, P., Leadley, R. J. Jr., Geer, P. G.: Natriuresis during atrial distension and a current decline in plasma atriopeptin. Am. J. Physiol. **255**, R259 – R267 (1988).
23. Goetz, K. L., Wang, B. C., Geer, P. G., Leadley, R. J. Jr., Reinhardt, H. W.: Atrial strech increases sodium excretion independently of release of atrial peptides. Am. J. Physiol. **250**, R946 – R950 (1986).
24. Gornall, A. G., Bardawill, C. J., David, M. M.: Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**, 751 – 757 (1949).
25. Heim, J. M., Kiefersauer, Fülle, H. J., Gerzen, R.: Urodilation and beta ANF: binding properties and activation of particulate cyclase. Biochem. Biophys. Res. Commun. **163**, 37 – 41 (1989).
26. Kangawa, K., Matsuo, H.: Purification and complete amino acid sequence of a human atrial natriuretic polypeptide (α-hANP). Biochem. Biophys. Res. Commun. **118**, 131 – 139 (1984).
27. King, K. A., Ledsome, J. R.: Atrial dynamics, atrial natriuretic factors, tachycardia and blood volume in anaesthetized rabbits. Am. J. Physiol. **261**, H22 – H28 (1991).
28. Knock, C. A.: Further evidence in vivo for a circulating natriuretic substance after expanding the blood volume in rats. Clin. Sci. **59**, 423 – 433 (1980).
29. Knox, F. G., Haas, J. A.: Factors influencing renal sodium reabsorption in volume expansion. Rev. Physiol. Biochem. Pharmacol. **92**, 75 – 113 (1982).
30. Lang, R. E., Thölhen, H., Ganten, D., Luft, F. C., Ruskoako, H., Unger, T.: Atrial natriuretic factor – a circulating hormone stimulated by volume loading. Nature Lond. **314**, 264 – 266 (1985).
31. Lendsome, J. R., Wilson, N., Courneya, C. A., Rankin, A. J.: Release of atrial natriuretic peptide by atrial distension. Can. J. Physiol. Pharmacol. **63**, 739 – 742 (1985).
32. Little, L. M.: Modified dyphenylamine procedure for determination of inulin. J. Biol. Chem. **180**, 747 – 754 (1949).
33. Nguyen, T. T., Lazure, C., Babinski, K., Chretieb, M., Ong, H., De Lean, A.: Aldosteron secretion inhibitory factor: a novel neuropeptide in borine chromaffin cells. Endocrinology **124**, 1591 – 1593 (1989).
34. Pearce J. W., Veress, A. T., Sonnenberg, H.: Time course of onset and decay of humoral natriuretic activity in the rat. Canadian J. of Physiol. and Pharmacol. **53**, 734 – 741 (1975).
35. Richards, A. M., Tonolo, G., Polonia, J., Montorsi, P.: Contrasting plasma atrial natriuretic concentrations during comparable natriuresis with infusion of atrial natriuretic factor and saline in normal man. Clin. Sci. Lond. **75**, 455 – 462 (1988).
36. Sakamoto, M., Nakao, K., Kihara, M., Norii, N., Sugawara, A., Shimokura, M., Kiso, Y., Yamori, Y., Imuro, H.: Existence of atrial natriuretic polypeptide in the kidney. Biochem. Biophys. Res. Commun. **128**, 1281 – 1287 (1985).
37. Sarille, M. A., Geer, P. G., Wang, B. C., Leadly, R. J. Jr., Goetz, K. L.: A high salt meal produces natriuresis is human without elevating plasma atriopeptin. Proc. Soc. Exp. Biol. Med. **188**, 387 – 393 (1988).
38. Schrier, R. W., de Wardener, H. E.: Tubular reabsorption of sodium ion: influence of factors other then aldosterone and glomerular filtration rate. N. Eng. J. Med. **285**, 1231 – 1303 (1971).
39. Schultz-Knappe, P., Forssmann, K., Herbst, F., Hock, D., Pipkorn, R., Forssmann, W. G.: Isolation and structural analysis of "urodilatin" a new peptide of the cardiodilatin-(ANP)-family extracted from human urine. Klin. Wochenschr. **66**, 752 – 759 (1988).
40. Schultz, H. D., Fater, D. C., Sunder, W. D., Geer, P. G., Goetz, K. L.: Reflexes elicited by acute strech of atrial vs. pulmonary receptors in conscious dog. Am. J. Physiol. **242**, H1064 – H1076 (1982).
41. Schwab, T. R., Edwards, B. S., Heublein, D. M., Burnet, J. C.: Role of atrial natriuretic peptide in volume expansion natriuresis. Am. J. Physiol. **251**, R310 – R313 (1986).
42. Seymour, A., A., Marsch, E. A., Mazach, E. K., Stabilito, I. I., Blaine, E. H.: Synthetic atrial natriuretic factor in conscious normotensive and hypertensive rats. Hypertension Dallas J. Suppl. T – 35, T – 42 (1985).

43. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., Graber, M.: The renal clearances of substituted hippuric acid derivated and other aromatic acids in dog and man. J. Clin. Invest. **24**, 388–404 (1945).
44. Sosa, R. E., Volpe, M., Marion, D. N., Atlas, S. A., Laragh, J. H., Vaugh, E. D. Jr., Maack, T.: Relationship between renal hemodynamic and natriuretic effects of atrial natriuretic factor. Am. J. Physiol. **250**, F520–F524 (1986).
45. Sudoh, T., Kangawa, K., Minamino, M., Matsuo, H.: A new natriuretic peptide in porcine brain. Nature Lond. **332**, 78–81 (1988).
46. Sudoh, T., Minamino, M., Kangawa, K., Matsuo, H.: C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. Biochem. Biophys. Res. Commun. **168**, 863–870 (1990).
47. Tremblay, J., Gerzer, R., Vinay, P., Pang, S. C., Béliveau, R., Hamet, P.: The increase in cGMP by atrial natriuretic factor correlates with the distribution of particulate guanonylate cyclase. FEBS Lett. **181**, 17–22 (1985).
48. Valentin, J. P., Sechi, L. A., Qiu, C., Schambelan, M., Humphreys, M. H.: Urodilatin displaces latelled atrial natriuretic peptide from renal binding sites and is equally potent in generating cyclic GMP. Hypertension Dallas **18**, 393–396 (1991).

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A novel spectrophotometric method for the enzymatic determination of NAD^+ and NADH

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The theory and practice of a novel spectrophotometric method for the enzymatic determination of NAD^+ and NADH is described. The method can not discriminate between NAD^+ and NADH, but determines the concentration of the sum of both nucleotides. The method is based on the bleaching of p-nitroso-N,N-dimethylaniline (NDMA) ($\varepsilon_{440\ nm}$ = 35400 $M^{-1}cm^{-1}$) with NADH, in the presence of ethanol and yeast alcohol dehydrogenase, under the conditions of enzymatic cycling (ethanol > NDNA > NAD/H). The initial rates of −NDMA bleaching are proportional to the concentration of NAD^+ or NADH, in a broad range from 10 nM to 100 μM.

Keywords: NAD^+, NADH, spectrophotometry, enzymatic determination, nucleotides, ethanol

Quantitative determination of NAD^+ (β-nicotinamide adenine dinucleotide) and NADH (β-nicotinamide adenine dinucleotide, reduced form) in micromolar concentrations is usually performed enzymatically. Detection and quantitation of NADH is based on its strong absorption band at 340 nm (ε = 6200 $M^{-1}cm^{-1}$) and of NAD^+ on its absorption band at 260 nm (ε = 18000 $M^{-1}cm^{-1}$); for the quantitative determination, NAD^+ is usually reduced enzymatically to NADH [1]. A 10 to 100-fold increase in sensitivity is obtained by measuring the concentrations of NADH fluorimetrically; excitation of NADH at 340 nm affords a strong fluorescence at 430 nm [2, 3].

Further increase in sensitivity is obtained by the method of enzymatic cycling; this method is reported to increase the sensitivity of determination of coenzymes into the picomolar range [1, 4]. The disadvantage of this method is the high cost of chemicals [1].

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In 1971, Dunn and Bernhard discovered a novel substrate of liver alcohol dehydrogenase – *p*-nitroso-*N,N*-dimethylaniline [5, 6]. Recently, we have found that NDMA is also a good substrate of yeast alcohol dehydrogenase [7]. NDMA has excellent spectral properties, with a strong absorption band at 440 nm (ε = 35400 M^{-1} cm^{-1}, pH-independent) and a low absorbancy at 340 nm (ε = 1000 M^{-1} cm^{-1}) [5, 7]. For this reason, NDMA is uniquely suited for the enzymatic determination of NAD^+ and NADH.

In this communication, we have described a novel spectrophotometric method for the enzymatic determination of NAD^+ and NADH, with NDMA and yeast alcohol dehydrogenase. The method is effective in a broad range of coenzyme concentrations, from 10 nM to 100 μM.

Materials and methods

Yeast alcohol dehydrogenase (EC 1.1.1.1), NAD^+ and NADH of the highest grade purity, were obtained from Boehringer GmbH, Mannheim (Germany). *p*-Nitroso-*N,N*-dimethylaniline was obtained from Aldrich-Chemie GmbH, Steinheim (Germany). All other chemicals were of analytical grade purity, obtained from commercial sources.

The concentration of enzyme protein in solution was estimated according to Hayes and Velick [8], the concentration of enzyme active sites was determined by the fluorescent method of Leskovac et al. [9], and expressed in μM in the text. Specific activity of enzyme on ethanol was 300 U/mg estimated at pH 9, according to Bergmeyer [1].

All reaction rates were determined in a self-recording double-beam spectrophotometer SPECORD UV VIS, Carl Ziess, Jena (Germany), in thermostated cuvette holders, at 25 °C. All kinetic measurements were performed in 0.1 M sodium phosphate buffer pH 6–8, 0.1 M sodium pyrophosphate pH 9, or 0.1 M sodium glycinate pH 10, supplemented with 0.5 mM EDTA.

Extraction of NAD^+ from the human blood was performed according to Bergmeyer [1]. Human blood was obtained from a voluntary blood donor in the University Hospital Novi Sad, and stabilized in a usual manner. One ml of fresh blood was pipeted directly into 5 ml of cold 0.6 N perchloric acid, and the protein precipitate removed by centrifugation. To 5 ml of the clear supernate, 1 ml of 1M K_2HPO_4 and 0.65 ml of 3N KOH were added to bring pH exactly to 7.0. The solid $KClO_4$ was centrifuged and the clear supernate was used as an extract of human blood for the enzymatic determination of NAD^+; the dilution factor human blood/extract was 1:7.75.

Results and discussion

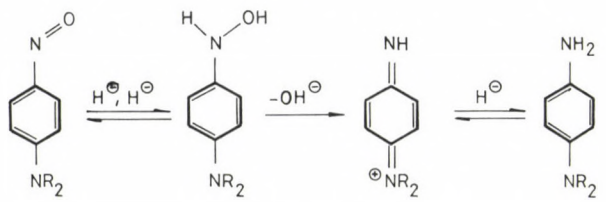
Chemical and kinetic properties of the enzymatic reaction

NDMA is very slowly reduced by NADH in neutral aqueous solutions, with a bimolecular rate constant of 0.4 M^{-1} s^{-1}. This redox reaction is greatly accelerated in the presence of yeast alcohol dehydrogenase [7].

In aqueous buffers, from pH 6–10, NDMA is enzymatically reduced by NADH into two types of stable reaction products: *p*-amino-*N,N*-dimethylaniline and

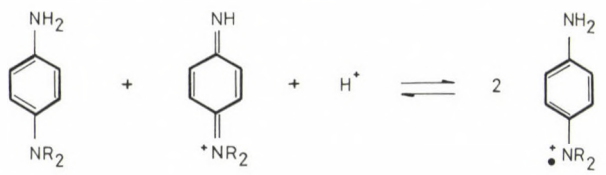
Abbreviations: NDMA, *p*-nitroso-*N,N*-dimethylaniline; ADMA, *p*-amino-*N,N*-dimethylaniline; DPD, *N,N*-dimethyl-*p*-phenyl-enediamine

N,N-dimethyl-*p*-phenylenediamin radical cation. Under the steady-state conditions, ADMA and DPD-radical are the only chemically and kinetically competent products of enzymatic reaction. The stoichiometry of the enzymatic reaction depends on the molar ratio of reactants; if the molar ratio NADH/NDMA > 2, the stoichiometry is: 2 NADH + H^+ + NDMA → 2 NAD^+ + ADMA + OH^-, and the reaction proceeds via the following mechanism [6, 7, 10]:



Scheme I

If the molar ratio of NADH/NDMA < 2, substantial concentrations of DPD-radicals develop in the steady-state below pH 7.5. DPD-radicals arise from the facile, non-enzymatic one electron transfer dismutation reaction [6, 11]:



Scheme II

The favourable spectral properties of this system allows the independent observation of NADH (absorption maximum at 340 nm) NDMA (440 nm) and DPD-radical (split peak at 515 nm and 555 nm); the molar extinction coefficient of DPD-radical at pH 7 was estimated at 8350 $M^{-1}cm^{-1}$ [11]. Formation of DPD-radicals is pH-dependent (Scheme II); below pH 7.5 they are stable for several minutes under the steady-state conditions, and above pH 7.5 their formation cannot be detected [10]. A detailed chemical and enzymatic mechanism of reactions presented in Schemes I and II is described elsewhere [10].

Determination of NAD+ and NADH by enzymatic cycling

The condition of enzymatic cycling [1, 4] is met when the yeast enzyme is mixed with NDMA, high concentrations of ethanol and low concentrations of NAD^+ or NADH. Under the conditions of enzymatic cycling (ethanol > NDMA > NAD/H), the chemical potential of the ethanol-acetaldehyde redox couple is used to recycle the catalytic amount of coenzyme present, and thus drive the reduction of NDMA to

completion [1, 5]. Under these conditions, the rate of the NDMA-bleaching at 440 nm is directly proportional to the concentration of coenzyme; this observation afforded the bases for the development of the novel spectrophotometric method for the enzymatic determination of NAD^+ and NADH.

Under the conditions of enzymatic cycling, the initial rate of NDMA-bleaching (v_o/e_o) is directly proportional to the concentration of NAD^+ or NADH, according to eqn (1):

$$\text{Log } (v_o/e_o) = A + B.\log [\text{coenzyme}] \quad (1)$$

where v_o = initial rate of NDMA-bleaching at 440 nm (M. s^{-1}) and e_o = the concentration of enzyme active sites (M). The linear relationship of eqn (1) is valid only in a restricted range of coenzyme concentrations, and is strongly pH-dependent; the slope factor B decreases slightly with increasing pH. Figure 1 shows such linear relationships obtained at pH 7 and pH 9.

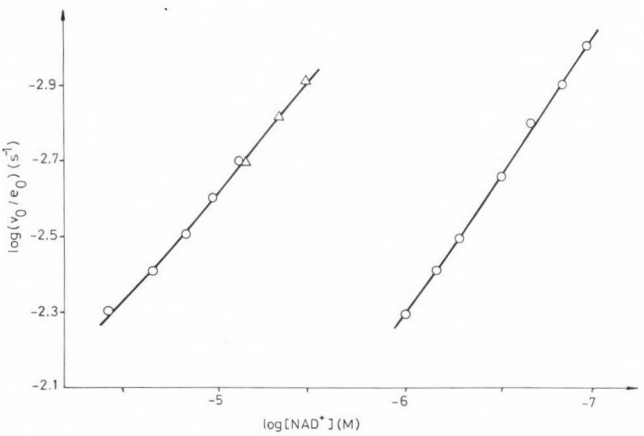


Fig. 1. Calibration graphs Left: NDMA (10.2 μM), ethanol (170 mM), enzyme (0.96 μM − o −; 3.6 μM − Δ −), and variable concentrations of NAD^+ (abscissa) were mixed at pH 9, and v_o/e_o determined from the rates of NDMA-bleaching at 440 nm (ordinate). Right: NDMA (25.2 μM), ethanol (170 mM), enzyme (2.1 μM), and NAD^+ (abscissa) were mixed at pH 7, and the values of v_o/e_o measured as above

Under the conditions of enzymatic cycling, a constant steady-state ratio of NAD^+/NADH is rapidly reached and maintained until the end of reaction [10]. Therefore, eqn (1) is obeyed irrespective of the redox state of coenzyme (NAD^+ or NADH), with which the reaction is started. Under the conditions of enzymatic cycling (NDMA > NAD/H), small concentrations of DPD-radicals appear below pH 7.5, which, however, does not invalidate eqn (1) and consequently does not interfere with the determination of coenzymes.

Practical application of the method

For the practical determination of NAD^+ or NADH, a self-recording double-beam spectrophotometer is required. The novel method is effective in a broad range of concentrations, from 10 nM to 100 μM NAD^+ or NADH. At pH 6, eqn (1) is valid in the concentration range 10–100 nm; at pH 7: 0.1–1.5 μM; at pH 8: 1–10 μM; at pH 9: 4–40 μM; and at pH 10: 20–100 μM. For the practical determination of NAD^+ an NADH concentrations, a calibration graph must be prepared for the desired concentration range, at a constant pH value; usually, 7–8 concentrations of coenzyme for one calibration graph are required (Fig. 1). The concentration of coenzyme must be determined twice, and a mean value calculated. The concentrations of NDMA, enzyme and ethanol in the test are arbitrary, but the recommended concentrations of ethanol are 100–200 nM and of NDMA 10–30 μM (Fig. 1).

The rates of NDMA-bleaching at 440 nm are strictly constant for at least 10 min, between pH 6 and pH 10, under experimental conditions of Fig. 1; this makes possible a very accurate determination of v_o in eqn (1).

Cur method cannot discriminate between NAD^+ and NADH; if a mixture of NAD^+ and NADH is measured, the method will give the sum of concentrations of both. NAD^+ and NADH can be separated and estimated independently by an acid or an alkaly treatment, respectively, as described by Bergmeyer [1]. The method is absolutely specific for NAD^+/NADH, and the presence of $NADP^+$/NADPH does not interfere.

Table I

Determination of NAD^+ in an acid extract of human blood

Volume of extract (ml)[a]	$v_o/e_o \times 10^3$ (s^{-1})	[NAD^+] in extract (μM)[b]
0.1	1.54	4.05
0.2	2.55	4.135
0.3	3.46	4.11
0.4	4.365	4.28
0.5	4.89	3.96
0.6	5.62	4.00
0.7	6.48	4.17
$\bar{x} \pm$ S.E.M.		4.10 ± 0.11

[a] Milliliters of extract in a total volume of 1.96 ml of test mixture; extract was prepared as described in the Methods section, and neutralized to pH 7.

[b] The unknown concentrations of NAD^+ in blood extracts were determined from eqn (1), using the constants A (2.05) and B (0.727) obtained independently from the calibration graph with known concentrations of NAD^+ at pH 7.0, shown in Fig. 1.

In order to test the precision of the method, the concentration of NAD^+ in the acid extract of the fresh whole human blood was determined. Table I shows the experimental data and the mean value ± standard error, from seven determinations.

An acid extract of blood destroys NADH [1, 12] and only NAD^+ is determined. If the concentration of NAD^+ in the acid extract (4.1 μM) is multiplied with the dilution factor (7.75, Methods section), the concentration of 30.8 μM of NAD^+ in the fresh whole human blood is obtained. The standard error of the method was calculated at ±2.7% (Table I), which indicates a satisfactory precision of this method.

Interference with the method

Aqueous or alcoholic solutions of NDMA are stable for two weeks in the cold. Traces of heavy metals are inhibitory for enzyme, but they are easily removed by including 0.1–1 mM EDTA in the buffer [13]. Thioalcohols (reduced glutathione, β-mercaptoethanol) readily reduce NDMA non-enzymatically, and their presence interferes with the method; the proteins rich in –SH groups, if present in high concentrations, are also able to slowly reduce NDMA. Yeast alcohol dehydrogenase, in concentrations above 5 mg/ml, reduces slowly NDMA, which usually does not interfere with the method, since the required concentrations of enzyme are much lower. The interference of other reducing agents has not been investigated.

Acknowledgement

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REFERENCES

1. Bergmeyer, H. U.: Methoden der enzymatischen Analyse, Verlag Chemie, Weinheim/Bergstasse, 1970.
2. Udenfriend, S.: Fluorescence Assay in Biology and Medicine, pp. 245–252, Academic Press, New York, 1962.
3. Westheimer, F.: in: Pyridine Nucleotide Coenzymes (Dolphin, D., Avramovic, O. and Poulson, R., eds), pp. 501–548, Wiley-Interscience, New York, 1987.
4. Lowry, O. H., Passoneau, J. V., Schulz, D. W., Rock, K. M.: J. Biol. Chem. **236**, 2746–2756 (1962).
5. Dunn, M. F., Bernhard, S. A.: Biochemistry **10**, 4569–4575 (1971).
6. Koerber, S. C., Schack, P., Au, A. M.-J., Dunn, M. F.: Biochemistry **19**, 731–738 (1980).
7. Trivic, S., Leskovac, V.: Biochem. Int. **25**, 669–675 (1991).
8. Hayes, J. H., Velick, S. F.: J. Biol. Chem. **207**, 225–232 (1954).
9. Leskovac, V., Trivic, S., Pantelic, M.: Anal. Biochem. **214**, 431–434 (1993).
10. Leskovac, V., Trivic, S., Anderson, B. M.: Forthcoming publication (1995).
11. Baetzold, R. C., Tong, L. K. J.: J. Am. Chem. Soc. **93**, 1347–1352 (1971).
12. Leskovac, V. Trivic, S., Cadzic, D., Marganovic, D.: Monatshefte für Chemie **118**, 1119–1128 (1987).
13. Leskovac, V., Pavkov-Pericin, D.: Biochem. J. **145**, 581–590 (1975).

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Different reactivity of two rabbit vessels under hypoxia

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The effect of hypoxia on isolated rabbit vessel reactivity to vasoconstrictive agents was studied. Short-lasting hypoxia (30 min.) enhanced the responses to noradrenaline, 5-hydroxytryptamine, histamine and endothelin in the ear artery. Increased reactivity was also found with KCl depolarization solution. Deendothelization of the ear artery did not influence the enhanced responses to noradrenaline and endothelin in hypoxia. In similar experimental conditions the femoral artery reactivity to noradrenaline was not affected by hypoxia, the constrictory responses to 5-hydroxytryptamine were decreased. It can be concluded that hypoxic facilitation of vasoconstrictive activity is probably independent on receptor-related mechanisms and/or on the presence of endothelium. The results obtained confirm also the difference in reactivity of rabbit vessels from two vascular beds to vasoconstrictive agents under hypoxia.

Keywords: rabbit ear and femoral arteries, hypoxia, enhanced reactivity, endothelium

Studies on reactivity of different types of vessels under hypoxia led to contradictory results. Earlier findings [8] have shown decreased reactivity of aorta to noradrenaline in hypoxia. Recently, several data have suggested that hypoxia increases the reactivity of vessels to various vasoconstrictive agents. Hypoxia or anoxia augmented the contractibility of isolated canine arteries and veins to noradrenaline, 5-hydroxytryptamine, PGF_{2alfa} and KCl [23, 5, 20]. Facilitation of vasoconstrictive responses under hypoxia was found in human, monkey and dog coronary arteries [22] and other types of vessels [15, 18]. Although hypoxia mostly causes facilitation of vasoconstrictive responses, in some studies hypoxia-induced vasodilatation was found [3, 17]. The cause of increased or decreased responses under hypoxia is not fully understood. In the last decade the role of endothelium in hypoxic responses has been thought into focus [5, 13, 22].

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In many studies isolated artery strips and rings were used for evaluating hypoxic responses. The perfusion method of isolated vessel segments has not been applied so frequently. It is considered to be more physiological than the method of strips and rings. Therefore, this work was designed to study the reactivity of rabbit vessels taken from two vascular beds (ear artery, femoral artery) to vasoactive agents in hypoxia by the method of in vitro vessel perfusion. Furthermore the evaluation of the role of endothelium in vascular responses to hypoxia was carried out.

Materials and methods

Male rabbits (Cincilla) weighing 2.5 – 3.0 kg were killed by cervical dislocation. The following experimental procedures were used:

Ear artery preparation: The artery was isolated after the ear had been amputated. Polyethylene cannula was introduced into the vessel lumen, leaving a 5 mm long free segment.

Femoral artery preparation: The artery was carefully removed and cannulated using a procedure that minimizes vessel damage [2]. The free segment was cut up to a constant length of 5 mm.

The preparations were mounted in vessel chamber and perfused at a constant flow with Tyrode solution (composition in mM: NaCl 137, KCl 2.7, $MgCl_2$ 1.1, NaH_2PO_4 0.32, $CaCl_2$ 0.9, $NaHCO_3$ 11.9, glucose 5.5). The perfusion solution was saturated with either 95% O_2/5% CO_2 (normoxia) or 95% N_2/5% CO_2 (hypoxia) in both the vessel chambers and reservoir, maintained at 37 °C and at pH 7.25 – 7.35. The pressure changes were measured by means of tensometric transducer (LDP 102, Tesla) and registered on recorder (TZ 4200, Tesla).

Experimental protocol: After an equilibration period of 30 minutes, the basal perfusion pressure was adjusted to 30 mm Hg in the ear arteries with the corresponding flow rate of 2.5 – 3 ml/min. In the femoral artery, the basal perfusion pressure was set to 18 – 20 mm Hg at a flow rate of 25 ml/min. Vasoactive agents (noradrenaline, histamine, 5-hydroxytryptamine, endothelin, acetylcholine) were injected directly into the cannula in a constant volume of 0.1 ml during normoxia, hypoxia and after deendothelization. Hypoxia was induced by a 30-minute lasting saturation of the perfusion solution with N_2/CO_2. Deendothelization of ear arteries was performed with air bubble perfusion for 10 minutes as described previously [2].

Isolated ear artery: The reactivity to series of noradrenaline (0.01, 0.05, 0.1 μg), histamine (0.01, 0.1, 1, 10 μg) and 5-hydroxytryptamine (0.1, 1, 10, 50, 100 μg) doses in both normoxia and hypoxia was evaluated. For evaluation of a functional state of endothelium, acetylcholine in a dose of 10 μg was administered to the vessel preconstricted with 2×10^{-6} M of noradrenaline (added to the reservoir solution) at the end of the experiment (Fig. 1).

Furthermore, reactivity to endothelin in intact and deendothelized vessels under hypoxia was studied with administration of a single bolus of endothelin (4×10^{-9} M – 4×10^{-6} M). Potassium chloride (85 mM) was added to evaluate the ear artery responses under normoxic and hypoxic conditions.

Isolated femoral artery: The reactivity to noradrenaline (0.1, 1, 5, 10 μg) and 5-hydroxytryptamine (0.1, 1, 10, 100 μg) in both normoxia and hypoxia was studied. In some experiments the free vessel segment was cut up for histological examination after 30-min. perfusion in hypoxic solution.

Immunochemical examination: The vessel specimens were fixed in formalin, processed with the routine paraffin technique and cut perpendicularly. Deparaffinized rehydrated 5 μm thick sections were digested for 10 min. by pronase (Dako) and immunochemically stained with anti-FVIII-RAg (Factor VIII-Related Antigen) antibody (Dako).

Drugs: Noradrenaline hydrogentartarate (Spofa), Histamine (Spofa), Acetylcholine chloride (Dispersa AG), 5-hydroxytryptamine (Sigma), Endothelin-1 (Sigma). The substances were dissolved in physiological saline solution. For dilution of drugs Tyrode's solution was used. All solutions were prepared fresh before each experiment.

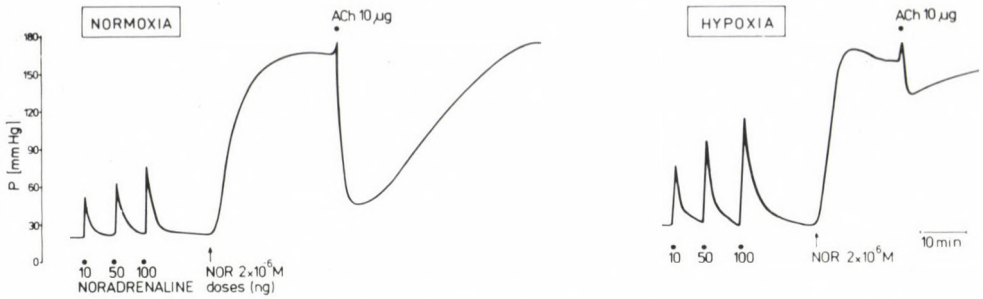

Fig. 1. Representative record from the perfusion experiment. Reactivity of the rabbit ear artery to single doses of noradrenaline and to acetylcholine in the vessel precontracted by noradrenaline (2×10^{-6} M) in both normoxic and hypoxic conditions. P = perfusion pressure, NOR = noradrenaline, ACh = acetylcholine

Evaluation of results: All results are expressed as mean ± standard error of the mean (S.E.M.). Statistical significance was determined by the paired Student's *t*-test. P values less than 0.05 were considered as significant.

Results

Ear artery

The reactivity of ear arteries to noradrenaline (Table I), histamine (Table II) and 5-hydroxytryptamine (Table III) in hypoxia increased significantly. More pronounced facilitation of responses in hypoxia was found to noradrenaline than to histamine and 5-hydroxytryptamine. The dilatory responses of ear arteries to acetylcholine were decreased during hypoxia to 27.9±3.8% if compared with the reactivity in normoxia (Fig. 1).

Table I

Responses of rabbit ear arteries (n = 6) to noradrenaline under normoxia and hypoxia (intact (+E) and deendothelized (−E) vessels)

Noradrenaline (dose in µg)	Normoxia (+E)	Hypoxia (+E) (responses in mm Hg)	Hypoxia (−E)
0.01	35.5 ± 5.92	72.5 ± 8.91***	74.0 ± 4.5
0.05	72.0 ± 10.76	116.5 ± 15.75***	107.0 ± 7.1
0.1	104.0 ± 14.89	142.5 ± 17.33***	129.0 ± 7.4

mean ± S.E.M., ***$p < 0.001$

Table II

Responses of rabbit ear arteries to histamine under normoxia and hypoxia

Ear arteries (n)	Histamine (dose in μg)	Normoxia (responses in mm Hg)	Hypoxia (responses in mm Hg)
8	0.01	23.25 ± 4.21	62.63 ± 5.63*
	0.1	24.75 ± 2.98	39.38 ± 4.29
	1.0	48.38 ± 4.52	69.75 ± 6.26*
	10.0	111.75 ± 6.07	140.06 ± 3.56*

mean ± S.E.M., *$p < 0.05$

Table III

Responses of rabbit ear arteries to 5-hydroxytryptamine (5-HT) under normoxia and hypoxia

Ear arteries (n)	5-HT (dose in μg)	Normoxia (responses in mm Hg)	Hypoxia (responses in mm Hg)
6	0.1	19.0 ± 1.78	60.0 ± 4.20**
	1.0	29.0 ± 2.23	65.0 ± 4.55**
	10.0	51.0 ± 2.97	86.5 ± 4.95**
	50.0	82.5 ± 4.34	112.0 ± 5.00***
	100.0	100.0 ± 5.20	129.5 ± 5.46**

mean ± S.E.M., **$p < 0.01$, ***$p < 0.001$

The removal of endothelium had no effect on hypoxia-induced increased reactivity to noradrenaline (Table I).

Constrictive responses of the ear arteries to endothelin were significantly enhanced in hypoxic conditions. Deendothelization did not produce further potentiation of the responses to endothelin (Fig. 2).

Potassium chloride-induced contractions of the ear arteries were significantly enhanced in hypoxia from 33.5±3.1 to 55±3.9 mm Hg ($n = 6$, $p < 0.001$).

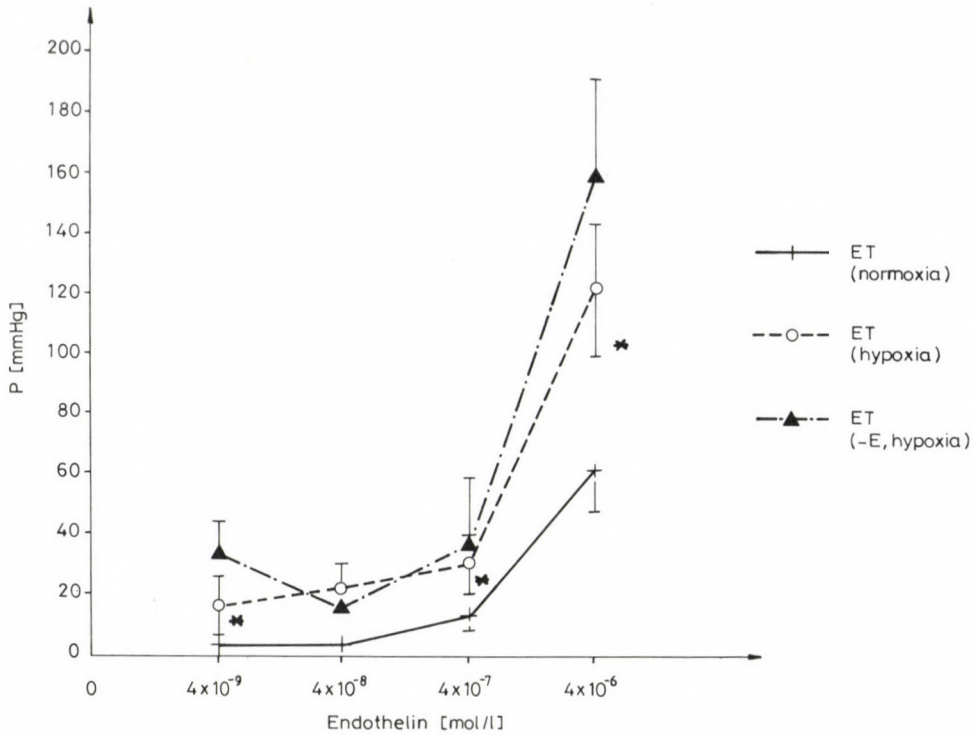

Fig. 2. Graph comparing the effect of endothelin (ET) on isolated rabbit ear arteries (n = 8) in normoxic and hypoxic conditions and after endothelium-removal (−E). Significant greater constrictory responses under hypoxia compared to that under normoxia (*$p < 0.05$)

Femoral artery

Contractile responses of femoral arteries to noradrenaline were not significantly higher in hypoxia when compared with the reactivity under normoxia (Table IV). The reactivity to 5-hydroxytryptamine significantly decreased during hypoxia (Table V).

Table IV

Responses of rabbit femoral arteries to noradrenaline under normoxia and hypoxia

Femoral arteries (n)	Noradrenaline (dose in μg)	Normoxia (responses in mm Hg)	Hypoxia (responses in mm Hg)
6	0.1	9.0 ± 1.29	9.92 ± 1.45
	1.0	16.0 ± 1.34	17.50 ± 1.03
	5.0	34.4 ± 2.3	40.25 ± 3.66
	10.0	55.5 ± 3.09	52.13 ± 5.47

mean ± S.E.M.

Table V

Responses of rabbit femoral arteries to 5-hydroxytryptamine (5-HT) under normoxia and hypoxia

Femoral arteries (n)	5-HT (dose in μg)	Normoxia (responses in mm Hg)	Hypoxia (responses in mm Hg)
	0.1	10.0 ± 1.0	3.5 ± 1.28
	1.0	21.25 ± 1.85	4.25 ± 0.99**
6	10.0	25.75 ± 2.6	5.5 ± 1.23**
	100.0	30.25 ± 2.63	5.25 ± 1.11**

mean ± S.E.M., **$p < 0.01$

The results obtained by immunohistochemical examination of the vessels showed immunopositivity of FVIII-RAg in endothelial cells and indicated that after 30 min. perfusion with hypoxic solution the endothelium was well preserved (Fig. 3).

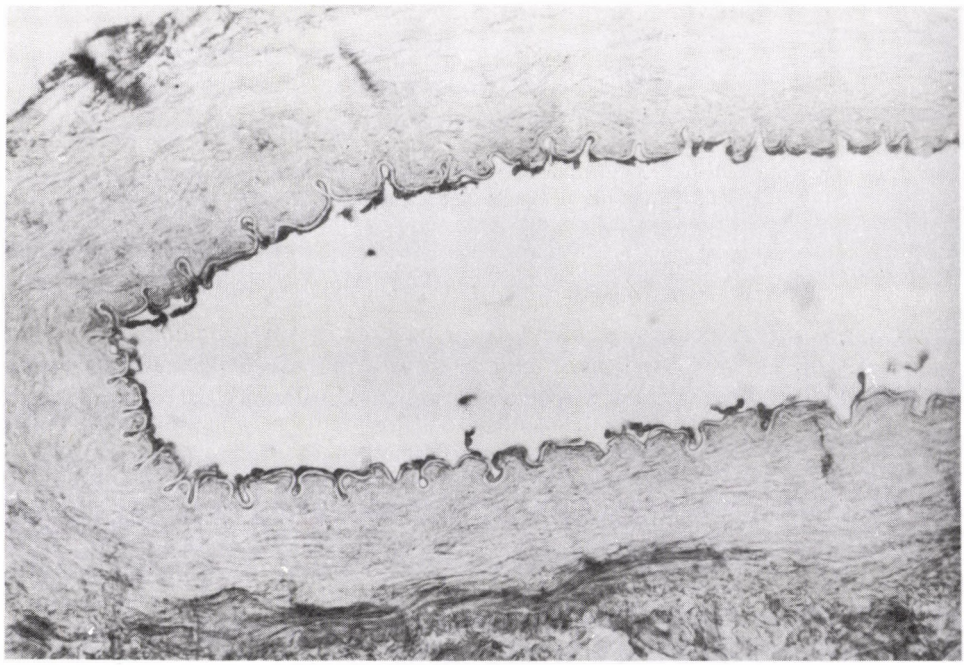

Fig. 3. Rabbit femoral artery after a 30-min. perfusion under hypoxia. Endothelium lining is well preserved. Anti-FVIII-RAg antibody, immuno-peroxidase technique, 200x

Discussion

Our results have shown that short-lasted hypoxia increased the reactivity of isolated rabbit ear artery to various vasoconstrictive agents. The finding of enhanced responses of the ear (cutaneous) artery during hypoxia was in agreement with the data found in literature [19]. Similar results were obtained in different types of vessels and to administration of several vasoactive agents. Facilitation of vasoconstrictive responses under anoxia was found in the canine femoral artery [5], under hypoxic conditions in human, monkey and dog coronary arteries [22] and in rat aorta and canine coronary arteries [15]. In the human internal mammary artery, the initial transient relaxation under hypoxia was followed by contraction of the vessel [18]. In another type of vessel – the rabbit femoral artery – under similar experimental conditions, we did not confirm the increased reactivity to noradrenaline, while the responses to 5-hydroxytryptamine decreased. These results were not surprising with regard to some data demonstrating that hypoxia induced also relaxation in some types of vessels, e.g. precontracted canine coronary arteries [3, 16], rabbit basilar and carotid arteries [17], and rabbit aorta [14]. The results obtained support the theory of organ differences in vessel reactivity to hypoxia and vasoactive agents.

We found increased reactivity not only to noradrenaline, 5-hydroxytryptamine and histamine, but also to endothelin and KCl. Hypoxic facilitation of vasoconstrictive responses suggests a more universal phenomenon which is not bound specifically to receptor-related mechanisms. This conception imply data on anoxic and hypoxic facilitation of responses to other substances, e.g. 5-hydroxytryptamine, PGF_{2alfa} [23, 5, 20], histamine [19].

The increased constrictive activity of the ear artery to K^+ depolarization solution indicates that hypoxia might affect the processes of excitation-contraction coupling in the vascular smooth muscle. Reduction of pO_2 (below 35 mmHg) causes depolarization and contraction in isolated canine carotid artery and human coronary artery, as Siegel et al. [21] demonstrated by measuring the membrane potential of smooth muscle cells in relation to the oxygen partial pressure. The effect of hypoxia on depolarization and ion transportation could be considered as another possible mechanism.

The vasoconstrictory effect of endothelin remarkably increased under hypoxia. This fact implied a speculation that the peptide, produced by endothelium, could mediate vasoconstrictive endothelium-dependent response in hypoxia. Hypoxia-induced increase in vessel reactivity to endothelin in our experiments was not significant after endothelium removal, thus it was not endothelium-dependent.

Since both, the isolated ear artery and the ear vascular bed, did not enhance responses to noradrenaline after deendothelization under normoxic conditions [12], the results obtained in this study of vessel reactivity in hypoxia support the theory of endothelium-independent hypoxic facilitation of reactivity. Some authors, using different types of vessels, have shown the endothelium-dependence of increased

reactivity in hypoxia [11, 13, 6]. It was previously supposed that endothelium releases vasoconstrictive substances during anoxia and hypoxia [5, 20, 11]. This theory was supported mainly by the results with the abolished hypoxic contraction of vessels by endothelium removal.

Besides the possibility of increased production of vasoconstrictive agents by endothelium under hypoxia, decreased production of endothelium-derived relaxatory factors could be involved in the mechanism of enhanced vasoconstrictive responses in hypoxia. As we found in our experiments, the relaxatory responses of rabbit ear artery to acetylcholine decreased during the short-lasting hypoxia. Inhibition of the endothelium-dependent relaxation to acetylcholine, and to other vasoactive agents as ADP, thrombin and vasopressin in hypoxia have been previously reported [4, 11]. Reduction in either generation or release of EDRF in hypoxia is closely related to the lower activity of guanylatcyclase and cGMP reduction in vascular smooth muscle cells [10]. It is unlikely that this is the only mechanism depending on the endothelium that would plausibly explain the increased reactivity of the ear arteries in hypoxia.

Results of the present study indicate that hypoxia-induced increase in reactivity of rabbit ear vessels could be related to multifactorial mechanisms. For further explanation of these mechanisms additional studies are going to be performed.

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REFERENCES

1. Babál, P., Kriška, M., Kristová, V., Jakubovský, J., Canová, R., Blahutová, V.: Changes of the vascular endothelium in rabbit fetuses under conditions of perfusion in vitro. Bratisl. Lek. Listy **91**, 335–340 (1990).
2. Babál, P., Kriška, M., Kristová, V., Caňová, R.: Evaluation of endothelium loss by in vitro vessel perfusion using Millipore filters. Physiol. Res. **41**, 359–362 (1992).
3. Busse, R., Bassenge, E.: Endothelium and hypoxic responses. Bibl. cardiol. No. **38**, 21–34 (1984).
4. De Mey J. G., Vanhoutte, P. M.: Interaction between Na^+, K^+ exchanges and the direct inhibitory effect of acetylcholine on canine femoral arteries. Circulat. Res. **46**, 826–836 (1980).
5. De Mey J. G., Vanhoutte, P. M.: Anoxia and endothelium dependent reactivity of the canine femoral artery. J. Physiol. **335**, 65–74 (1983).
6. Demiryurek, A. T., Wadsworth, R. M., Kane, K. A., Peacock, A. J.: The role of endothelium in hypoxic constriction of human pulmonary artery rings. Amer. Rev. Resp. Dis. **147**, 283–290 (1993).
7. Elliott, D. A., Ong, B. Y., Bruni, J. E., Bose, D.: Role of endothelium in hypoxic contraction of canine basilar artery. Brit. J. Pharmacol. **96**, 949–955 (1989).
8. Furchgott, R. F.: Metabolic factors that influence contractibility of vascular smooth muscle. Bull. N. Y. Acad. Med. **42**, 996–1005 (1966).

9. Harrison, D. G.: Physiological aspects of vascular endothelial cell interactions in hypertension and atherosclerosis. Acta Anaesth. Scand. 37, Suppl. **99**, 10–15 (1993).
10. Johns, R. A., Linden, J. M., Peach, M. J.: Endothelium-dependent relaxation and cyclic GMP accumulation in rabbit pulmonary artery are selectively impaired by moderate hypoxia. Circulat. Res. **65**, 1508–1515 (1989).
11. Katušic, Z. S., Vanhoutte, P. M.: Anoxic contraction in isolated canine cerebral arteries: Contribution of endothelium-derived factors, metabolites or arachidonic acid and calcium entry. J. Cardiovasc. Pharmacol. **8**, S97–S101 (1986).
12. Kristová, V., Kriška, M., Hejdová, E., Caňová, R., Dobrocký, P.: Comparison of vessel reactivity from two vascular beds after deendotheliazation. Čs. Fysiol. **41**, 63 (1992).
13. Lin, P. J., Pearson, P. J., Schaff, H. V.: Vasoconstriction to hypoxia of the human internal mammary artery. Chang Keng I Hsuen (Taivan) **14**, 141–148 (1991).
14. Moreland, S., Coburn, R. F., Baron, C. B., Moreland, R. S.: Mechanical and biochemical events during hypoxia-induced relaxations of rabbit aorta. Advanc. Exp. Med. Biol. **304**, 147–157 (1991).
15. Muramatsu, M., Iwama, Y., Shimizu, K., Asano, H., Toki, Y., Miyazaki, Y., Okumura, K., Hashimoto, H., Ito, T.: Hypoxia-elicited contraction of aorta and coronary artery via removal of endothelium-derived nitric oxide. Amer. J. Physiol. **263**, H1339–H1347 (1992).
16. Myers, P. R., Muller, J. M., Tanner, M. A.: Effects of oxygen tension on endothelium dependent responses in canine coronary microvessels. Cardiovasc. Res. **25**, 885–894 (1991).
17. Pearce, W. J., Ashwal, S., Long, D. M., Cuevas, J.: Hypoxia inhibits calcium influx in rabbit basilar and carotid arteries. Amer. J. Physiol. **262**, H106–H113 (1992).
18. Pearson, P. J., Lin, P. J., Evora, P. R. B., Schaff, H. V.: Endothelium-dependent response of human internal mammary artery to hypoxia. Amer. J. Physiol. **264**, H376–H380 (1993).
19. Randall, M. D., Griffith, T. M.: Modulation of vasodilatation to levcromakalim by hypoxia and EDRF in the rabbit isolated ear: a comparison with pinacidil, sodium nitro-prusside and verapamil. Brit. J. Pharmacol. **109**, 386–393 (1993).
20. Rubanyi, G. M., Vanhoutte, P. M.: Hypoxia releases a vasoconstrictor substance from the canine vascular endothelium. J. Physiol. **364**, 45–56 (1985).
21. Siegel, G., Schnalke, F., Schaarschmidt, J., Müller, J., Hetzer, R.: Hypoxia and vascular muscle tone in normal and arteriosclerotic human coronary arteries. J. Vasc. Med. Biol. **3**, 140–149 (1991).
22. Toda, N., Matsumoto, T., Yoshida, K.: Comparison of hypoxia-induced contraction in human, monkey, and dog coronary arteries. Amer. J. Physiol. **262**, H678–H683 (1992).
23. Van Nuetten, J. M., Vanhoutte, P. M.: Effect of Ca^{2+} antagonist lidoflazin on normoxic and anoxic contractions of canine coronary arterial smooth muscle. Europ. J. Pharmacol. **64**, 173–176 (1980).

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On the possibilities of existence of enzymatic hydrolysis in the membranes themselves of the intestinal epithelial cells

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On the basis of literary data is made a profound survey of the place of the final stages of the sugars and peptides hydrolysis in the small intestine. It is pointed out that whereas it has been proved for the oligosaccharides to be situated on the surface of the enterocyte membranes the discussion on the cytoplasmic and membrane localization of the most dipeptidases has not ended up yet. In our own experimental investigation it has been established that the repeated washing of the small intestine with Ringer solution reduces the maltase activity whereas the glycyl-glycin dipeptidase activity does not change. This confirms the surface membrane localization of the disaccharidase while the dipeptidase is evidently not there. Due to the low pH in the cytoplasm the glycyl-glycin-dipeptidase is considered hardly likely to be localized intracellularly. A hypothesis is advanced according to which this enzyme is structurally related to the enterocyte membranes, his catalytic centre being in the membranes themselves. In this way hydrolysis is realized in the membrane pores as well, which allows the supposition that there exists intramembrane digestion.

Keywords: intramembrane digestion, disaccharidases, dipeptidase, transmembrane proteins, membrane pores

Nowadays it is considered as already proved that the enzymes realizing the intermediate and final stages of carbohydrate hydrolysis are localized in the premembrane structures of the apical enterocyte membranes. It is marked using the methods of preparative isolation of the brush border surface at high speed gradient centrifugation and with electronic microscopy that these structures in higher animals and man contain up to 90% of the disaccharidase activity of the enterocytes [7, 8, 10]. On the basis of these proofs of surface localization of oligosaccharidases the

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conception for the intracellular hydrolysis of oligo- and disaccharidases has lost its practical significance.

For more than 25 years, however, has continued the discussion on the exact localization of some dipeptidases, and respectively, on the place of the final phases of the corresponding substrate hydrolysis. Some of the authors think that the dipeptidases are situated mainly on the outer surface of the apical enterocyte membranes [4], others claim that they have cytoplasmic localization [9], and still others assume that there is a double localization of the enzymes – both on the membranes and intracellularly [5].

Directed investigations have established that the membrane peptidases are more resistant to a number of unfavourable factors of the environment (high temperature, detergents and others) than the enzymes of cytosol but the latter are less sensitive to the changed conditions of nutrition [6]. These data show that the membrane and intracellular peptidases are different in some of their basic characteristics, which according to us, makes their identity doubtful.

According to some authors the intracellular enzymes are proenzymes of the membrane ones and that is why they cannot have an essential nutritious importance [1]. Apart from that a statement has been made according to which the intracellular peptidases take part mainly in the catabolism of the plasmic proteins, i.e. these are enzymes related to the cellular metabolism.

It is known that the pH of the intracellular space is in the range of 3.5–5.5, which is necessary for the optimal function of the lysosomal hydrolytic enzymes [1]. It is accepted that the optimum of peptidase activity is at pH about 7.5–8.0. Such is pH on the surface of the enterocyte membranes [3]. Consequently, it is hardly probable for the intracellular peptidases to take part in the hydrolysis of intact peptides, penetrated intracellularly as the peptidases function only at low values of pH. Our experiments with alkalic-acid models also registered inhibition of the dipeptidases activity at low levels of pH [12].

All these data lead to the conclusion that the intracellular peptidases have a double function: part of them are proenzymes of the membrane enzymes and their presence in the cytoplasm is justified as a place of synthesis and not of action; the other part is connected with the catabolism of the plasmic proteins. In both cases, however, the peptidases do not have a real significance in the digestive process.

It is interesting to note that most of the authors have an opinion that glycyl-glycin-dipeptidase is one of the most probable enzymes with an intracellular localization. According to us this is the least probable as in all our experimental investigations using the method of accumulating preparation of mucosa [14] we practically prove that it is not possible for this enzyme to be localized intracellularly, because this method registers the enzymatic action only in the premembrane layers of the intestinal mucosa or in the membrane structures of the enterocytes. In order to demonstrate the membrane localization of some disaccharidases an dipeptidases we made our own investigation in this direction.

Materials and methods

The experimental investigation was carried out on white rats "Wistar" with average weight 180–200 g. For marking of the enzymatic activity was used the method of accumulating preparation of mucosa [17]. In order to prepare the preparations of intestinal mucosa the experimental animal is decapitated after which its small intestine is quickly extracted and put on glass in ice bath. Beginning from the proximal end the intestine is cut into segments with a length of about 3 cm. Then every segment is turned with the mucosa outside and both its ends are tied with thread. The incubation of the preparations takes-place in 5 mmol solutions of maltose and glycyl-glycin at a t° of 37 °C for a period of 60 min using continuous aeration of the medium with oxigen. The maltase and glycyl-glycin-dipeptidase activity is registered by the increase of the accumulated glucose and glycin in the intestinal mucosa as a result of the enzymatic hydrolysis of maltose and glycyl-glycin. The quantitative analysis on the accumulated glucose and glycin is determined according to the modificated methods of Ugolev et al. [16]. The data from the experiment are statistically processed using the method of variational analysis and are to be found in Table I.

Results

The method of accumulating preparation of mucosa requires after the separation of the small intestine from the abdominal cavity to be washed twice with cooled Ringer solution for washing away the adsorbed enzymes. In our experiment the small intestines of the controlled group were washed 5 times, and those of the other 10-times with cooled Ringer solution. The investigation of the activities of the disaccharidases and dipeptidases established that the maltase activity is reduced with the number of washings while the glycyl-glycin-dipeptidase activity does not change even after the 10-times washing of the small intestine (Fig. 1).

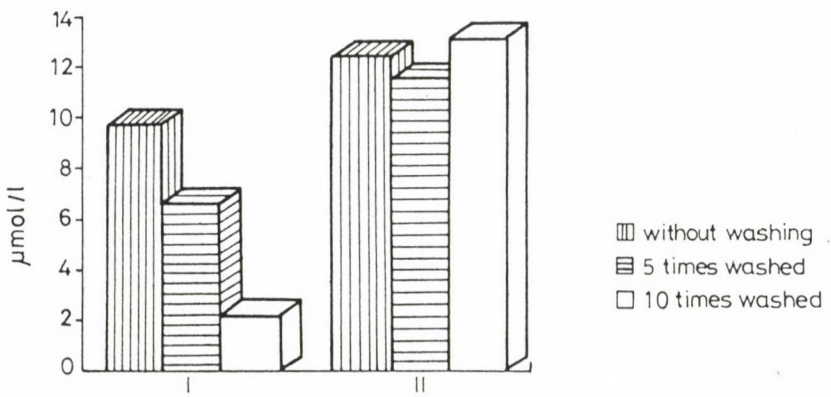

Fig. 1. Disaccharidase and dipeptidase activities in the small intestine without and after its washing

Intramembrane digestion

Maltase activity

	n	x ± SD	t	p
1. Control (without washing)	6	9.7 ± 0.81	–	–
2. Experiment (5-times washed)	6	6.6 ± 0.54	3.16	< 0.01
3. Experiment (10-times washed)	6	2.2 ± 0.39	8.3	< 0.001

Glycyl-glycin-dipeptidase activity

	n	x ± SD	t	p
1. Control (without washing)	6	12.4 ± 0.54	–	–
2. Experiment (5 times washed)	6	11.5 ± 0.71	1.01	> 0.05
3. Experiment (10 times washed)	6	13.1 ± 0.67	0.81	> 0.05

Discussion

The data from the experiment show that the repeated washing with Ringer solution separates the lightly fixed maltase from the surface of the intestinal mucosa which undoubtedly speaks for its premembrane localization. The lack of changes in the dipeptidase activity shows that the enzyme is strongly fixed to the enterocyte membrane or it is not there at all.

The difficulties we met in the interpretation of these data were reduced by using the investigations in this field of Ugolev et al. [18]. In a methodology developed by them the entire separation of the apical glycocalyx from the plasmatic membrane is achieved. In their investigation they noted that in it there were not more than 10% dipeptidases, glycyl-glycin-dipeptidase being absent among them.

This means that as this enzyme is not to be found on the surface of the enterocyte membranes and having in mind that we have excluded its practical importance for the intracellular digestion, the only place for its optimal action should be somewhere in the middle, i.e. in the lipoprotein membranes themselves.

Our conception for the intramembrane localization of some dipeptidases respectively of the existence of intramembrane digestion has been confirmed so far by some indirect data. In this aspect we could apply the anoxic criterion which includes the following arguments: if the degradation of some dipeptid precedes its absorption then the absence of active transport in the conditions of hypoxia will not affect the intensity of its hydrolysis. If, however, there exists active transport of intact dipeptide, the inhibition of this process at hypoxia will be accompanied by a decreasing in the rate of its hydrolysis. The application of hypoxic models in our experiments led to the conclusion that the hypoxia inhibits the activity of glycyl-

glycin-dipeptidase [13]. This criterion confirms our opinion that the enzyme or its catalytic centre is localized in depth from the apical surfaces of the enterocyte membranes where is realized the hydrolysis of the intact dipeptidases penetrated through the membrane pores.

A serious argument in favour of the intramembrane localization of some peptidases are the investigations of Peters [10] who using differentiated centrifugation of homogenate of intestinal mucosa from experimental animals found out that the activity of most dipeptidases is concentrated to the side of greater density in the intestinal homogenate. Namely in the membranes themselves the density is greatest.

With the conception of intramembrane digestion it is assumed that the enterocyte membranes are not only the place of transport of nutrients but also of enzymatic hydrolysis. This possibility is confirmed by the modern molecular structure of the membrane enzymes for which it is accepted that they are transmembrane proteins, part of them partially included, and others fully included in the lipoprotein membranes [2]. It is known also that the enterocyte membranes have an excess of negative charges, which have an adverse effect on the catalytic parts of the enzymes situated on the surface [15]. On the other hand, if the active centres of the enzymes are in contact with the intracellular space, their function will be inhibited by the low values of pH there. So, according to us, the localization of the catalytic part of the enzymes in the membranes themselves is most favourable for their optimal function because it is exactly there that they are best defended against adverse external and internal influences.

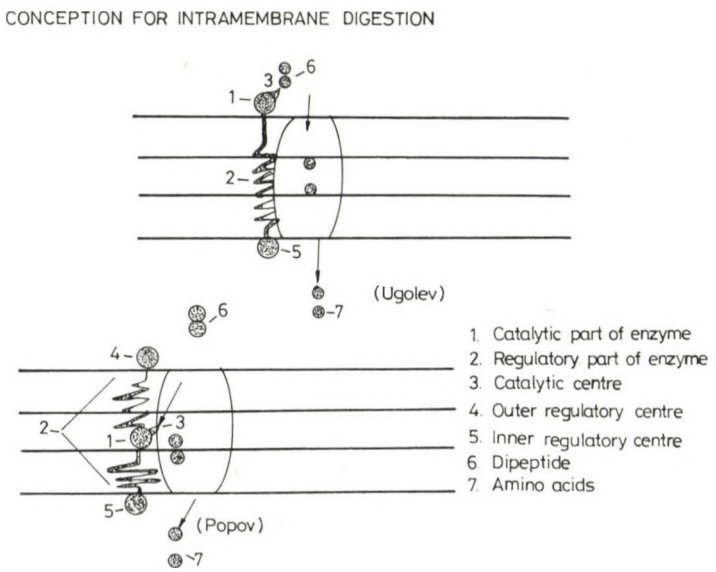

Fig. 2. Two models for premembrane and intramembrane localization of the catalytic part of the enzyme

In one of his latest works Ugolev [19] assumes that some of the enzymes have an intramembrane localization but he considers that their active centre is on the surface of the membranes. According to our conception, the active centre of the enzymes is localized in the membranes themselves and the realized digestion is principally not premembrane but intramembrane (Fig. 2).

With the conception for intramembrane hydrolysis the chain of successive digestive processes is not interrupted and the integration of the hydrolytic and transporting processes is preserved at the different levels. Using a precise experimental technique in the future which would allow the isolation of "pure" enterocyte membranes, the enzymatic spectrum in them realizing this principally new type of digestion will be exactly determined.

REFERENCES

1. Bairton, D.: The discovery of lysosomes. J. Cell. Biol. **91**, 66–76 (1981).
2. Feracci, I., Maroux, S.: Rabbit intestinal aminopeptidase purification and molecular properties. Biochem. Biophys. Acta **599**, 448–463 (1980).
3. Fogel, M., Adibi, S.: Varied alteration in transport of free amino acids and dipeptidases by the acidic intraluminal pH in human intestine. Gastroenterology **62**, 747 (1972).
4. Kim, T., Brophy, E.: Rat intestinal brush border membrane peptidases. J. Biol. Chem. **251**, 3199–3205 (1976).
5. Kushak, P.: Digestive-transport system of the enterocytes. Riga p. 304 (1988).
6. Nicholson, J., Kim, J.: The response of rat intestinal brush border and cytosol peptide hydrolase activities to variation in dietary protein content. J. Clin. Invest. **54**, 890–898 (1974).
7. Nishi, J., Takesve, J.: Electron microscope studies on the subunits of small intestinal sucrase-isomaltase complex. J. Electron Microscopy **3**, 197–198 (1976).
8. Overton, J., Crane, R.: Studies on the organization of the brush border in intestinal epithelial cells. J. Cell. Biol. **26**, 693–706 (1965).
9. Peters, T., Ponlon, J.: The subcellular localization and specificity of intestinal peptide hydrolases. In: Transport across the intestine, Edinburgh, London pp. 153–167 (1972).
10. Peters, T.: Biochemical structure of the enterocyte. J. Gastroenterology Moscow Medicine pp. 50–69 (1988).
11. Pokrovsky, A.: Lysosomes. Moscow, Nauka p. 383 (1976).
12. Popov, B., Ilutchev, D. et al.: Investigation of some enzyme an transport activities of the small intestines under the condition of experimental alkalosis and acidosis. XIII Int. Congress of Nutrition, Brighton UK (1985).
13. Popov, B., Ilutchev, D., Stoyanov, Z.: Effect of experimental hypoxia on peptide and sugar degradating and transporting systems in the intestines. Fifth European Nutrition Conference, Warsaw (1987).
14. Popov, B.: Enteral membrane digestion – theoretico-methodological, alimentary and hygienic aspects. D. Sc. Dissertation, Sofia Bulgaria (1991).
15. Sementsa, G.: Glucosides in the brush border of the small intestine. Moscow, Medicine p. 408 (1980).

16. Ugolev, A., Iesuitova, I. et al.: Investigation of the digestive system in man. Sanct Peterburg, Nauka, p. 215 (1969).
17. Ugolev, A., Nurks, E.: Accumulating preparation of mucosa – a new method for investigations the initial stages of nutrients transfer through intestinal wall. Russian Journal of Physiology **56**, 1638–1641 (1970).
18. Ugolev, A.: Characteristic of the digestive enzymes of enterocytes. Russian Journal of Physiology **68**, 1731–1743 (1982).
19. Ugolev, A.: Membrane hydrolysis and transport – new data and hypotheses. Sanct Peterburg, Nauka, p. 240 (1986).

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Evaluation of selected parameters of a metabolic profile and levels of cadmium in reproductive organs of rabbits after an experimental administration

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Selected serum parameters of metabolic profile and distribution of cadmium in reproductive organs in rabbits were studied after an acute and subchronic administration.

After an i.p. application of cadmium (1.5 $mg.kg^{-1}$) a significant increase of glucose (5.90 $mmol.l^{-1}$) and cholesterol (2.22 $mmol.l^{-1}$) was observed. There were no significant differences in these parameters after the subchronic (5 month) p.o. application. The concentration of AST and ALT significantly decreased after a subchronic p.o. administration (1.0 $mg.kg^{-1}$) but not after an acute i.p. application. There were no significant differences in the level of total proteins and total lipids.

The highest accumulation in reproductive organs (testis, ovary, uterus) was observed after i.p. administration. The level in the testis was 40 times higher than in the control group (0.04 $mg.kg^{-1}$; 1.93 $mg.kg^{-1}$), 174 times higher in the ovary (0.03; 5.21) and 65 times higher in the uterus (0.04; 2.59). After a subchronic p.o. application, the concentration was only 2.5 times higher in the testis (0.04; 0.10), 16 times higher in the ovary and 6 times higher in the uterus than in the control group.

Keywords: rabbits, cadmium, metabolic profile, reproductive organs

Cadmium is a relatively volatile element and is, from present knowledge, not essential for plants, animals and human beings. Since eight times more cadmium has been consumed in the last years than in the entire history of mankind before, problems associated with cadmium have accelerated only since about 1950. Global emission of cadmium compounds into the atmosphere is estimated to be around 7000 metric tons per year with more than 90% coming from anthropogenic sources [16].

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Cadmium as metal has mainly been used as an anticorrosive, electroplated onto steel. The absorption of cadmium compounds through skin is negligible, the main routes of exposure for human, being are via respiration and ingestion. Due to the long half-life of cadmium in the critical organs and the irreversibility of the critical effect, primary prevention is essential [3, 4].

Long-term cadmium exposure may lead to disturbance of calcium metabolism, osteoporosis and osteomalacia. This has been seen both after occupational and general environmental exposure. The syndrome of cadmium induced proteinuria, glucosuria, and osteomalacia and/or osteoporosis was epidemic in the 1950s in a cadmium polluted area of Japan and it was called Itai-itai disease [3]. The most typical feature of chronic cadmium intoxication is kidney damage. Cadmium affects reabsorption function of the proximal tubuli. Glomerular damage causes enhanced excretion of high molecular weight proteins in urine. Tubular damage can be recognized by an increased excretion of low molecular weight proteins in urine due to the decreased reabsorption of these proteins that can pass the glomerular filter and occur in the primary urine [16]. The liver is the major storage organ for cadmium and may be adversely affected by this metal. Single i.p. injection of cadmium chloride at doses 2.5 or 3.75 $mg.kg^{-1}$ to male Wistar rats produced significant inhibition of aniline hydroxylase and nitroreductase activity and also lowered the microsomal cytochrome P-450 content to 50% of the control value [19].

An accumulation of cadmium occurs in many tissue, and particularly long half-lifes, 10–30 years, have been reported for muscles [3]. Many authors have described that cadmium accumulates in the liver and kidneys [3, 4, 14]. Cadmium injected subcutaneously accumulates at high levels and is retained in the liver and kidneys [25]. From 50 to 60% of ingested cadmium is stored mainly in the liver and kidneys [9]. Very strong relationship of the concentration of cadmium in liver and the and the administered dose in rats were reported [5]. The highest levels were reported in kidneys (10.0–35.0 $\mu g.g^{-1}$ of tissue) and in the liver (0.5–5.0 $\mu g.g^{-1}$). By comparing the amount of cadmium in the kidney of several species of farm animals the highest levels were accumulated in the kidneys of horses [14]. In acute poisoning, higher concentration in liver than in kidneys was recorded and after chronic poisoning the opposite occurred.

The aim of this study was to study selected parameters of a metabolic profile and the distribution of cadmium in the reproductive organs of rabbits after an acute and subchronic application.

Material and method

All experiments were conducted in rabbits (Hyla, VÚŽV Nitra). All animals were devided is three groups (K, A, B). Eight female and eight male rabbits received cadmium intraperitoneally (i.p.) (1.5 $mg.kg^{-1}$ body weight). These animals (group A) where killed 48 hours after an application of cadmium ($CdCl_2$, Sigma Chemical Company, St. Louis). A chronic experiment (group B) was conducted with the same number of animals. Cadmium was administered the dose of 1.0 $mg.kg^{-1}$ b.w. for five

month in their pelletized food. The food and the water was available for all animals ad libitum. All animals were killed after an application period. Cadmium was diluted with the physiological solution to the claimed concentration. The last group (K) served as control, without administration of cadmium.

During killing, the blood from the vena jugularis was used for evaluation of selected parameters of metabolic profile (glucose, cholesterol, AST, ALT, total proteins, total lipids). The serum was obtained by centrifugation at 3000 rpm for 30 minutes. All parameters were rated by spectrophotometry (Unicam SP 1800). Levels of cadmium in selected reproductive organs of rabbits were determined by atomic absorption spectrophotometry (AAS, Perkin Elmer mod. 760).

From the final data, basic statistical characteristics were calculated (Std Dev, Mean) and an analysis of variance by Scheffe's test for each variable was done (SAS).

Results

Changes in selected parameters of the metabolic profile

In a comparison of average values of the glucose concentration, we found a significant increase of the concentration in group A (i.p.) in comparison with group K and group B. Levels in group K and group B were very similar.

The same image was seen in the evaluation of the cholesterol concentration. The highest concentration (2.22 m.mol.l^{-1}) was in group A which was significantly ($P < 0.05$) higher than in group K and group B (1.78 and 1.80 m.mol.l^{-1}).

Determination of AST showed that the level was highest in group K (0.70 μkat.l^{-1}), followed by group A (0.64 μkat.l^{-1}) with the lowest level found in

Table I

Average values of selected parameters of metabolic profile

Metabolite	Group		
	control	acute	subchronic
Glucose (mmol.l^{-1})	4.90	5.90*	4.88
Cholesterol (mmol.l^{-1})	1.78	2.22*	1.80
AST (μkat.l^{-1})	0.70	0.64	0.33*
ALT (μkat.l^{-1})	0.51	0.51	0.40*
Total proteins (g.l^{-1})	64.20	63.23	65.57
Total lipids (g.l^{-1})	1.72	2.41	2.95

* $P < 0.05$

group B (0.33 μkat.l^{-1}). A significant decrease was in the group B in comparison with the group K and also with the group A.

The average values of ALT were the same in group K and group A (0.51 μkat.l^{-1}). In group B, the level was the lowest (0.40 μkat.l^{-1}) and furthermore this value was significantly lower than that in group K and group A.

The highest concentration of total proteins was found in group B (65.57 g.l^{-1}). Lower levels were found in group K (64.20 g.l^{-1}) and group A (63.23 g.l^{-1}). This decrease was not significant.

The level of total lipids proved to be similar in all groups (from 1.72 to 2.95 g.l^{-1}) and there were no significant differences (Table I).

Distribution of cadmium in reproductive organs

We found that in the control group the concentration of cadmium was the lowest in the ovary (0.03 mg.kg^{-1} wet tissue) and the concentrations in the testis and the uterus were nearly the same (0.04 mg.kg^{-1}). After an i.p. administration of cadmium (1.5 mg.kg^{-1}), the level in the testis was 48 times higher, in the ovary 174 times higher, and in the uterus the concentration of cadmium was 65 times higher than in the control group. Exact data are recorded in Table II. After a chronic p.o. application of cadmium for 5 month daily (1.0 mg.kg^{-1}) the highest concentration was measured in the ovary (0.47 mg.kg^{-1}), followed by the uterus (0.25 mg.kg^{-1}), and the lowest accumulation was found in the testis (Table II).

Table II

Average concentration of cadmium in reproductive organs

Organ	Group		
	control (mg.kg^{-1})	acute (mg.kg^{-1})	subchronic (mg.kg^{-1})
Testis	0.04	1.93	0.10
Ovarium	0.03	5.21	0.47
Uterus	0.04	2.59	0.25

Discussion

Our study has shown a significant increase of glucose and cholesterol after in i.p. administration of cadmium. Generally, it is known that the hyperglycaemia is caused by a stress situation [23], which might be present in an i.p. application. Stress causes sympaticotonia. This tonia of sympatic nerves urge the function of the cortex of adrenals, which is manifested by immediate secretion of adrenaline. On the other

hand, higher levels of cholesterol are a sign of liver disturbance (damage of parenchyma, cirrhosis).

Significantly lower concentrations of AST and ALT after chronic application are evident, but the decrease of enzymatic activity has no diagnostic importance [23].

Our results concerning the distribution of cadmium in reproductive organs confirm the data of early reports [3, 4] and have described the increasing tendency of cadmium accumulation. The age dependent accumulation in the human ovary has already been reported [22]. A linear increase between 30 and 65 years of age was found. A rapid incorporation of cadmium into the corpora lutea of the ovary was described [11]. The average level of cadmium in the human ovary was detected to be $0.31-0.75\ \mu g.g^{-1}$ wet tissue [10, 17, 20]. In the study of distribution of cadmium in ovaries, adrenals and pituitary gland after chronic administration of cadmium the lowest level was found in ovaries and cadmium accumulated time- and dose-dependently [21]. Our results show that the accumulation of cadmium in ovaries is dependent on time and dose, when the level increases after a high single i.p. dose. After subchronic p.o. application the concentration is much lower (Table III).

Table III

Concentration of cadmium in reproductive organs [10]

Tissue	Mean range		'Best' mean	
	Caucasian	Japanese	Caucasian	Japanese
Testis	0.11 – 1.2	0.5 – 1.9	0.25	1.0
Ovarium	0.10 – 0.65	0.75 – 1.3	0.25	0.8
Uterus	0.09 – 0.52	–	0.15	–

Many authors described very relevant embryotoxic effects of cadmium. Toxaemia of pregnancy is phenomenally induced by cadmium salts [13]. Malformations of fetuses found during the poisoning of the maternal organism by cadmium are caused by a deficiency of zinc in developing fetuses [24]. Fetuses with limb defects (oligodactyly, polydactyly), exencephaly, open eyes, but their incidence differed in relation to the day of administration of cadmium [15]. After relatively high levels of cadmium (100 ppm $30\ mg.kg^{-1}$) all of examined fetuses had malformations 2.39 and 5.62%, respectively [8]. At a dose of cadmium 12 and 24 $\mu mol.kg^{-1}$ 29.7 and 70.6% of malformed fetuses were reported, from which 24.1 and 68.4% respectively, had limb defects [6]. Fetal death is not the result of direct effects of cadmium on the fetus. Histologic examination of placentae indicated that the placental necrosis, fetal death and utero-placental blood flow alterations occurred over a similar time period. It is not apparent whether placental necrosis was a cause of an effect of the decrease in utero-placental blood flow [7]. A possible ability of cadmium to pass from the yolk-sac cavity into the primitive gut before the closure of the vitelline duct but not

later was described [2]. Cadmium appeared to inhibit the cleavage of embryo from the 8-cell to 16-cell stage, but not from the 4-cell to 8-cell stage [27]. The declining ratio of cadmium in progrediating pregnancy is probably the result of the increasing synthesis of metallothionein which binds cadmium [18].

The uterus of a rat is more sensitive to the effects of estrogens between birth and puberty [1]. On Day 4 of pregnancy, a significant drop in secretion rate and in the level of progesterone was detected [12]. Cadmium given on the Day 8 of pregnancy in the hamster heavily accumulated in the primitive gut of embryos, the decidua, the yolk sac, the ectoplacental cone and later in the chorioallantoic placenta; possibly disturbing the maternal-embryonic relationship and fetal nutrition [2].

In our experiment the level of cadmium in testes ranged from 0.04 to 1.39 $mg.kg^{-1}$. Biochemical changes that occur in the rat or mouse testis after a single s.c. injection of a sub-toxic dose of $CdCl_2$ include decrease in the content of DNA, RNA and in the activity of certain Zn^{2+}-containing enzymes. The protein content is increased, although the rate of protein synthesis is reduced. Uptake of Cd^{2+} by the testis is small and does not cause the displacement of Zn^{2+} [26]. The degeneration of the seminiferous epithelium and all the biochemical and physiological changes known to occur in the testis following cadmium treatment can be considered rather a secondary than a direct effect. The are supported to be induced by ischemia.

Our results show that the accumulation of cadmium in the testis, ovary and uterus is depending on the dose and the time. The concentration after an i.p. injection is much higher than after a p.o. application.

REFERENCES

1. Cibulka, J.: Pohyb olova, kadmia a rtuti v biosféře. Akademia Praha, 1991.
2. Dencker, L.: Possible mechanisms of cadmium fetotoxicity in golden hamsters and mice: Uptake by the embryo, placenta and ovary. J. Reprod. Fert. **44**, 461–471 (1975).
3. Friberg, L., Kjellström, T., Nordberg, G. F.: Cadmium. In: Friberg, L., Nordberg, G. F., Vouk, V.: Handbook on the Toxicology of Metals. Elsevier Sci. Publishers B. V., 1986, 131–184.
4. Ginter, E., Nagyová, A.: Kadmium: metabolizmus a mechanizmus toxického pôsobenia. Čs. fyziológie, **40**, 575–582 (1991).
5. Klaassen, C. D., Kotsonis, F. N.: Biliary Excretion of Cadmium in the Rat, Rabbit and Dog. Tox. Appl. Phar. **41**, 101–112 (1977).
6. Layton, W. M., Layton, M. W.: Cadmium Induced Limb Defects in Mice: Strain Associated Differences in Sensitivity. Teratology **19**, 229–236 (1979).
7. Levin, A. A., Miller, R K.: Fetal Toxicity of Cadmium in the Rat: Decreased Utero-Placental Blood Flow. Tox. Appl. Phar. **58**, 297–306 (1981).
8. Machemer, L., Lorke, D.: Embryotoxic Effects of Cadmium on Rats upon Oral Administration. Toxicol. Appl. Pharmacol. **58**, 438–443 (1981).
9. Neumann, J., Lopuchovský, J., Zapletal, O.: Chemizace zemědelství, farmakologie a toxikologie. SZN Praha. 1989.
10. Nriagu, J. P.: Cadmium in the environment. Part II: Health Effects. John Wiley & Sons 353–510 (1981).

11. Paksy, K., Náray, M., Varga, B., Kiss, I., Folly, I., Ungváry, G.: Uptake and Distribution of Cd in the Ovaries, Adrenals, and Pituitary in Pseudopregnant Rats: Effect of Cd on Progesterone Serum Levels. Environ. Res. **51**, 83–90 (1990).
12. Paksy, K., Náray, M., Varga, B., Olajos, F., Folly, G.: Altered ovarian progesterone secretion induced by cadmium fails to interfere with embryo transport in the oviduct of the rat. Reprod. Toxicol. **6**, 77–83 (1992).
13. Pařízek, J.: The peculiar toxicity of cadmium during pregnancy – an experimental "toxaemia of pregnancy" induced by cadmium salts. J. Reprod. Fertil. **9**, 111–112 (1965).
14. Piskač, A., Kačmár, P.: Veterinární toxikologie. SZN, Praha 87–92 (1985).
15. Soukupová, D., Dostál, M.: Development Toxicity of Cadmium in Mice. I. Embryotoxic Effects. Func. Devel. Morphol. **1, 2**, 3–9 (1991).
16. Stoeppler, M.: Cadmium. In: Merian, E.: Metals and Their Compounds in the Environment. VCH Weinheim-NY-Basel-Cambridge p. 803–852 (1991).
17. Sumino, K., Hayakawa, K., Shibata, T., Kitamura, S.: Heavy metals in normal Japanese tissues. Amount of 15 heavy metals in 30 subjects. Arch. Environ. Health **30**, 487–494 (1975).
18. Šimoník, I., Pavelka, J.: Retence kadmia v placente skotu. Veterinářství **39**, 4, 167–168 (1989).
19. Teare, F. W., Jasansky, P., Renaud, L., Read, P. R.: Acute effect of cadmium on hepatic drug-metabolizing enzymes in the rat. Toxicol. Appl. Pharmacol. **41**, 57–65 ( ).
20. Tipton, I. H., Cook, M. J.: Trace elements in human tissue. Part II. Adult subjects from the United States. Health Phys. **9**, 103–145 (1963).
21. Varga, B., Paksy, K., Náray, M.: Distribution of cadmium in ovaries, adrenals and pituitary gland after chronic administration in rats. Acta Physiol. Hungarica **78**, 221–226 (1991).
22. Varga, B., Zsolnai, B., Paksy, K., Náray, M., Ungváry, G.: Age dependent accumulation of cadmium in the human ovary. Reprod. Toxicol. **7**, 225–228 (1993).
23. Vrzgula, L.: Poruchy látkového metabolizmu hospodárskych zvierat a ich prevencia. Príroda, 458–485 (1990).
24. Vrzgula, L., Kováč, G.: Vnútorné choroby prežúvavcov. Príroda Bratislava, 232–233 (1991).
25. Webb, M.: Persistence of stored Cd^{2+} in the livers and kidneys of female rats during pregnancy. J. Reprod. Fertil. **30**, 99–103 (1972).
26. Webb, M.: Biochemical effects of Cd^{2+}-injury in the rat and mouse testis. J. Reprod. Fert. **30**, 83–98 (1972).
27. Yu, H. S., Chan, S. T. H.: Cadmium toxicity on mouse pre-implantation zygotes in vitro: Interactions of cadmium with manganese, zinc and calcium ions. Toxicology **48**, 261–272 (1988).

Acta Physiologica Hungarica, Volume 83 (3), pp. 275–278 (1995)

BOOK REVIEWS

Regulation of breathing

2nd Edition, revised and expanded Lung Biology in Health and Disease. Series 79.
Edited by Jerome A. Dempsey and Allen I. Pack.
Marcel Dekker Inc., New York, Basel, Hong Kong
ISBN: 0-8247-9227-0 1244 pp. 1995. $ 195.00

The reviewer has just finished his chapter on the regulation of respiration in a textbook of medical physiology, and deeply regrets that he did nod see this volume somewhat earlier. It is an excellent source of information in a difficult field, where the gathering of data started nearly 200 years ago but the final picture has not emerged yet.

Part I has the title: Overview, and the single chapter it contains is entitled:... "An uninhibited survey from the perspective of comparative physiology". It is a fascinating text and it really gives impetus for reading the chapters to follow. The questions how and why are raised and mostly also answered.

Part II deals with central nervous control. It gives an excellent coverage of both anatomical organization and description of events at the cellular level, including electrophysiology, transmitters and receptors. It is an extra advantage that it goes beyond the usual brain stern approach and higher parts of the CNS are widely covered: there are separate chapters on the hypothalamus and the cerebral cortex. The schemes illustrating pathways and relays are self-evident and informative.

Part III deals with afferent systems. Cellular and subcellular analysis of chemoreceptor function, both peripheral and central is given. Non-chemical afferentation is covered by looking at the responses they elicit. In this part are the responses to hypoxia detailed. Fortunately, human aspects are also covered and I enjoyed also a drawing on the famous experiment of the Rev. Stephen Hales of 1773 (Chapter 15., "Respiratory modulation of autonomic outflow").

Part IV is on developmental and hormonal influences. It focuses very much on human data and much neonatal pathology is included. It is in this Part that a detailed analysis of sex steroid action is given. Part V has the title of "Loads, sensations and failure". The part is heterogeneous but interesting. Part VI, "Metabolic state effects" includes in effect much more than the title promises, including respiratory changes during REM sleep, but metabolic correlates are also well treated. Exercise is covered in several chapters, but this is more of advantage than a drawback.

There are some more merits of the book. The list of references is extensive, starting with classical data and ending with the most recent ones. The indexes (authors an subjects) are adequate.

The price of the book is certainly not low, but it is an indispensable volume for those who do research in the field as well as for those who reach physiology at university level. It is a work on broad biological basis, but also of a medical approach.

A. FONYÓ

David Male, Brian Champion, Anne Cooke and Michael Owen

Advanced Immunology

2nd edition, Gower Medical Publishing, London, New York 1991.
ISBN 0-397-44586-5. 250 pages, 228 figures (incl. 6 colour figs). Price: 24.95 GBP.

The new, second edition of Advanced Immunology is a useful textbook. The book, containing also glossary and index, is a concise, well-illustrated publication. It consists of five parts and 18 chapters. The first part contains the introductory chapter. The second part (Chapters 2–5) discusses the structure of various antigen binding molecules. In the third part (Chapters 6–10) the initiation of the immune response is analysed while in the fourth part (Chapters 11–13) the authors deal with the interactions of immunologically active cells. The final part (Chapters 14–18) writes about the immunological effector systems. The authors successfully realized their declared purpose to provide the latest information for people possessing the basic knowledge in immunology. Since the first edition in 1987 the text has not only been revised but completely rewritten following the rapid development in certain fields of immunology and missing other areas faded from prominence. Most of the chapters are improved by selecting particular experiments from journals to acknowledge the work of the most important authors showing their way which led to their results. A short Summary found at the end of each chapter is very useful for the reader, and who is interested in greater depth in a given area, will find a long list of references, up to 1990. The reviewer, being a biochemist, found mot interesting the chapters discussing the MHC molecules, lymphocyte activation and cytokines and immunoregulation, but other parts like advances in immune recognition and in molecular mechanisms of leukocyte migration are also noteworthy for the experts in these fields.

In Summary: this excellent, concise book is a useful help for English-speaking students, their teachers and theoretical or clinical immunologists to deepen their knowledge in basic immunology acquired during their education. It is advisable to have it on the shelves of our libraries.

A. HRABÁK

David Male

Immunology (an illustrated outline)

2nd Edition, Gower Medical Publishing, London New York. 1991
ISBN 0-397-44825-2, 128 pages, 97 figures Price: 9.95 GBR

This excellent, short, well-illustrated book was written by David Male, one of the authors of "Advanced Immunology". The author pointed out the double aim of his work; on the one hand, it is a glossary of immunology, on the other hand it is a concise revision guide or study aid. The book consists of six chapters each of which discusses related topics; The Immune System; Immune Recognition; Immune Responses; Inflammation and Cell Migration; Immunopathology, Immunological Tests and

Techniques. The reader can easily find anything by using the term index at the beginning of the book. This small book is recommended to all English-speaking Hungarian students in medicine or biology, it is an excellent help during preparation for an examination on basic principles of immunology. Although the price seems to be high for a Hungarian student, those can buy it, should not by all means miss it. This pocket book is also an important item in the libraries of our University departments together with its similarly edited counterparts (Clinical Chemistry, Biochemistry, Medical Genetics).

A. HRABÁK

Endotoxin and the lungs

Lung Biology in Health and Disease. Series 77. Edited by Brigham KL. Marcel Dekker, Inc., New York, Basel, Hong Kong, 1994. 549 pp., 175 US$

"Endotoxin and the lungs" is the 77th volume of the series on "Lung biology in health and disease", edited by Claude Lenfant (NIH, USA). The 48 contributors, all of them acknowledged experts of experimental and clinical research, mostly from the USA, others from Canada, Japan and Germany, discuss the subject in 18 chapters. They accomplish this task in a comprehensive way, starting from the structure of endotoxins as far as designing therapy.

Lipopolysaccharides (LPS) are dominating constituents of cell membrane of Gram negative bacteria. Interaction between free lipid A, the endotoxically active centre of LPS and a defined cellular recognition molecule (LPS binding protein = LBP) represent the main event in endotoxin action and are responsible for deleterious effects in Gram negative sepsis and ARDS. LPS–LBP complex activates macrographes via CD14 antigen: This provides possibility of early host–defense response as well as it can initiate inflammatory action, vascular complications, hypotension and DIC. LPS induces rapid expression of different gene products in susceptible cells (i.e. vascular endothel in the lung) through signal transducing pathways which is not yet completely understood and needs further research. Human pulmonary circulation responds to endotoxin by vasoconstriction and ventilation/perfusion mismatch. Obstruction and occlusion of pulmonary vessels by inflammatory cells and thrombosis result increased pulmonary resistance. Increased lung vascular permeability deteriorates gas exchange. Long-term effect of endotoxins on airway function may involve structural changes (inflammation, edema, wall-thickening). Metabolites of arachidonic acid cascade (cyclo-oxigenase, lipoxigenase) are involved in oxygen-free radical formation but their relationship in ARDS needs further clinical research. Haemostasis disorders (microvascular thrombosis, DIC) and other alterations associated with Gram negative sepsis in ARDS can combine with hyaline membrane formation in alveoli: Mechanism of this phenomenon was examined in primate model. A delicate balance between oxidants and antioxidants is essential for the normal function of the lung since oxygen radicals can damage vital cellular DNA and protein molecules. There are experimental and clinical evidences that in acute lung injury (ARDS) oxidant activity is increased, antioxidant enzymes are perturbed. On the other hand, recent evidence suggests that endotoxin can

reduce damages to oxidants through stimulating tolerance mechanism. Similarly, phagocytes which are critical in the host – defence mechanism of lung may also contribute to lung injury.

For the clinician novel therapeutic approaches are the most exciting parts of the book. Conventional therapy of Gram negative sepsis and its complications (ARDS...) too, often fail to improve/restore patients' condition. Immunological therapy is at its onset: cross-reactive endotoxin antibodies and antagonists of host-generated cytokines produced contradictory results. Administration of exogenous surfactant proved beneficial in infant RDS, in adults' experiences are limited but promising, mainly in aerosol form. Highly invasive therapies of acute lung injury (extracorporeal membrane oxigenization or carbon dioxide removal, intravascular oxigenization, etc.) have serious side-effects but such heroic techniques are under consideration as implantable artificial lung or lung transplantation, too.

The final chapter refers to the hopeful future dealing with the prospects of gene therapy. Genes cloned to protect lungs from endotoxin injury may provide an entirely new category of therapy.

In summary, it can be concluded that we are halfway in progress on understanding pulmonary effects of endotoxin. Considerable knowledge has been accumulated in the past decade on pathomechanism and its biochemical background. The next step would be the practical application of this knowledge. Abundant references complete all chapters. Indexes of authors and subjects facilitate the use of the book. Treatment of subjects is concise and clear, even the clinician reader, who was afraid of the relatively abstract subject, enjoyed the book. Taking into consideration the great number of contributors, the uniform structure of the chapters is remarkable and praises the editor. There are no more overlappings than unavoidable considering again the multi-author character. "Endotoxin and the lungs" provides a good example how basic research can contribute to clinical investigation and its integration into clinical practice.

The book is highly recommended for investigators and clinicians, mainly for intensive therapists.

I. VADÁSZ

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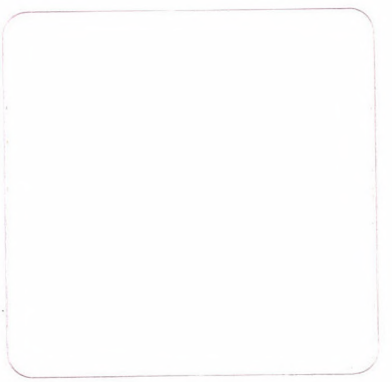

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Transformation and recovery of rat skeletal muscles in pathologic hormonal states[1]

T. **Bányász**, Ágota **Vér***, T. **Kovács**

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* 1st Department of Chemistry-Biochemistry, Semmelweis University of Medicine, Budapest, Hungary

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There are numerous controversial data published on muscle transformation in pathologic endocrine states. Information is consistent in respect of slow muscle sensitivity to diabetes, but data concerning characteristics of fast muscles are contradictory under the same conditions.

Sensitivity of fast and slow muscles to diabetes was examined and compared in this study. Diabetes was induced by a single intravenous dose of Streptozotocin in Wistar rats. Contractile parameters of soleus and extensor digitorum longus muscle were determined by in vitro measurements. We collected data on reversibility of diabetic transformation using exogenous thyroxine and insulin treatment. Insulin was used both to prevent and revert diabetic transformations.

We found that, according to the data previously published, soleus muscle is more sensitive to diabetes than the extensor digitorum longus (EDL). Changes in the EDL muscle proved not to be statistically significant, but the comparison of hyperthyroid and diabetic muscles to controls led us to the conclusion that there is a sublimital transformation in the EDL in diabetes.

T4 and insulin were effective in preventing diabetic muscle transformation. Application of insulin not only prevented diabetic complications, but its use in animals suffering from diabetes for a longer time (8 weeks) resulted in a successful recovery of the muscle transformation.

Keywords: diabetes, skeletal muscle, contraction, rat

Diabetes mellitus is frequently associated with transformation of skeletal muscles. Prolongation of the contraction curve was found to be characteristic to insulin

This work was supported by research grants from the Hungarian Scientific Research Fund (OTKA 1466 and T 12962) and from Hungarian Ministry of Public Welfare (T 02 746/93).

[1] This paper dedicated to the memory of Professor Tibor Kovács (1929–1994)

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dependent diabetes [2, 4, 6, 12]. It is widely accepted that slow muscles, like soleus are more sensitive than fast ones, like extensor digitorum longus (EDL). These differences are supported by morphological and biochemical abnormalities explored by comparative analysis of slow and fast muscles in diabetic animal models [4, 14]. Presence and absence of altered contractility was reported in soleus and the EDL muscle, respectively, under diabetic conditions [2, 4, 12]. In some cases altered biochemical functions are reported in both EDL and soleus muscles [5, 7, 15]. It is hard to understand why mechanical parameters are found to be unaltered if EDL is subject to biochemical changes during diabetes?

In medical practice insulin therapy is widely used in diabetes to successfully maintain normal plasma glucose level. Normalized plasma glucose provides safe and valuable life style to diabetic patients. In spite of the efforts invested into diabetes care, complications (hypertension, myopathy, glomerulopathy) of diabetes will ascend sooner or later. Usefulness of insulin therapy in prevention or reversion of diabetic complications is not explored in detail. There are numerous publications reporting successful insulin therapy in different complications of diabetes [3, 9, 13], but in respect of muscle transformation, reversibility remains to be determined. Furthermore, some authors claim specific insulin effects on skeletal muscle [1, 8, 10, 11] which makes the problem even more complicated.

Hyperthyreosis is known to accelerate contraction process. Shortened twitch time of the hyperthyroid muscle is caused by altered mechanical properties of contractile proteins, and modification in the function of the second messenger chain of myocytes. It would be worthwhile to see if induction of hyperthyreosis can revert or not diabetic changes in skeletal muscles.

Considering gaps listed above we felt obliged to take steps to cease discrepancies and hiatus. Using Streptozotocin (STZ) treated rat as experimental model, we compared the time to peak (ttP) and half relaxation time (HRT) in soleus and EDL muscle. Diabetic animals (D8) were compared to age matched controls (YC).

The goal of this study was to compare the sensitivity of the EDL and soleus to diabetes and hyperthyreosis, and testing the reversibility of changes found.

Methods

Animal model

Adult, randomly selected Wistar rats from both sexes weighing 120–150 g were used in the experiments. Animals were fed on standard laboratory diet (ALTROMIN, produced by LATI) receiving water and food *ad libitum* and maintained under controlled environmental conditions (natural light and dark cycle 24 °C constant temperature).

Induction of diabetes

Rats chosen to be made diabetic were treated with 65 mg/kg Streptozotocin (Zanosar^{R}, Upjohn) i.v. into the tail vein. Control animals were injected with vehicle. Hyperglycaemia was confirmed by blood glucose test using enzymatic technique. The first blood glucose test was performed 48 hours after STZ

treatment, then the test was repeated weakly. Animals treated with STZ exhibited a blood glucose level higher than 10 mM. Rats were sacrificed at the 8th week following STZ treatment in all cases, except group R8, in which experiments were performed at the 16th week (8 weeks diabetes + 8 week insulin therapy).

Induction of hyperthyreosis and thyroxine treatment

To assess the sensitivity of EDL and soleus to T4, and testing the effect of thyroxine on diabetic muscles, two separate groups were used. In the first group (hyperthyroid: HT) exogenous T4 was administrated to healthy animals as long as 8 weeks, followed by the measurement of muscle mechanical parameters. In the other group (thyroxine treated diabetic: DT), from the second day of STZ treatment T4 was applied for 8 weeks to prevent the onset of diabetic transformation. Thyroxine (SIGMA) was freshly dissolved, just before administration and injected intraperitonially once a day. The daily dose was 300 microgram/kg b.w. in either hyperthyroid and thyroxine treated diabetic group.

Insulin treatment

Bovine insulin (ULTRALENTE, HUMAN) was employed to prevent diabetic muscle transformation in some cases. Following the confirmation of diabetes insulin was supplied subcutaneously in a dose of 4 units/day/animal. Blood glucose was tested weekly, and the daily insulin dosage was adjusted to maintain 5 mM blood glucose concentration. Effectiveness of insulin therapy was tested by two ways. In the first insulin treated group, referred to as insulin prevented group (P8), insulin therapy was introduced on the day following STZ treatment. Animals in this group virtually never displayed elevated plasma glucose concentration. The goal with this group was to test the possibility of prevention of diabetic muscle transformation. In the other insulin treated group, insulin therapy was introduced 8 weeks after STZ treatment. During this 8-week period in all of the animals collected in this group an elevated plasma glucose level was found. Insulin therapy was continued in another 8-week long period. The aim of this particular experiment was to study whether diabetic alterations following their complete improvement (R8) are reversible.

Measurement of contractility

Following ether anaesthesia rats were decapitated and bled out. Muscles were removed and transferred to the preparation dish. Short loops from surgical thread were applied onto both ends linking the muscle to a capacitive transducer in the experimental chamber. The chamber was thermoregulated (32 °C) and continuously perfused with oxygenated Tyrode solution (NaCl 150 mM, KCl 5 mM, $MgCl_2$ 1.5 mM, $CaCl_2$ 2.5 mM, HEPES 5 mM, pH: 7.24) refreshing the chamber volume 9–11 times per minute. Parallel platinum electrodes were used for field stimulation (40 mA/cm^2), pacing the muscle continuously with 2 ms long electric square pulse at a constant rate. Muscle length was adjusted for maximal twitch tension. The capacitive transducer used in our experiment provides analogous electric signal proportional to the isometric tension of the muscle in a range of 1–100 mN.

Analogous electric signals were digitalized and evaluated by a computer. Cardinal contractile parameters (developed peak tension [P], time interval between stimulation and peak tension [ttP], rising rate of developed tension [dP/dt], time interval between peak tension and 50% relaxation [HRT], and area under the contraction curve [Int]) were calculated and stored for further analysis.

Experiments were repeated on 7–15 animals and results were united. Collected data were averaged and standard error was calculated to illustrate scattering. ANOVA and Student-Newsman-Keels test were used to compare groups. All statistical analyses were done on IBM compatible PC using SIGMASTAT (Jandel) software.

This investigation conformed to the guidelines for the care and use of laboratory animals published by the US National Institute of Health as well as the principles outlined in the Declaration of Helsinki.

Results

Results for soleus muscles are shown in Figs 1–2. Both ttP and HRT was found to be lengthened by 8 weeks of diabetes, while T4 treatment resulted in significant shortening in the contraction curve. Thus one might conclude that, soleus muscle is a sensitive organ, to either diabetes or hyperthyreosis. T4 treatment of diabetic animals resulted in an intermediate state between diabetes and hyperthyreosis. Both ttP and HRT was found to be similar to the control group. This recovery in contractile parameters suggests that the slowing down of the contraction observed in diabetes was fully prevented by T4 treatment.

Despite the successfully prevented muscle transformation, T4 treatment failed to normalize several other biological parameters of the experimental animals. Blood glucose concentration was still increased in DT group and loss in body weight was also considerable (data are not shown). Cardinal symptoms of diabetes (polyuria, polydipsia, polyphagia) were still present in thyroxine treated animals in a great extent.

Contraction parameters were normalized in both insulin treated groups. Insulin therapy seems to be successful in both prevention and reversion of diabetic muscle transformation. Indeed, average values of ttP and HRT were found to be less than those in YC. This shortening was statistically not significant, but it undeniably existed. Pairwise multiple comparison revealed that there is no statistically significant difference among insulin or T4 treated and control groups, while diabetes or hyperthyreosis established the base of muscle transformation.

Insulin treatment successfully normalized blood glucose concentration, and body weight. Chief symptoms of diabetes were also diminished.

EDL muscles were found to be less sensitive to diabetes or hyperthyreosis than soleus (Figs 3–4). Both ttP and HRT were prolonged in diabetes, and shortened in hyperthyreosis, but these differences proved not to be statistically significant. In a first approach one might deduce that no muscle transformation occurred. This conclusion is mathematically highly supported, but some other observations seem to contrast this statement. There were manifest differences between groups T4 and D8, which was strongly significant statistically. Moreover, while P8 and R8 groups were found to be identical, both of them displayed dissimilarity comparing to D8 groups in respect of ttP. HRT failed to show a similar relation between D8 and insulin treated groups. It is hard to understand, if hyperthyreosis and diabetes caused no alteration in muscle function, where the difference between T4 and D8 groups came from? In addition, what is the origin of the significant alterations of ttP between either D8 and R8 or D8 and P8?

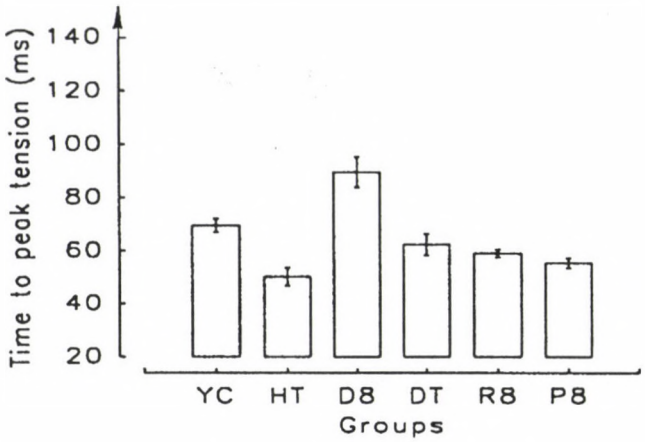

Fig. 1. Alteration in time to peak (ttP) value of rat soleus muscle in different endocrinological states. (YC: control; HT: hyperthyroid; D8: diabetic; DT: T_4 treated diabetic; R8: diabetic with insulin reversion; P8: diabetic with insulin prevention) In diabetes, but not in hypothyreosis ttP was found to be lengthened. This prolongation coinciding with diabetes was found to be fully reversible by administration of either T_4 or insulin

There were apparent differences in sensitivity between soleus and EDL. HRT and ttP displayed parallel and proportional alterations in soleus muscle, nevertheless hyperthyreosis affected HRT in greater extent than ttP. In case of the EDL muscle, HRT was found to be altered exclusively by T4 treatment, while ttP was sensitive to the diabetic condition.

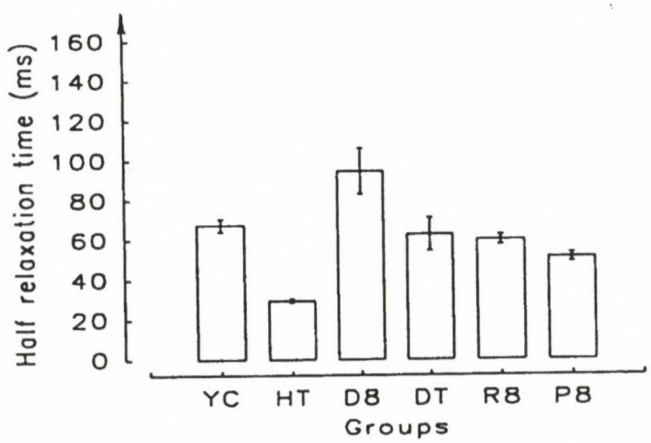

Fig. 2. Alteration in half relaxation time value of rat soleus muscle in different endocrinological states. For abbreviations see Fig. 1. Similar to ttP, HRT was found to be sensitive to both hyperthyreosis and diabetes. T4 and insulin can successfully return HRT to control value in diabetic animals

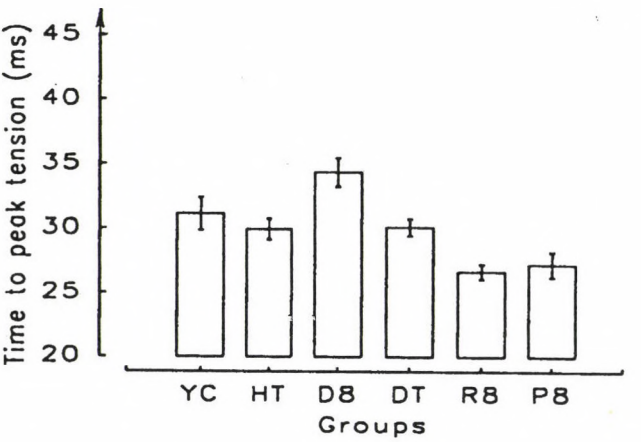

Fig. 3. Alteration in time to peak value of rat extensor digitorum longus muscle in different endocrinological states. For abbreviations see Fig. 1. Hyperthyreosis and diabetes were found not to alter this parameter. Nevertheless, there were significant differences between insulin or T4 treated groups and D8. This shows, muscle transformation has to be present in diabetes

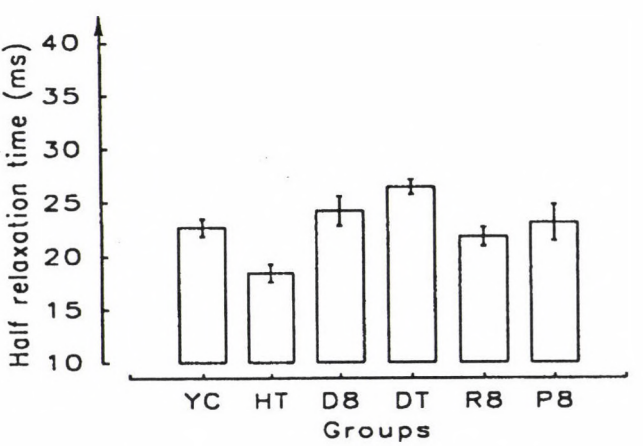

Fig. 4. Alteration in half relaxation time value of rat extensor digitorum longus muscle under different endocrinological states. For abbreviations see Fig. 1. HRT displayed similar changes like ttP, but only T4 and not insulin treatment turned out to be successful in producing muscle transformation

Discussion

Our findings can be summarized as follows. The time-course of contractions is subject to characteristic alterations under both diabetes and hyperthyreosis in slow and fast skeletal muscles of rat. EDL and soleus muscles were found to be responding in parallel direction but in various extent. Hyperthyreosis resulted in shortening of contraction curve in both EDL and soleus. Under diabetic conditions contraction curves were found to be lengthened. Detailed analysis revealed that changes are unmistakable in soleus muscle, while in case of EDL the transformation remained sublimital. Application of both thyroxine and insulin to diabetic animals uncovered that functional parameters of both soleus and EDL are sensitive to diabetes.

Our observation is in apparent contradiction with earlier publications [4, 12] reporting studies in respect of skeletal muscle contractility in diabetes. There are other observations published supporting insensitivity of EDL. Stephenson and her coworkers [14] reported altered calcium sensitivity in diabetic muscles, which was more pronounced in soleus than in EDL. Lacking effect of diabetes on EDL was pointed out in Cameron's paper [2]. All of these authors agreed in that twitch time of EDL is not altered by diabetes. These conclusions led to the conclusion that slow skeletal muscles, but not fast ones, are subject to muscle transformation during diabetes.

In spite of these consistent reports, we believe that EDL is subject to muscle transformation during diabetes, but individual fluctuations of ttP and HRT in animals obscure differences, making changes invisible. Our observations are supported by numerous publications reporting definitive alterations in EDL muscle during diabetes. Eibschutz and his group found altered Ca^{++} uptake on diabetic EDL muscle [5]. Defective Ca^{++} homeostasis was found in hamstring muscle of diabetic rat [15]. Hamstring muscle is known to contain predominantly fast fibres. Ca^{++} stimulated ATP-ase activity was reported to be increased in soleus, hamstring and gastrocnemius muscles [7]. Gastrocnemius muscle, like hamstring and EDL, is also known to be fast type. These observations, listed above are highly consistent with our conclusion that fast muscle function is altered by diabetes.

Another less doubtful finding revealed by our experiment concerns the reversibility of diabetes. Insulin treatment, applied either before or following the development of diabetes, resulted in complete recovery in the parameters studied. There were no signs of specific insulin effect resulting in transmutation different from the control state. Streptozotocin is a popular drug in induction of experimental diabetes, but the mechanism of its effect is not fully understood. Successful insulin reversion of diabetic transformations is a direct proof of the usefulness of the STZ model. Full recovery from all symptoms is an explicit evidence that all diabetic alterations manifested in STZ diabetes are the consequence of insulin deficiency. Furthermore, our results clearly show that these changes are reversible, and preventable on the time scale tested in our work.

Concentration of thyroid hormones are reported to be decreased in the sera of diabetic animals, while insulin treatment can increase plasma T_4 and T_3 contents even

more than the control value [16]. Thus, diabetic animals can be regarded as hypothyroids. One might suppose that diabetic muscle transformation is caused by the hypothyreosis present in diabetes. We have found that exogenous T_4 can protect skeletal muscles against diabetic transformation. This observation strongly supports the concept that hypothyreosis is a cardinal component of those elements which play a key role in muscle transformation present in diabetes mellitus.

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Thanks are due to Katalin Láber and Katalin Horváth for their excellent technical assistance.

REFERENCES

1. Brozinick, Joseph T., Etgen, Garret J., Yaspelkis Benedict, B., III, and Ivy John, L.: The effects of muscle contraction and insulin on glucose-transporter translocation in rat skeletal muscle. Biochem. J., **297**, 539–545 (1994).
2. Cameron, Norman E., Cotter Mary, A. and R. Sharon: Changes in skeletal muscle contractile properties in streptozotocin-induced diabetic rats and role of polyol pathway and hypoinsulinemia. Diabetes, **39**, 460–465 (1990).
3. Challis, R. A. John, Vranic, Mladen and Radda, George K.: Bioenergetic changes during contraction and recovery in diabetic rat skeletal muscle. Am. J. Physiol., **256**, E129–E137 (1989).
4. Cotter, M. A., Cameron, N. E., Robertson, S. and Ewing, I.: Polyol pathway-related skeletal muscle contractile and morphological abnormalities in diabetic rats. Exp. Phys., **78**, 139–155 (1993).
5. Eibschutz, Barry, Lopaschuk, Garry D., McNeill, John H. and Katz Sidney: Ca^{2+}-transport in skeletal muscle sarcoplasmic reticulum of the chronically diabetic rat. Research Communications in Chem. Path. and Pharm., **45**, 301–304 (1984).
6. Ganguly, P. K., Mathur, S., Gupta, M. P., Beamish, R. E. and Dhalla, N. S.: Calcium pump activity of sarcoplasmic reticulum in diabetic rat skeletal muscle. Am. J. Physiol., **251**, E515–E523 (1986).
7. Ganguly, P. K., Taira, Y., Elimban, V., Roy, M. and Dhala, N. S.: Altered contractile proteins in skeletal muscle of diabetic rats. Am. J. Physiol., **253**, E395–E400 (1987).
8. Hougen, Thomas J., Hopkins, Barry E. and Smith, Thomas W.: Insulin effects on monovalent cation transport and Na-K-ATPase activity. Am. J. Physiol., **234**, C59–C63 1978.
9. Kenji Okumara, Takuya Nishiura, Kiyokazu Shimizu, Yoshio Iwama, Junichiro Kondo, Hidekazu Hashimoto and Takayuki Ito: Fatty acid composition in 1,2-diacylglycerol of diabetic and insulin-treated diabetic rat hearts. Jpn Heart J., **32**, 667–673 (1991).
10. Machicao, E. and Wieland, O. H.: Evidence that the insulin receptor-associated protein kinase acts as a phosphatidylinositol kinase. FEBS Letters, **175**, 113–116 (1984).
11. Omatsu-Kanbe, Mariko and Kitasato Hiroshi: Insulin stimulates the translocation of Na^+/K^+-dependent ATPase molecules from intracellular stores to the plasma membrane in frog skeletal muscle. Biochem. J., **272**, 727–733 (1990).
12. Paulus, S. F., Grossie, J.: A comparison of isometric contractions in fast and slow muscle. Diabetes, **32**, 1035–1039 (1983).

13. Schmidt, R. E., Plurad, S. B., Olack, Barbara J. and Scharp, D. W.: The effect of pancreatic islet transplantation and insulin therapy on experimental diabetic autonomic neuropathy. Diabetes, **32**, 532–540 (1983).
14. Stephenson, Gabriela M. M., O'Callaghan, Alexia, and Stephenson D., George: Single-fiber study of contractile and biochemical properties of skeletal muscles in streptozotocin-induced diabetic rats. Diabetes, **43**, 622–628 (1994).
15. Taira, Y., Hata, T., Ganguly, Pallab, K., Elimban, V. and Dhalla, N. S.: Increased sarcolemmal Ca^{2+} transport activity in skeletal muscle of diabetic rats. Am. J. Physiol., **260**, E626–E632 (1991).
16. Sundersean, P. R., Sharma, V. K., Gingold, S. I., Banerjee, S. P.: Decreased β-adrenergic receptors in rat hear in streptozotocin-induced diabetes: role of thyroid hormones. Endocrinology, **114**, 1358–1363 (1984).

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Characteristics of stretch-release induced tension development in arterial smooth muscle[1]

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Stretching helical strips of porcine a. carotis comm. to 1.7 times the resting length increases myosin light chain phosphorylation from 0.22 to 0.67 mol phosphate/mol light chain. While stretch is maintained, the stretch induced light chain phosphorylation decreases and reaches the resting level over a period of 60 minutes.

The fully stretched arterial strips containing maximally phosphorylated myosin light chain cannot generate active tension which indicates, according to the sliding filament theory, the lack of overlap between actin and myosin filaments.

When stretch is released from these muscle strips active tension develops. The amplitude of this stretch-release induced tension depends on the level of myosin light chain phosphorylation related to the time interval between stretch and release as well as on the extent of the overlap zone related to the length of the released strip.

Three-dimensional graphic representation of both measured and interpolated data points in an active tension–light chain phosphorylation–muscle length space reveals a contractility surface which demonstrates that active tension appears as a sigmoidal function of light chain phosphorylation, on the one hand and shows maximum as a function of muscle length, on the other hand.

Keywords: active tension, a. carotis, light chain phosphorylation, passive tension, sliding filament theory, smooth muscle, stretch-release

Bayliss proposed first that stretch may activate constriction of blood vessels [1]. Since then changes in both electrical and mechanical properties of vascular smooth

[1]This paper is dedicated to the memory of Professor Tibor Kovács (1929–1994)

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muscles during or following stretch have been reported in numerous cases (for reviews see [7, 13]). The biochemical events behind these myogenic reactions were clarified by Bárány and coworkers [3, 4, 14]. They demonstrated the following: stretch induces phosphorylation of 20 kDa light chain of myosin (LC); the extent of phosphorylation is a function of the applied stretch reaching a maximum at 1.7 times the resting length; the stretch induced phosphorylation is catalyzed by myosin light chain kinase, not by protein kinase C and decays as a function of time. The fully stretched arterial strips cannot generate active tension even in the presence of any exogenous stimulating agents which indicates the lack of overlapping filaments. When stretched arterial strips are released active tension develops without any exogenous stimulating agents.

During our experiments reported in this paper helical strips from pig carotid arteries were activated by stretching them to 1.7 times their resting length then the stretched muscle strips were released. The developing active tension was recorded. The extent of release as well as the time interval between stretch and release were varied systematically. In this way we were able to investigate the active tension development at different muscle lengths and at different myosin light chain phosphorylation levels. Our results demonstrate that the amplitude of the stretch-release induced active tension is a monotonous function of light chain phosphorylation and shows a maximum as a function of muscle length. These data are presented in a three-dimensional active tension–light chain phosphorylation–muscle length space.

Materials and Methods

Carotid arteries were obtained from freshly slaughtered pigs. Helical strips were prepared and mounted in a water jacketed organ bath [14] containing physiological salt solution (130 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 1.6 mM $CaCl_2$, 14.9 mM $NaHCO_3$, 5.5 mM glucose, 0.03 mM $CaNa_2EDTA$ equilibrated with 5% CO_2–95% O_2, pH 7.4 at 37 °C) [6]. A resting tension appropriate to each strip's geometrical dimensions was applied to simulate 100 mmHg mean arterial pressure [8]. The length of strips was measured and considered as resting length (L_0).

Following a 30 minute incubation the strips were stimulated with K^+ challenge solution (100 mM KCl and 35 mM NaCl in the above-described physiological salt solution). The K^+ challenge-induced active tension was considered as a reference tension of the given muscle strip [3].

The strips were washed with physiological salt solution until the active tension disappeared then they were stretched to 1.7 times the resting length by means of the device shown in Fig. 1. Stretch was maintained for different periods of time then it was partly released. Stretch-release induced active tension was recorded and its amplitude was expressed as a percentage of the K^+ challenge induced tension [3].

For quantitation of myosin light chain phosphorylation the muscle strips were frozen by immersion in liquid N_2, then pulverized and solubilized. The solubilized samples were subjected to two-dimensional gel electrophoresis. The gels were stained with Coomassie Brillant Blue and the staining intensity of the myosin light chain spots was scanned. Light chain phosphorylation was calculated as described previously [2, 5, 6].

The curves fitted through experimental data were calculated using the cubic spline interpolation method [17].

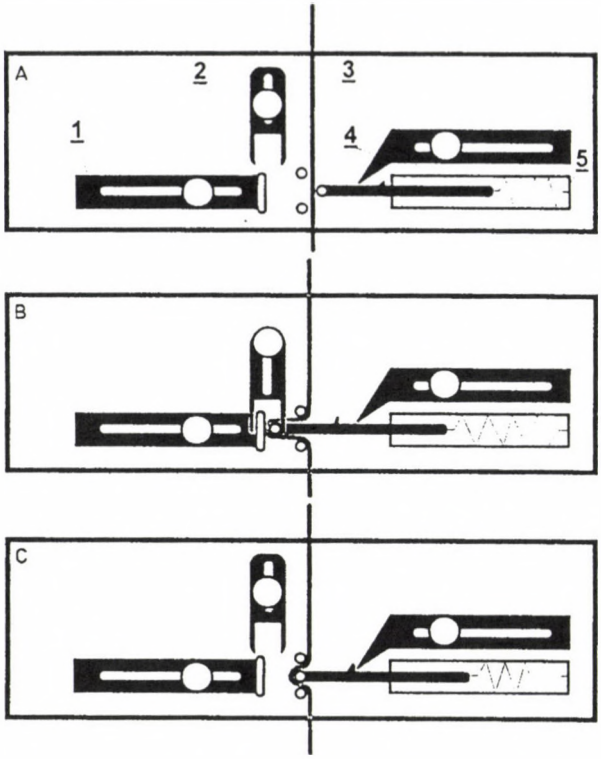

Fig. 1. Scheme of the stretch-release device. 1: stretch controller, 2: locker, 3: thread which connects the muscle srip to the isometric transducer, 4: release controller, 5: spring. A: start state, B: stretched state, C: released state. Proper positioning of the stretch controller allows stretching arterial strips exactly to 1.7 times their resting length. In the stretched strips light chain phosphorylation can reach maximal level and the overlap between the thin and thick filaments is abolished. The stretched state can be maintained by means of the locker, meanwhile myosin light chain phosphorylation spontaneously decreases. Release of the stretch provides an overlap zone between the actin and myosin filaments. The extent of this zone can be varied systematically by changing the position of the release controller at any phosphorylation level

Results

Figure 2 shows the tension record of a typical organ bath experiment. As a reference, maximal active tension was induced by K^+ depolarization of an arterial smooth muscle strip at resting length. This tension was taken as 100%. The K^+ challenge was removed by rinsing the strips with physiological salt solution several times. The relaxed arterial strip was then stretched to 1.7 times its resting length over 3–4 seconds and released to 1.4 times its resting length within 15 seconds. Consequently, active tension developed which attained 56% of the reference tension. Spontaneous active tension generation was followed by tension maintenance for at least 30 min (data not shown).

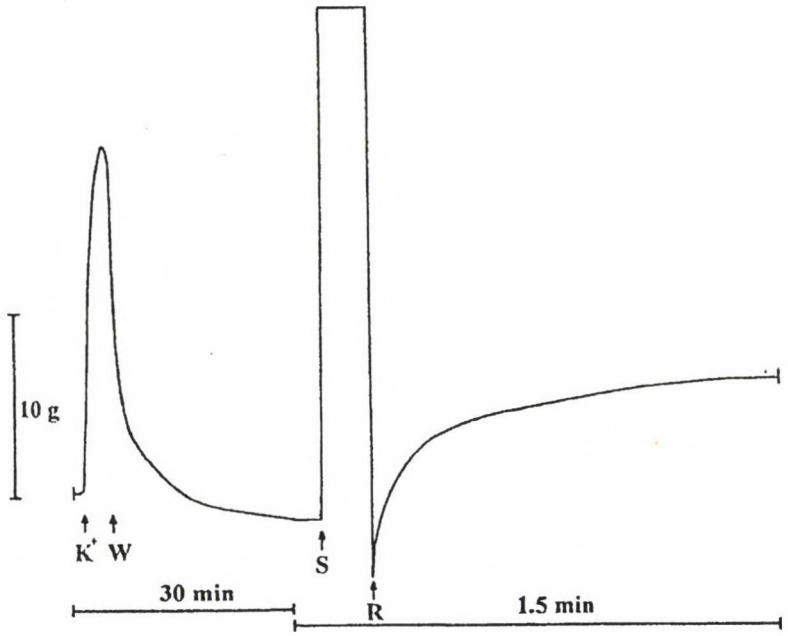

Fig. 2. A representative tension record. After adjusting the resting tension and measuring the resting length, the arterial smooth muscle strip was contracted with the K^+ challenge solution (K^+). When maximal active tension developed the strip was washed (W) until tension declined to resting tension. The strip was stretched to 1.7 times its resting length (S), then the stretched strip was released to 1.4 times its resting length (R). The amplitude of stretch-release induced tension reached 56% of the amplitude of the K^+ challenge induced tension and remained maintained during the observed 30 min period (not shown)

The stretch-release device (Fig. 1) constructed in our laboratory allowed to investigate the stretch-release induced tension as a function of release length. Our experimental data presented in Fig. 3 can be considered as length-tension curve and imply the sliding filament theory for arterial smooth muscle.

Our stretch-release device also allowed us to investigate the stretch-release induced tension as a function of time interval between stretch and release. Figure 4 demonstrates that the longer the interval between stretch and release, the smaller the stretch-release induced active tension.

We have supposed that spontaneous decay of stretch induced myosin light chain phosphorylation already described [3] is responsible for the observed time dependence of stretch-release induced tension. To verify this hypothesis arterial strips were stretched, their stretch was maintained for different periods of time then the myosin light chain phosphorylation was determined. Figure 5 indicates that myosin light chain phosphorylation exponentially decays reaching the resting value over 60 min.

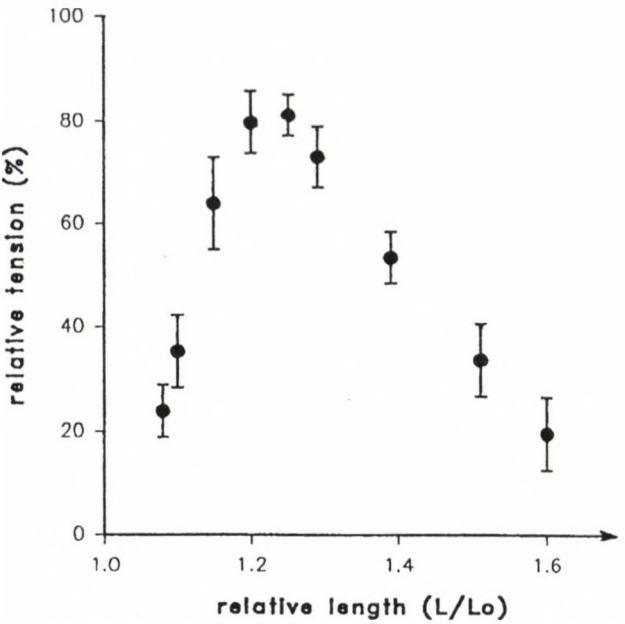

Fig. 3. Stretch-release induced active tension as a function of relative muscle length. Arterial smooth muscle strips were stretched to 1.7 times their resting length over 2–4 s. The stretched strips were kept at this length for 12–14 s, then each strip was released to the length shown on the abscissa. The stretch-release induced active tension was monitored and expressed as a percentage of K^+ challenge induced active tension. (L_0: resting length, at which passive tension is equal to about 100 mm Hg mean arterial blood pressure, L: release length.) Each symbol represents the mean of 3–9 separate experiments, the error bars indicate the standard error

The experimental data presented on Figs 4 and 5 provided the possibility to replot stretch-release induced tension as a function of myosin light chain phosphorylation. In Fig. 6, relative tension appears as a sigmoidal function of LC phosphorylation.

The above-mentioned results clearly indicate that stretch-release induced tension of arterial smooth muscle depends on both myosin light chain phosphorylation related to the time interval between stretch and release and on the extent of overlap between actin and myosin filaments related to the release length. To show these relationships as a two-variable function three additional length-tension curves were constructed using stretch-release induced tension values measured after 10, 30 and 60 min stretch maintenance, respectively. Cubic splines were fitted through experimental points in order to generate a 25 rows by 25-column matrix containing data points for Fig. 7 which introduces the stretch-release induced active tension as a two-variable function. The independent variables are the time interval (left panel) or light chain phosphorylation (right panel) and muscle length, the dependent variable is the relative tension on both panels.

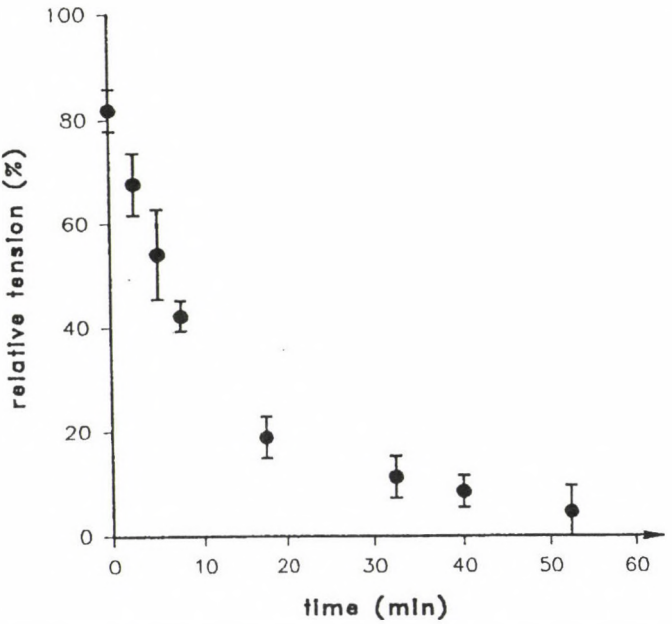

Fig. 4. Stretch-release induced tension as a function of time interval between stretch and release. Arterial smooth muscle strips were stretched to 1.7 times their resting length over 2–4 s. The stretched strips were kept at this length, then at the time shown on the abscissa each strip was released to 1.2 times its resting length. The stretch-release induced active tension was monitored and expressed as a percentage of K^+ challenge induced active tension. Each symbol represents the mean of 3–9 separate experiments, the error bars indicate the standard error

Discussion

The arterial smooth muscle normally responds to stretch by means of mechanical, structural and biochemical modifications.

The stretch-induced mechanical response has two components: passive (i.e. Ca^{2+} independent) tension and active (i.e. Ca^{2+} dependent) tension. Passive tension has been termed either viscoelasticity (if rate dependent) or plasticity (if not rate dependent) [16]. The active component of the stretch-induced tension is a suitable material for investigating the mechanism underlying the myogenic theory of blood flow regulation [1, 15]. In our experiments excessive elongation of helical strips from porcine carotid artery produces only passive tension (S in Fig. 2).

The absence of stretch-induced active tension is the consequence of the stretch-induced structural modification. According to Huxley's sliding filament theory [12] the lack of an overlap zone between the thick and thin filament is responsible for the absence of active tension [3, 14]. The stretch-induced structural modification is reversible: the release of the stretch provides the necessary overlap between actin and myosin filaments for active tension development (R in Fig. 2).

The amplitude of stretch-release induced active tension depends on the release length. The length-tension curve presented in Fig. 3 is similar to that determined in Bárány's [14] and Murphy's [11] laboratory and further supports the sliding filament theory for smooth muscle. The descending limb of the curve reflects a decrease in the number of crossbridges with increasing length. The ascending limb (i.e. the decline in isometric active tension at short length) may also occur through a reduction in the number of attached crossbridges caused by excessive contractile filament overlap and steric hindrance of crossbridge attachment [10].

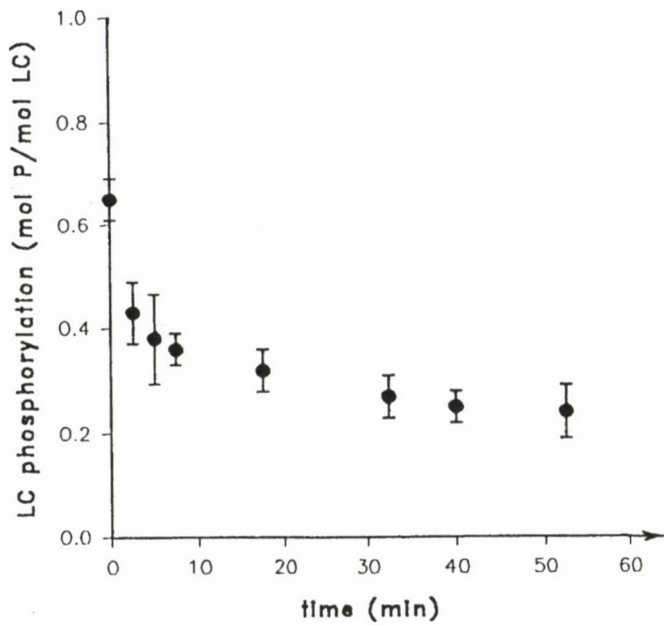

Fig. 5. Spontaneous dephosphorylation of stretched arterial strips. Arterial smooth muscle strips were stretched to 1.7 times their resting length over 2–4 s. The stretched strips were kept at this length, then at the time shown on the abscissa each strip was frozen. Myosin light chain phosphorylation was measured as described in Experimental procedures. Each symbol represents the mean of 3–9 separate experiments, the error bars indicate the standard error

Among the stretch-induced biochemical modifications phosphorylation of the myosin regulatory light chain in the key event necessary to initiate active tension development in arterial smooth muscle [3, 14]. The stretch-induced light chain phosphorylation catalyzed by Ca^{2+}, calmodulin dependent myosin light chain kinase [4] decreases over time (Fig. 5 and Ref. 3) while stretch-release induced active tension remains maintained (Fig. 2). The maintenance of active tension can be explained by the latchbridge hypothesis which postulates that tension is maintained by dephosphorylation of attached, phosphorylated crossbridges thereby forming attached, dephosphorylated crossbridges (latchbridges) that maintain tension but do not cycle [9, 16]. This model also account for the observed nonlinear active tension-light chain phosphorylation relation in arterial smooth muscle (Fig. 6).

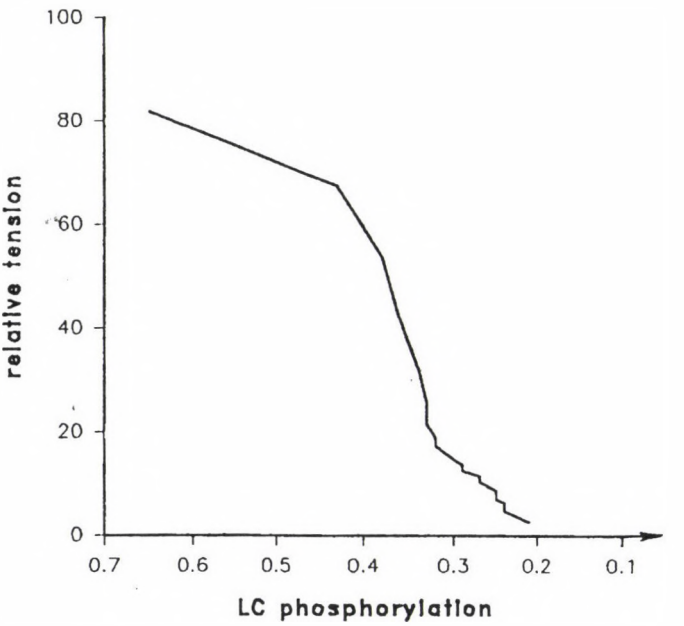

Fig. 6. Stretch-release induced active tension as a function of myosin light chain phosphorylation. Curves were fitted through the experimental data points presented in both Figs 4 and 5 by means of the cubic spline interpolation method. The curve fitted through the points of Fig. 4 was replotted replacing the time values on the abscissa by the corresponding phosphorylation levels obtained from the curve fitted through the experimental points of Fig. 5

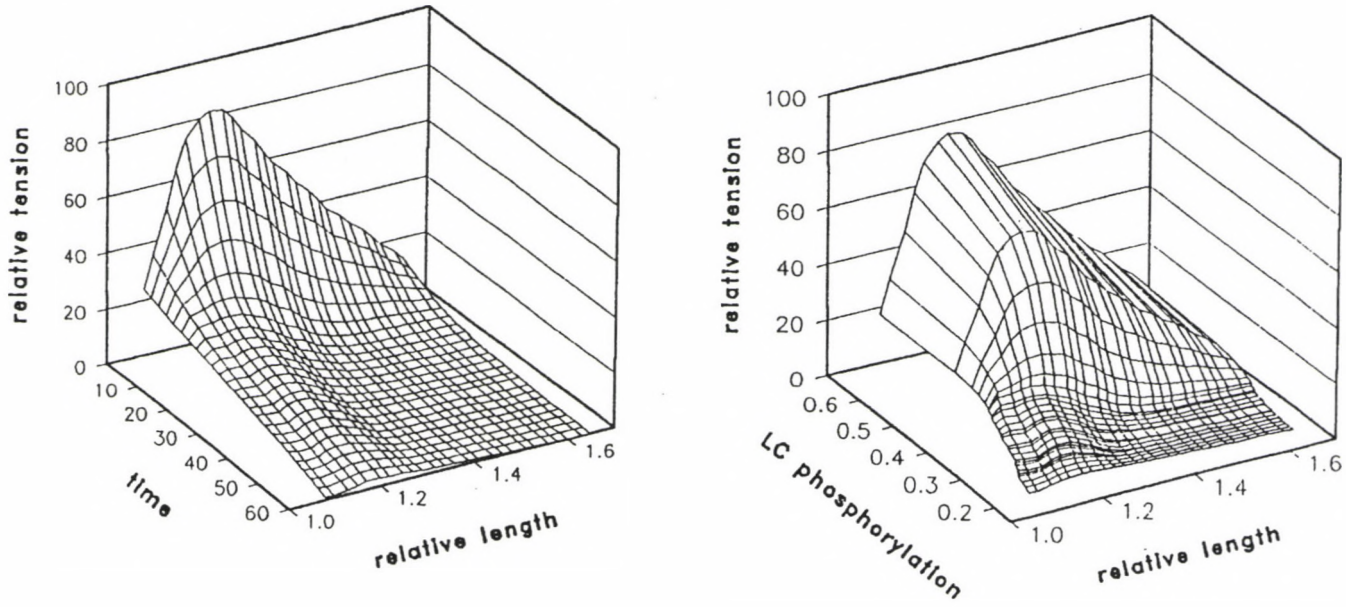

Fig. 7. 3-dimensional presentation of contractility surface of stretch-activated arterial smooth muscle. Left panel: the contractility surface is shown in a time–relative length–relative tension space. Right panel: the contractility surface is shown in a LC phosphorylation–relative length–relative tension space

Figure 7 summarizes both the light chain phosphorylation and the length dependence of stretch-release induced active tension of porcine arterial smooth muscle. It would be of interest to compare contractility surfaces in the case of smooth muscle tissues from different species and/or with different physiological or pathological background.

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REFERENCES

1. Bayliss, W. M.: On the reactions of the arterial wall to changes in internal pressure. J. Physiol., **28**, 220–231 (1902).
2. Bárány, K., Csabina, S. and Bárány, M.: The phosphorylation of the 20,000-dalton myosin light chain in uterus. Advances in Protein Phosphatases II, 37–58 (1985).
3. Bárány, K., Ledvora, R. F., Mougios, V. and Bárány, M.: Stretch-induced myosin light chain phosphorylation and stretch-release-induced tension development in arterial smooth muscle. J. Biol. Chem., **260**, 7126–7130 (1985).
4. Bárány, K., Rokolya, A. and Bárány, M.: Stretch activates myosin light chain kinase in arterial smooth muscle. Biochem. Biophys. Res. Comm., **173**, 164–171 (1990).
5. Csabina, S., Bárány, M. and Bárány, K.: Stretch-induced myosin light chain phosphorylation in rat uterus. Archives Biochem. Biophys., **249**, 374–381 (1986).
6. Csabina, S., Bárány, M. and Bárány, K.: Comparison of myosin light chain phosphorylation in uterine and arterial smooth muscles. Comp. Biochem. Physiol., **87B**, 271–277 (1987).
7. D'Angelo, G. and Meininger, G. A.: Transduction mechanisms involved in the regulation of myogenic activity. Hypertension, **23**, 1096–1105 (1994).
8. Dobrin, P. B.: Mechanical properties of arteries. Physiol. Reviews, **58**, 397–460 (1978).
9. Hai, C. M. and Murphy, R. A.: Ca^{2+}, crossbridge phosphorylation and contraction. Ann. Rev. Physiol., **51**, 285–298 (1989).
10. Harris, D. E. and Warshaw, D. M.: Am. J. Physiol., **260** (Cell Physiol. 29) C1104–C112 (1991).
11. Herlihy, J. T. and Murphy, R. A.: Length-tension relationship of smooth muscle of hog carotid artery. Circ. Res., **33**, 275–283 (1973).
12. Huxley, H. E.: The mechanism of muscle contraction. Science, **164**, 1356–1366 (1969).
13. Johnson, P. C.: The myogenic response. In: Handbook of Physiology. Bohr, D. F., Somlyo, A. P and Sparks, H. V. Eds, American Physiological Society, Bethesda, 1980 vol. II, pp. 409–442.
14. Ledvora, R. F., Bárány, M. and Bárány, K.: Myosin light chain phosphorylation and tension development in stretch-activated arterial smooth muscle. Clinical Chemistry, **30**, 2063–2068 (1984).
15. Nakayama, K., Suzuki, S. and Sugi, H.: Physiological and ultrastructural studies on the mechanism of stretch-induced contractile activation in rabbit cerebral artery smooth muscle. Jap. J. Physiol., **36**, 745–760 (1986).
16. Rembold, C. M.: Resistance to stretch, $[Ca^{2+}]_i$ and activation of swine arterial smooth muscle. J. Muscle Res. Cell Mot., **13**, 27–34 (1992).
17. Valkó, P. and Vajda, S.: SPLINE functions in signal processing. In: Advanced Scientific Computing in BASIC with Applications in Chemistry, Biology and Pharmacology, pp. 235–246, Elsevier, Amsterdam (1989).

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Interference of the sulphonylurea antidiabeticum gliquidone with mitochondrial bioenergetics in the rat under *in vitro* conditions[1]

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The hypoglycaemic sulphonylurea gliquidone and glibenclamide exerted a partial uncoupling effect on mitochondrial respiration of liver under *in vitro* conditions using various citrate cycle intermediates as substrates. Besides the uncoupling effect, gliquidone and glibenclamide caused a direct inhibition of ATP – as well as DNP – stimulated oxigen consumption. Both phenomena proved to be dose dependent. Respiratory control ratio decreased progressively with increasing concentrations of sulphonylureas mainly through the inhibition of ADP-stimulated respiration. Basal and DNP-stimulated ATP-ase activity of isolated mitochondria changed similarly to the respiratory parameters. Changes in membrane permeability of mitochondria and the inhibition of substrate uptake further support the assumption of structural and functional alteration of mitochondria by the hypoglycaemic compounds tested.

Keywords: sulphonylurea antidiabetica, gliquidone, glibenclamide, mitochondrial bioenergetics, respiration, ATP-ase, mitochondrial swelling, substrate uptake

In the last decades several sulphonylurea antidiabetic drugs have been synthetized which effectively lowered the level of blood glucose, inhibited the hepatic gluconeogenesis and influenced the peripheral glucose utilization [6, 9, 10, 14, 15, 27, 34–36].

[1] This paper is dedicated to the memory of Professor Tibor Kovács (1929–1994)

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Most peroral antidiabetica have a lipophyl character, thus they may cause disturbances in the structure and function of biomembranes. These compounds, depending on their concentration applied either *in vitro* or *in vivo*, may influence the mitochondrial oxidative processes and the energy household of the cells [3, 8, 12, 21, 23].

The widely used sulphonylurea, glibenclamide inhibited the pyruvate carboxylase in liver mitochondria [12] and this effect was associated with the reduction of mitochondrial ATP-ase activity [8]. The sulphonylureas could also interfere with the mitochondrial substrate uptake processes [23].

The side-effects mentioned above have been already described in the case of most sulphonylurea antidiabetica including glibenclamide. Interestingly, no data are available, whether the newer sulphonylurea compound gliquidone might similarly influence the mitochondrial bioenergetic processes. This question seems to be important, since the effective dose of gliquidone is at least 5 times higher, than that of glibenclamide [5, 36].

The present study was designed to examine a presumable interference of gliquidone with mitochondrial bioenergetics. The widely known hypoglycaemic agent, glibenclamide served as a reference substance.

Materials and Methods

Animals

Male CFY rats weighing 150–180 g (LATI, Gödöllő, Hungary) were used for experiments. The animals were appropriately housed and fed by corresponding laboratory diet supplemented with vitamin premix and water *ad lib*.

Chemicals

Gliquidone (1-Cyclohexyl-3-[<p-[2-(3,4-dihydro-7-methoxy-4,4-dimethyl-1,3-dioxo-2(1H)-isochinolyl)ethyl] phenyl>sulphonyl]-urea) and glibenclamide (N-4-[2-(5-chloro-2-methoxybenzmido)-ethyl]-phenyl-sulphonyl-N-cyclohexylurea) were obtained from Dr. Karl Thomae GmbH, Biberach an der Riss, Germany. L-(U^{14}C)-malic acid (40 mCi/mmol) was purchased from Amersham, England, and (1-4^{14}C)-succinic acid (40 mCi/mmol) was obtained from New England Nuclear, Dreieich, Germany. All other chemicals and biochemical products were of the highest purity commercially available.

Preparation of liver mitochondria

Mitochondria from rat livers were isolated by the method of Schneider [29] with minor modifications. Briefly, the livers were washed in preparation solution (0.25 M sucrose, 0.005 M Tris-HCl, 0.001 M EGTA, pH 7.2), cleaned and minced. Homogenization was carried out by 6 to 10 strokes in a glass/teflon homogenizer. Nuclei and non-disrupted cells from the 10 per cent homogenate were sedimented by 500×g for 10 min. The supernatant was centrifuged for 20 min at 4000×g. The pellet was resuspended in the preparation solution, homogenized and recentrifuged at 4000×g for 15 min. This washing procedure was repeated once more. The final pellet was resuspended in a minimal volume of preparation solution. After determination of protein content (20), the final concentration was adjusted to 60 mg protein/ml. Only

those preparations were used, where the respiratory control ratio reached the minimal value of 6 with glutamate + malate as respiratory substrates.

Measurement of the mitochondrial respiration

O_2 uptake was measured polarographically using a Clark type oxygen electrode. The registration was carried out in 2.0 ml of standard medium (80 mM KCl, 20 mM Tris-HCl 5 mM KH_2PO_4, 1 mM EGTA, pH 7.2) previously equilibrated with air. 2.5 mg mitochondrial protein was pipetted into the glass chamber which was kept in thermostat at 37 °C. Further additions were performed by microsyringes through the central hole of the stopper in a minimal volume. The following substrates were used in the experiments: 5 mM glutamate + 1 mM malate, 5 mM pyruvate + 1 mM malate; 5 mM succinate with 1.5 μM rotenone. ADP and DNP were added at final concentrations of 0.4 mM and 40 μM, respectively. Gliquidone and glibenclamide were dissolved in dimethylsulphoxide (DMSO), if not otherwise indicated 10 μl of dissolved drugs or DMSO alone were added to the glass chamber.

Determination of ATP-ase activity of mitochondria

ATP-ase activity was measured in 2.0 ml standard respiratory medium without KH_2PO_4 supplemented with 5 mM $MgCl_2$ and 5 mM Na-ATP, pH 7.2 and 40 μM DNP if indicated. The reaction was initiated by the addition of 0.5 mg mitochondrial protein. After 10 min incubation at 37 °C 1.0 ml 20 per cent TCA was added and the P_i liberated was estimated in the protein free filtrate according to Lohmann and Jendrassik [19].

Measurement of mitochondrial swelling

Mitochondrial osmotic volume changes were estimated by measuring the apparent absorbance changes at 540 nm spectrophotometrically. The measurements were carried out in 3.0 ml of appropriate isoosmotic media at 25 °C as indicated in the legends to the Figures.

Measurement of substrate uptake

Mitochondria were suspended in a loading medium containing 225 mM mannitol, 75 mM sucrose, 20 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 5 mM malonate. The suspensions were preincubated for 20 min at 0 °C. After centrifugation with 4500×g for 10 min the pellets were suspended in the above-mentioned medium at a concentration of 50 mg/ml. The substrate uptake of mitochondria was measured in 0.3 ml reaction volume containing 0.27 ml incubating solution (15 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 50 mM Tris-HCl pH 7.4, 1 μM Na_2 arsenite, 0.2 μM mersalyl and 1 μM rotenone), 30 μl mitochondrial suspension (1.5 mg protein), 10 μl of 30 mM ^{14}C-malate or ^{14}C-succinate and 3 μl glibenclamide or gliquidone dissolved in dimethylsulphoxide. After a 5 or 20 s incubation at 0 °C, 2.7 ml incubating medium (containing 25 mM phenylsuccinate) was added to the mixtures and the media were filtered through Whatman glass GF/F filters and the radioactivity of the filters was measured by liquid scintillation. Substrate uptake was calculated from the difference of radioactivity between the 20 s and 5 s values.

Results

The influence of gliquidone and glibenclamide on mitochondrial respiration capacity

To test whether gliquidone as well as glibenclamide influence the mitochondrial ATP generation and therefore the energy dependent metabolic pathways of the cells, their effects were investigated on respiration capacity of isolated rat liver mitochondria.

Under *in vitro* conditions both gliquidone and glibenclamide exerted a dual effect on mitochondrial respiration. As it is shown in Fig. 1 after the addition of the drugs at increasing concentrations, the basal respiration was progressively enhanced in a concentration dependent manner, under the same condition the ADP – as well as DNP – stimulated O_2 uptake was gradually decreased reaching the value of the basal respiration when glutamate and malate substrates were used. Although the basal respiration by 50 μM gliquidone was only slightly affected, gliquidone at this concentration caused an approximately 50 per cent inhibition of ADP-stimulated respiration. 100 μM gliquidone produced more than 70 per cent inhibition of ADP-dependent O_2 uptake, and increased the basal respiration threefold. These changes indicated a certain uncoupling of the mitochondrial oxidative phosphorylation besides the direct inhibition of the respiratory capacity. As a result, the respiratory control ratio (RCR) also progressively decreased, causing a 50 per cent reduction at about 50 μM gliquidone (Fig. 1). The ADP/O quotient decreased similarly (data not shown). Glibenclamide elicited a similar, but somewhat more pronounced effect on the respiration parameters than gliquidone. 100 μM glibenclamide caused a total uncoupling of oxidation from phosphorylation. Similar gliquidone – as well as glibenclamide – induced alterations were observed when the oxidation of pyruvate + malate (Fig. 2) and that of succinate (in the presence of rotenone) was measured (Fig. 3).

Effect of gliquidone and glibenclamide on the mitochondrial ATP-ase activity

Gliquidone and glibenclamide induced similar changes of the ATP-ase activity of liver mitochondria to those of respiration.

Under *in vitro* conditions both gliquidone and glibenclamide exerted a dual effect on mitochondrial ATP-ase activity. After the addition of the drugs at increasing concentrations, the basal ATP-ase activity was progressively enhanced in a concentration dependent manner, under the same conditions the DNP-stimulated ATP-ase activity decreased gradually reaching the basal value. These changes supported further a potential damage of structural integrity of mitochondria due to sulphonylureas investigated. No significant difference was observed between gliquidone and glibenclamide in their inhibitory actions upon mitochondrial ATP-ases (Fig. 4).

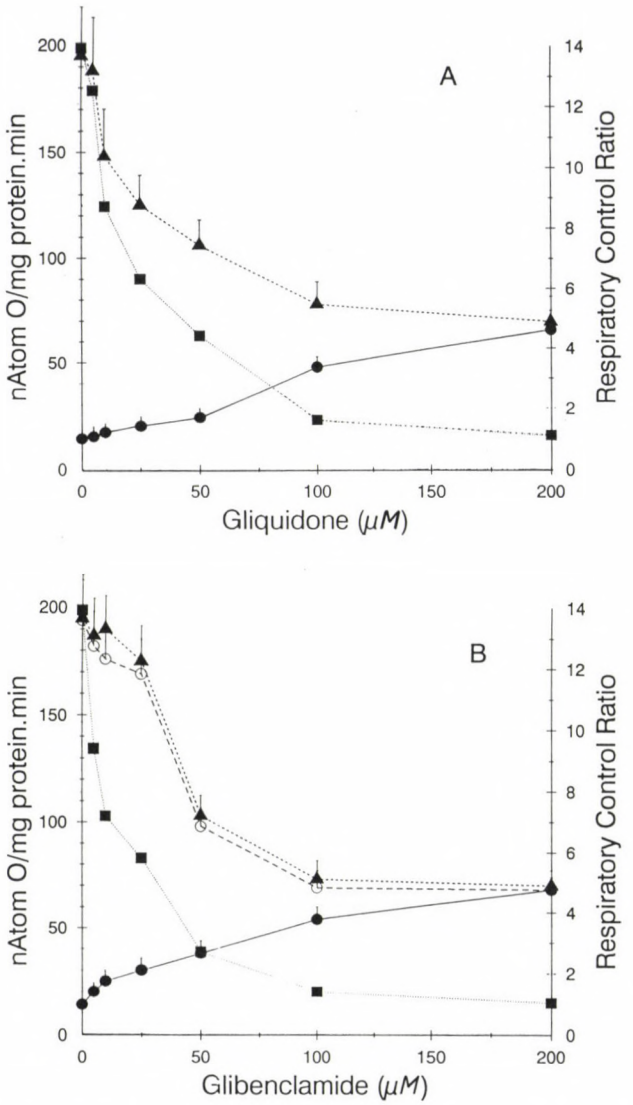

Fig. 1. Effect of gliquidone and glibenclamide on the mitochondrial respiration in the presence of glutamate + malate. A: effect of gliquidone; B: effect of glibenclamide; Basal respiration •---•; ADP-stimulated respiration ▲---▲; DNP-stimulated respiration o --- o ; respiratory control ratio (RCR) ■---■; The details of the experimental conditions were as described in **Methods**

Effects of gliquidone and glibenclamide on mitochondrial swelling

Both sulphonylurea compounds investigated caused a slight but measurable progressive swelling of isolated mitochondria in isoosmotic KNO_3 indicating an enhanced K^+-permeability. However, this swelling was not comparable to the effect of the K^+-ionophore valinomycin (Fig. 5).

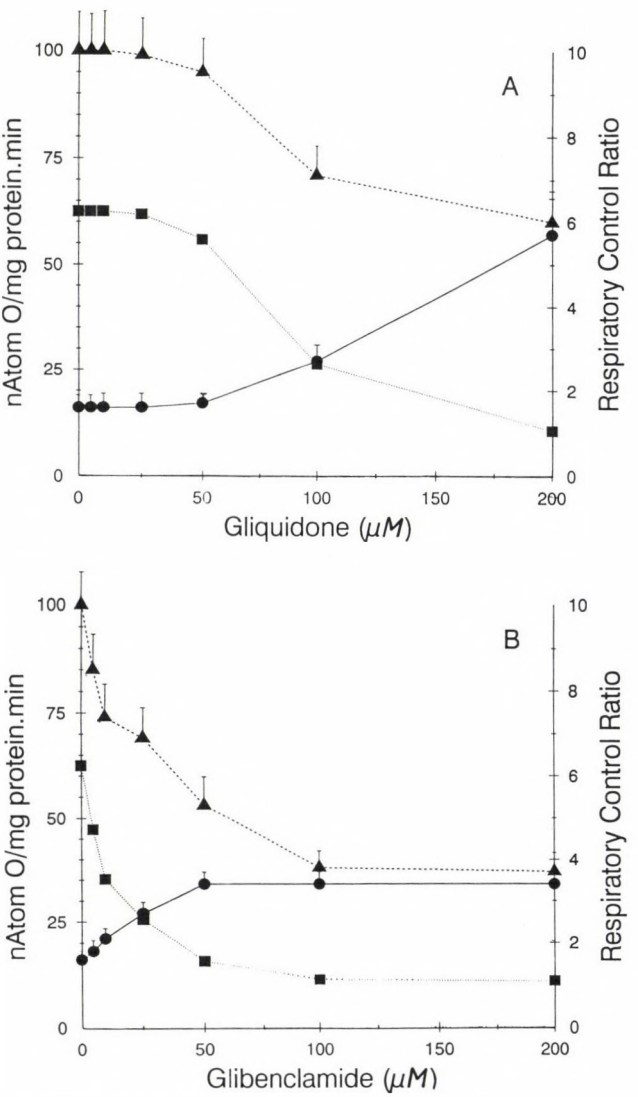

Fig. 2. Effect of gliquidone and glibenclamide on the mitochondrial respiration in the presence of pyruvate + malate. For symbols and experimental details see the legend of Fig. 1

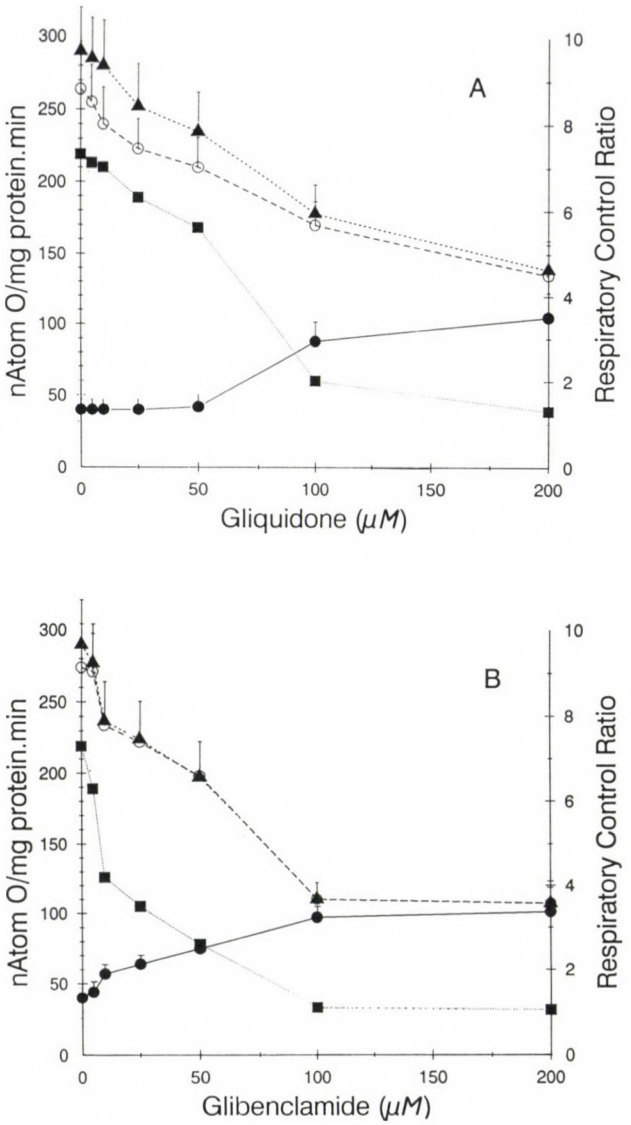

Fig. 3. Effect of gliquidone and glibenclamide on the mitochondrial respiration in the presence of succinate with rotenone. For symbols and experimental details see the legend of Fig. 1

When the mitochondria were suspended in isoosmotic $NaNO_3$ no significant swelling could be observed (not shown). Mitochondrial swelling can be also observed when the mitochondria are suspended in isotonic ammonium phosphate. NH_3 can

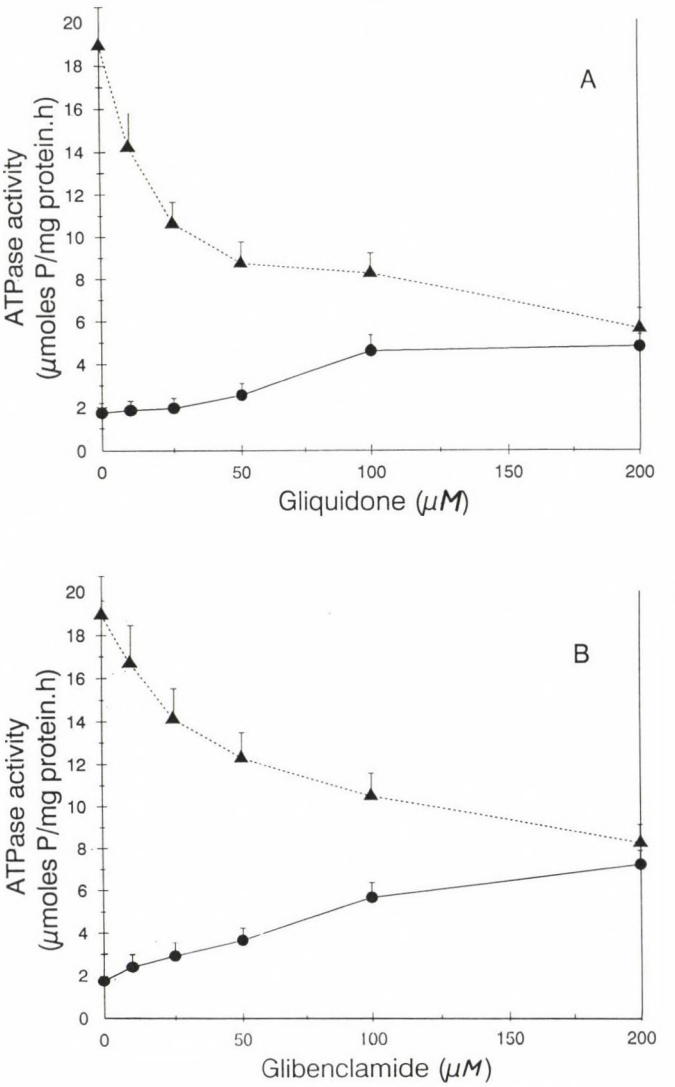

Fig. 4. Influence of gliquidone and glibenclamide on the mitochondrial ATP-ase activity. A: effect of gliquidone; B: effect of glibenclamide; basal ATP-ase activity •---•; DNP-stimulated ATP-ase activity ▲---▲; The experimental details were as described in **Methods**

penetrate the membrane freely, and $H_2PO_4^-$ will be transported together with a proton via the phosphate carrier system [11]. When the phosphate carrier is blocked by mercurials or N-ethylmaleimide (NEM) no osmotic volume change can occur. Gliquidone and glibenclamide caused only a partial inhibition of the swelling in ammonium phosphate medium indicating a depressed penetration of phosphate into mitochondria which may also contribute to the decreased activity of oxidative phosphorylation (Fig. 6).

Effect of gliquidone and glibenclamide on the substrate uptake of mitochondria

Ten μM gliquidone caused an approximately 10 per cent inhibition of malate and succinate uptake into mitochondria. Fifty per cent inhibition was produced by 50 μM gliquidone while 70 per cent inhibition was observed in the presence of 100 μM. 0.5 mM gliquidone practically abolished the substrate uptake into mitochondria. No significant difference was found in the gliquidone sensitivity of malate and succinate uptake (Fig. 7). Glibenclamide resulted in similar changes in substrate uptake processes as gliquidone (Fig. 8).

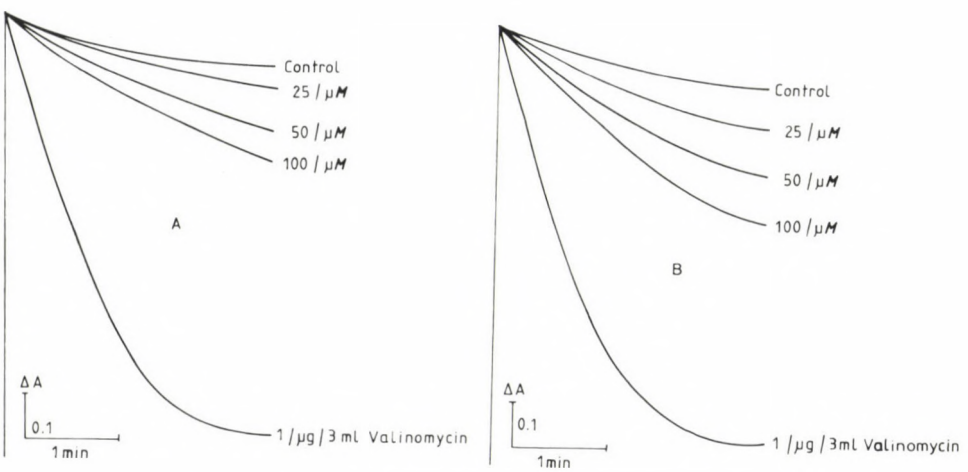

Fig. 5. Influence of gliquidone and glibenclamide on the mitochondrial swelling in isoosmotic KNO_3 medium A: effect of gliquidone; B: effect of glibenclamide; mitochondria (1 mg protein) were suspended in 3 ml medium containing 5 mM Hepes, 135 mM KNO_3, 0.1 mM EDTA, and 2 μM rotenone (pH 7.2). All other experimental details were as described in **Methods**

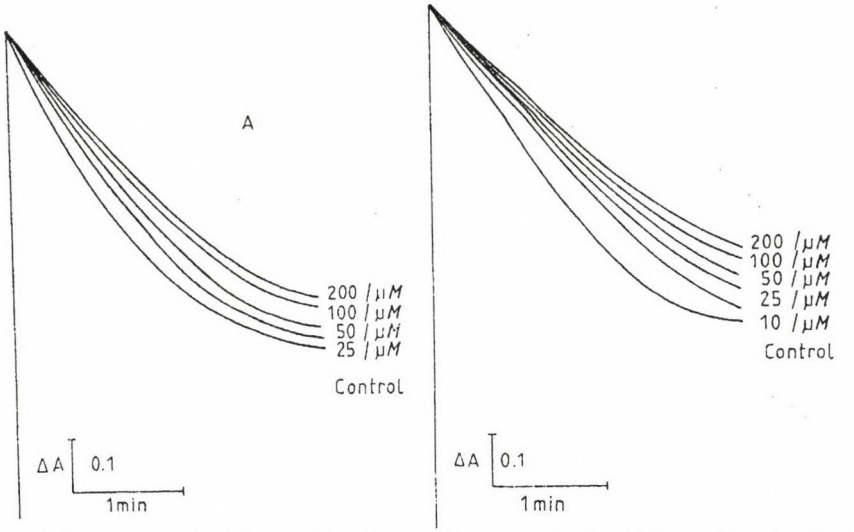

Fig. 6. Influence of gliquidone and glibenclamide on mitochondrial swelling in isoosmotic ammoniumphosphate medium. A: effect of gliquidone; B: effect of glibenclamide; mitochondria (1 mg protein) were suspended in 3 ml medium containing 5 mM Hepes, 135 mM $(NH_4)_3PO_4$, 0.1 mM EDTA and 2 μM rotenone (pH 7.2). All other experimental details were as described in **Methods**

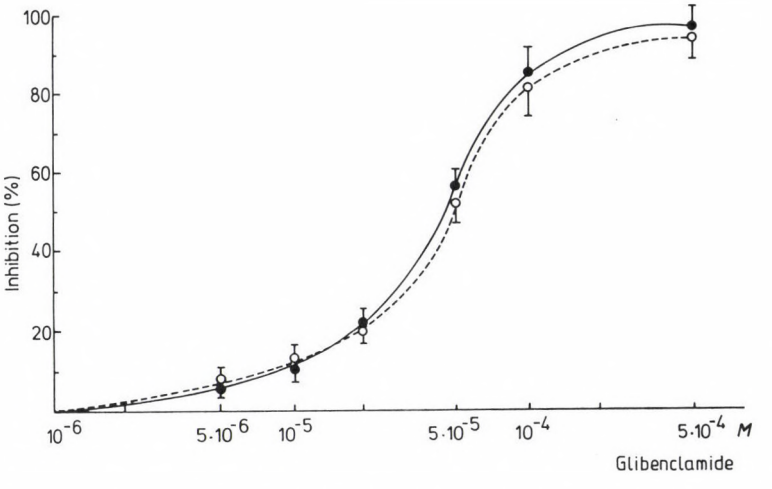

Fig. 7. Effect of glibenclamide on succinate and malate uptake into mitochondria; succinate •---•; malate o --- o ; The experimental details were as described in **Methods**

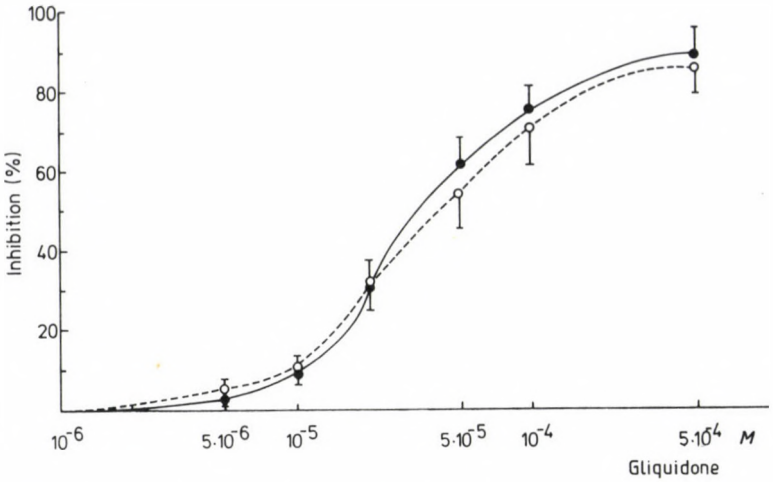

Fig. 8. Effect of gliquidone on succinate and malate uptake into mitochondria; For symbols and experimental details see the legend of Fig. 7

Discussion

In spite of many detailed investigations, the exact mechanism of the antidiabetic action of sulphonylurea compounds remains to be clarified. Besides the effects on beta-cells of pancreas [5, 13, 16, 19], different extrapancreatic actions of sulphonylurea compounds were also demonstrated in various tissues [2, 17, 18, 28, 31]. Among these effects the potentiation of insulin action and the reduction of glucose production of liver seem to be undoubtedly the most important [1, 4, 7, 16]. Since the gluconeogenesis is a highly energy consuming process, it can be supposed that the reduction of gluconeogenesis by sulphonylureas may be partially due to the available energy sources [30]. In this connection the inhibitory action of antidiabetic sulphonylureas on the bioenergetic processes in the liver should be taken into consideration.

The antidiabetic sulphonylurea compounds tested in this study caused a direct inhibition of ADP-stimulated respiration and exerted a partial uncoupling effect on oxidative phosphorylation in liver under *in vitro* conditions using various citrate cycle intermediates as substrates. In this respect not only the damage of citrate cycle but the inhibition of the oxidation of long chain fatty acids by the sulphonylurea compounds seems to be also important [26]. In an earlier study we could detect glibenclamide in liver and kidney mitochondria even one hour after intra-peritoneal administration of the compound [32]. The mitochondrial effects of sulphonylureas seem not to be very specific, because they are not limited to the electron–transport chain. These hypoglycaemic agents can interfere with mitochondrial substrate uptake processes [23]. In this study we have also demonstrated the inhibition of substrate uptake process by

gliquidone and glibenclamide. Furthermore, changes in the K^+-permeability of mitochondrial inner membrane under influence of both sulphonylureas produce further evidence that due to their lipophylic character these compounds may cause disturbances in the structure and functions of the mitochondria.

The question arises whether the alterations in the mitochondrial functions described here could take place also under *in vivo* conditions. In this connection the chronic treatment with hypoglycaemic sulphonylureas should be taken into consideration especially in the case of liver damage. In the forthcoming paper we try to give an appropriate answer to this problem [33].

Acknowledgements

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REFERENCES

1. Altan, N., Altan, M., Mikolay, L., Larner, J., Schwartz, C. F. W.: Insulin-like and insulin enhancing effects of the sulphonylurea glyburide on rat adipose glycogen synthase. Diabetes, **34**, 281–286 (1985).
2. Bachmann, W., Bottger, I., Haslbeck, M. and Mehnert, H.: Extrapancreatic action of sulphonylureas: effect of gliquidone on insulin and glucagon binding to rat liver plasma membranes. Eur. J. Clin. Invest., **9**, 411–415 (1979).
3. Belfiore, F., Rabuazzo, A. M., Iannello, S., Campione, R., Castorina, S. and Urzi, F.: Extrapancreatic action of glibenclamide in man: reduction in vitro of the inhibitory effect of glucagon and epinephrine on the hepatic key glycolytic enzymes phosphofructokinase (PFK) and pyruvate kinase (PK). Eur. J. Clin. Invest., **19**, 367–371 (1989).
4. Blumenthal, S. A.: Potentiation of the hepatic action of insulin by chlorpropamide. Diabetes, **26**, 485–489 (1977).
5. Borthwick, L. J. and Wilson, S.: Diabetic control with gliquidone – a short acting sulphonylurea. Eur. J. Clin. Pharmacol., **26**, 475–479 (1984).
6. Boyd, A. E. III., Aguilar-Bryan, L. and Nelson, D. A.: Molecular mechanisms of action of glyburide on the beta cell. Am. J. Med., **89** (Suppl. 2A), 3S–10S (1990).
7. Davidoff, F.: Hepatic effect of oral hypoglycemic drugs. Fed. Proc., **36**, 2724–2727 (1977).
8. Debeer, L., Mannaerts, G. and deSchepper, P. J.: Metabolic effects of hypoglycaemic sulphonylureas V. In vitro effects of sulphonylureas on mitochondrial adenosine triphosphatase activity. Biochem. Pharmacol., **23**, 251–258 (1974).
9. Debeer, L. J., Mannaerts, G. and deSchepper, P. I.: Effect of sulphonylureas on triglyceride metabolism in the rat liver, possible role of the lysosomes in hepatic lipolysis. J. Clin. Invest., **59**, 185–192 (1977).
10. Flier, I. S. and Matsouka, P.: Insulin action and the glucose transporter gene, in: Insulin Action, eds Draznin, B., Melmed, S. and Le Roith, D., Alan R. Liss, Inc. New York, 1972, pp. 175–182.
11. Fonyó, A. and Bessmann, S. P.: Inhibition of inorganic phosphate penetration into liver mitochondrial by p-mercuribenzoate. Biochem. Med., **2**, 145–163 (1968).

12. Foy, J. M. and Standing, V. H.: The effect of glibenclamide on two enzymes important in gluconeogenesis. Arzneim. Forsch., **24**, 1279–1282 (1974).
13. Garrino, M. G., Meissner, H. P. and Henquin, J. C.: The non-sulphonylurea moiety of gliquidone mimics the effects of the parent molecule on pancreatic B-cell. Eur. J. Pharmacol., **124**, 309–316 (1986).
14. Happ, I., Beyer, I., Nest, E., Fröhlich, A., Althoff, P. H. and Schöffling, K.: Blutzucker, Seruminsulin, unveresterte Fettsäuren und Wachstumshormon im Tagesprofil bei Erwachsenen-Diabetiker unter Mono-therapie mit verschiedenen Sulfonylharnstoffen. Arzneim. Forsch., **24**, 1228–1234 (1974).
15. Kolterman, O. G., Gray, R. S., Shapiro, G., Scarlett, I. A., Griffin, I. and Olefsky, I. M.: The acute and chronic effects of sulphonylurea therapy in type II diabetic subjects. Diabetes, **33**, 346–354 (1984).
16. Krall, L. P.: Sulphonylurea therapy, in: The Diabetes Annual/5, eds Alberti, K. G. M. M. and Krall, L. P., Elsevier, Amsterdam, 1990, pp. 110–127.
17. Lebovitz, H. E.: Oral hypoglycaemic agents, in: The Diabetes Annual/1, eds Alberti, K. G. M. M. and Krall, L. P., Elsevier, Amsterdam, 1985, pp. 93–110.
18. Lebovitz, H. E.: Oral hypoglycemic agents, in: The Diabetes Annual/3, eds Alberti, K. G. M. M. and Krall, L. P., Elsevier, Amsterdam, 1987, pp. 72–93.
19. Lohmann, K. and Jendrassik, L.: Kolorimetrische Phosphorsäurebestimmungen im Muskelextrakt. Biochem. Z., **178**, 419–426 (1926).
20. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J.: Protein measurement with the folin phenol reagent. J. Biol. Chem., **193**, 265–275 (1951).
21. Mannaerts, G., Debeer, L., Ceuterick-Goethala, A. M. and deSchepper, P. J.: Metabolic effects of hyperglycaemic sulphonylureas IV. Interference of sulphonylureas with mitochondrial oxidative phosphorylation. Biochem. Pharmacol., **23**, 239–250 (1974).
22. Mc Cormick, K., Williams, M. C., Sicoli, R. and Chen, L.: Effect of tolazamide on basal ketogenesis, glycogenesis and gluconeogenesis in liver obtained from normal and diabetic rats. Endocrinology, **119**, 1268–1273 (1986).
23. Mc Guinness, D. P., Cherrington, A. D.: Effect of glyburide on hepatic glucose metabolism. Amer. J. Med., **89**, (Suppl. 24) 2A–26S–37S (1990).
24. Monge, L., Mojena, M., Ortega, J. L., Samper, B., Cabello, M. A., Feliu, J. E.: Chlorpropamide raises fructose-2,6-bis-phosphate concentration and inhibits gluconeogenesis in isolated rat hepatocytes. Diabetes, **35**, 89–96 (1986).
25. Patel, T. B.: Effects of tolbutamide on gluconeogenesis and glycolysis in isolated perfused rat liver. Am. J. Physiol., **250**, E82–E86 (1986a).
26. Patel, T. B.: Effect of sulphonylureas on hepatic fatty acid oxidation. Am. J. Physiol., **251**, E241–E246 (1986/b).
27. Porte, D. Jr. and Kahn, M. B.: Mechanism for hyperglycaemia in type II diabetes mellitus: therapeutic implications for sulphonylurea treatment – an update. Am. J. Med., **90** (Suppl. 6A), 8S–14S (1991).
28. Rinninger, F., Kirsch, D., Haring, H. U. and Kemmler, W.: Extrapancreatic action of the sulphonylurea gliquidone: post-receptor effect on insulin-stimulated glycogen synthesis in rat hepatocytes in primary culture. Diabetologia, **26**, 462–465 (1984).
29. Schneider, W. C.: Intracellular distribution of enzymes III. The oxidation of octanoic acid by rat liver fractions. J. Biol. Chem., **176**, 259–266 (1948).
30. Sherratt, H. S. A.: The inhibition of gluconeogenesis by non-hormonal hypoglycaemic compounds. in: Short-term Regulation of Liver Metabolism, eds Hue, L., Werve, Van der, Elsevier, Amsterdam, 1981, p. 199.

31. Sherratt, H. S. A. and Alberti, K. G. M. M.: New oral hypoglycaemic drugs, in: The Diabetes Annual/5, eds Alberti, K. G. M. M. and Krall, L. P., Elsevier, Amsterdam, 1990, pp. 125–141.
32. Somogyi, J., Vincze, I., Willig, F. and Schmidt, F. H.: The subcellular distribution of ^{14}C-glibenclamide in different tissues of rat. Horm. Metab. Res., **6**, 181–183 (1974).
33. Somogyi, J., Vér, Á., Trója, G., Végh, E., Bányász, T., Csermely, P., Popovic, S., Kovács, T.: Interference of sulphonylurea antidiabetica with mitochondrial bioenergetics under in vivo conditions. Acta Physiol. Hung. **83**, 313-321 (1995).
34. Taylor, R. and Agius, L.: The biochemistry of diabetes. Biochem. J., **250**, 625–640 (1988).
35. Venkatesh, N., Lamp, S. T. and Weiss, J. N.: Sulphonylureas, ATP-sensitive K^+ channels and cellular K^- loss during hypoxia, ischemia, and metabolic inhibitor in mammalian ventricle. Circulation Res., **69**, 623–637 (1991).
36. Zilker, Th., Ermler, R., Henderkott, U. and Bottermann, P.: Untersuchungen zum Wirkungsmechanismus von Gliquidon. Dtsch. med. Wschr., **100**, 1733–1741 (1975).

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Interference of sulphonylurea antidiabetica with mitochondrial bioenergetics under *in vivo* conditions[1]

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Sulphonylurea antidiabetica effectively inhibits the basal hepatic glucose production. Since it has been firmly established that lipophylic sulphonylurea drugs exerted an uncoupling effect on mitochondrial oxidative phosphorylation, a relationship between the reduction of hepatic gluconeogenesis and the insufficient energy supply due to sulphonylureas could be supposed. In this study we have investigated the effects of glibenclamide and gliquidone on mitochondrial bioenergetics in liver after peroral treatments of normal rats with different doses. The treatment of rats with 5 mg/kg glibenclamide or gliquidone daily for 14 days elicited only a marginal inhibition on mitochondrial oxidation capacity and remained without any effect on mitochondrial ATPase activity. Only the supermaximal dose 50 mg/kg for 14 day produced a significant damage in the mitochondrial functions. The basal respiration increased with 60–80 per cent, whereas the ADP- or DNP-stimulated oxygen consumption significantly decreased independently from the respiratory substrates investigated. Similar alterations were found in the mitochondrial ATPase activity after treatment with these drugs. No essential differences have been observed in the actions between glibenclamide and gliquidone.

However, the lowest dose applied in this study is many times higher than the usual therapeutic dose. Consequently, glibenclamide and gliquidone do not interact with mitochondrial bioenergetic processes under therapeutic conditions. On the other hand, in different liver and kidney damages we have no sufficient knowledge whether these drugs can be accumulated in these organs and therefore their elevated concentration may interfere with the mitochondrial energy metabolism.

Keywords: sulphonylurea antidiabetica, glibenclamide, gliquidon, mitochondrial bioenergetics, *in vivo* effects

[1] This paper is dedicated to the memory of Professor Tibor Kovács (1929–1994)

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Sulphonylurea antidiabetic drugs are widely used in treatment of non-insulin dependent diabetes mellitus to stimulate insulin release from pancreatic cells [1, 2]. This effect is associated with an increased number of insulin receptors [3, 18, 42, 49] and an enhanced insulin mediated glucose utilization [2, 5, 14, 26, 31, 37]. Furthermore, sulphonylureas reduce the basal hepatic glucose production [5, 26].

Not only the anti-hyperglycaemic action of sulphonylurea but their pharmacokinetics and metabolism have been also intensively studied [6, 7, 10, 11, 21, 22, 28, 43, 44]. The widely used sulphonylureas gliquidone and glibenclamide are rapidly absorbed, their elimination from plasma occurs also quickly [13, 28, 46]. The biological half-life proved to be similar in both cases [10, 20, 28]. Both drugs are extensively metabolized in the liver. Three main metabolites of glibenclamide and four derivates of gliquidone were isolated. The metabolites had no hypoglycaemic effect. Elimination of glibenclamide is equally mediated by the urine and faeces. However, excretion of gliquidone occurs in 90 per cent via bile, in the urine no more than 5 per cent of its inactive metabolite can be detected [13, 28, 36].

In our previous paper the possible interference of gliquidone and glibenclamide with mitochondrial bioenergetics was studied [45]. We have established that under *in vitro* conditions these sulphonylureas exerted a partial uncoupling effect on mitochondrial respiration of liver and they inhibited the DNP-activated ATPase and the substrate uptake into mitochondria. These effects proved to be dose dependent [45]. Although many changes in mitochondrial functions under *in vitro* conditions have been published [15, 32, 34, 45, 47], a detailed analysis of *in vivo* alteration of mitochondria following sulphonylurea treatment has not been performed so far. In this study the effects of glibenclamide and gliquidone on the mitochondrial bioenergetics of rats are investigated after peroral treatment with different doses of sulphonylurea tested.

Materials and Methods

Male CFY rats weighing 150–180 g (LATI, Gödöllő, Hungary) were used for experiments. The animals were appropriately housed and fed a corresponding laboratory diet supplemented with vitamin premix and water *ad lib.*

The rats were treated with a single dose of 50 mg/kg of the respective sulphonylurea intraperitoneally, suspended freshly in 1 per cent methylcellulose (Aldrich Chemical Co.). Two hours after the drug administration the rats were killed and liver mitochondria were isolated. Other groups of rats were treated with 5 mg or 50 mg/kg drugs *per os* for two weeks once daily. To control animals only methylcellulose was administered. All other methods used in this study were described previously [45].

Results

The influence of gliquidone and glibenclamide on mitochondrial respiration capacity

To test whether gliquidone as well as glibenclamide influence the mitochondrial ATP generation and therefore the energy dependent metabolic pathways of the cells under *in vivo* conditions, their effects were investigated on respiration capacity of rat liver mitochondria after treatments with different doses of the drugs.

An administration of 5 mg/kg glibenclamide or gliquidone to the rats for 14 days, did not alter the basal as well as the ADP- or DNP-stimulated respiration in comparison with the control values using different respiratory substrates (Tables I–III).

When a supermaximal dose (50 mg/kg) of gliquidone or glibenclamide was administered to the rats intraperitoneally, the respiratory parameters of the isolated liver mitochondria were only slightly affected two hours after the treatment. Glibenclamide caused a little but measurable increase in the basal respiration, gliquidone treatment was without effect in the case of all respiratory substrates tested. In the ADP- as well as DNP-stimulated O_2-uptake no changes was observed at these doses of both drugs (Tables I–III).

Table I

Mitochondrial respiration of liver mitochondria with glutamate + malate

No. of experiments	Pretreatment	Oxygen uptake (nAtom O/mg protein/min) Basal	ADP-stimulated	DNP-stimulated	RCR
10	Control	14 ± 2	195 ± 20	188 ± 19	13.9
6	5 mg/kg glibenclamide p.o. 14 days	15 ± 2	196 ± 21	186 ± 18	13.1
10	50 mg/kg glibenclamide i.p. 2 hours	17 ± 3	198 ± 19	191 ± 19	11.6
6	50 mg/kg glibenclamide p.o. 14 days	23* ± 3	157* ± 17	146* ± 15	6.8
6	5 mg/kg gliquidone p.o. 14 days	14 ± 2	196 ± 18	187 ± 18	14.0
12	50 mg/kg gliquidone i.p. 2 hours	15 ± 2	199 ± 18	191 ± 19	13.3
6	50 mg/kg gliquidone p.o. 14 days	23* ± 3	170* ± 17	164* ± 17	7.4

* Significance: $p < 0.05$

Table II

Mitochondrial respiration of liver mitochondria with pyruvate + malate

No. of experiments	Pretreatment	Oxygen uptake (nAtom O/mg protein/min) Basal	ADP-stimulated	DNP-stimulated	RCR
10	Control	10 ± 2	102 ± 9	98 ± 10	10.2
6	5 mg/kg glibenclamide p.o. 14 days	11 ± 2	102 ± 2	98 ± 10	9.3
10	50 mg/kg glibenclamide i.p. 2 hours	12 ± 2	98 ± 10	86 ± 9	8.9
6	50 mg/kg glibenclamide p.o. 14 days	17* ± 3	75* ± 9	69* ± 8	4.4
6	5 mg/kg gliquidone p.o. 14 days	11 ± 2	102 ± 9	98 ± 9	9.3
12	50 mg/kg gliquidone i.p. 2 hours	11 ± 2	104 ± 10	83 ± 9	9.5
6	50 mg/kg gliquidone p.o. 14 days	18* ± 3	76* ± 8	71* ± 8	4.2

* Significance: $p < 0.05$

Table III

Mitochondrial respiration of liver mitochondria with succinate (in the presence of rotenone)

No. of experiments	Pretreatment	Oxygen uptake (nAtom O/mg protein/min) Basal	ADP-stimulated	DNP-stimulated	RCR
10	Control	40 ± 5	290 ± 31	264 ± 28	7.3
6	5 mg/kg glibenclamide p.o. 14 days	41 ± 6	293 ± 32	264 ± 27	7.1
10	50 mg/kg glibenclamide i.p. 2 hours	44 ± 5	301 ± 32	264 ± 27	6.8
6	50 mg/kg glibenclamide p.o. 14 days	47* ± 6	239* ± 26	201* ± 23	5.1
6	5 mg/kg gliquidone p.o. 14 days	41 ± 5	291 ± 30	265 ± 28	7.1
12	50 mg/kg gliquidone i.p. 2 hours	41 ± 4	297 ± 30	239 ± 25	7.2
6	50 mg/kg gliquidone p.o. 14 days	46* ± 5	283* ± 31	231* ± 25	6.2

* Significance: $p < 0.05$

Table IV

ATPase activity of isolated liver mitochondria

No. of experiments	Pretreatment	ATPase activity (μmoles P_i/mg protein/h) Basal	DNP-stimulated
8	Control	1.74 ± 0.21	18.93 ± 2.11
6	5 mg/kg glibenclamide p.o. 14 days	1.72 ± 0.19	18.84 ± 2.06
6	50 mg/kg glibenclamide i.p. 2 hours	1.89 ± 0.34	18.61 ± 2.43
6	50 mg/kg glibenclamide p.o. 14 days	2.27 ± 0.41*	12.39 ± 2.08*
6	5 mg/kg gliquidone p.o. 14 days	1.69 ± 0.20	18.96 ± 2.10
6	50 mg/kg gliquidone i.p. 2 hours	1.65 ± 0.33	18.90 ± 2.11
6	50 mg/kg gliquidone p.o. 14 days	1.97 ± 0.34	12.35 ± 1.83*

* Significance: $p < 0.05$

After a treatment of 14 days with the same doses of both glibenclamide and gliquidone substantial alterations were seen in the mitochondrial respiration. The basal respiration increased by 60–80 per cent when glutamate or pyruvate with malate were used as substrates. Interestingly, the one-step oxidation of succinate was elevated less than 20 per cent under the same conditions (Tables I–III). The ADP- as well as DNP-stimulated respiration was reduced by 25 per cent maximally. As a result of the opposite changes of the respiration values with and without added ADP, the respiratory control ratios decreased significantly. With glutamate or pyruvate in the presence of malate, the decrease of RCR value exceeded 50 per cent, while the change in the RCR value with succinate was less pronounced (Tables I–III).

Effect of gliquidone and glibenclamide on the mitochondrial ATPase activity

Gliquidone and glibenclamide induced similar changes of the ATPase activity of liver mitochondria to those of the respiration. After two hours of the intraperitoneal administration of 50 mg/kg glibenclamide or gliquidone, no substantial alteration of mitochondrial ATPase activity was observed. Similarly, no changes in ATPase activity of mitochondria were observed when 5 mg/kg of the drugs were administered to the rats for 14 days (Table IV). A treatment of 14 days with supramaximal dose (50 mg/kg) of the drugs caused a 15–30 per cent increase in the basal ATPase activity and a near 40 per cent reduction in the DNP-stimulated ATPase activity (Table IV). No significant difference was observed between gliquidone and glibenclamide in their

inhibitory potential upon mitochondrial ATPase. The alterations in mitochondrial ATPase activities observed supported further a potential damage of structural integrity of mitochondria due to sulphonylureas under *in vivo* conditions.

Discussion

In the last more than 30 years sulphonylurea compounds have been widely used in the treatment of patients with non-insulin-dependent diabetes mellitus. These drugs act at both pancreatic and extrapancreatic sites. However, it seems likely that the initial and quantitatively most important action of the sulphonylureas is the stimulation of insulin secretion [3, 9, 12, 19, 27, 30]. Among the extrapancreatic effects the reduction of hepatic glucose production and the increase of the insulin dependent glucose uptake into the insulin sensitive tissues are the most important [2–4, 8, 11, 16, 40, 48]. Using cultured rat hepatocytes the stimulation of glycogenesis and lipogenesis under influence of sulphonylurea drugs was also observed [14, 17, 37, 38, 39, 41]. In the hepatic glucose production the gluconeogenesis plays the cardinal role. This process involves both the mitochondrial and cytosolic compartments of the cells and can be influenced at many different sites. The transport of metabolites between cytosol and mitochondria, the availability of precursor molecules and reducing equivalents, and the supply with ATP are probably the most important factors which can alter the intensity of the gluconeogenesis. On the other hand, the actual concentration of fructose 2,6-bisphosphate determines the direction of metabolic processes to synthesis or to degradation of glucose. For the synthesis and breakdown of fructose 2,6-bisphosphate the same bifunctional enzyme is responsible, the phosphorylated state of this enzyme determines in which direction the enzyme acts. It was demonstrated from many sites that sulphonylureas can stimulate fructose 2,6-phosphate formation in liver even in streptozotocin induced diabetic rats [4, 23–25, 33, 35].

The energy supply of gluconeogenesis meet demands by the intensive oxidation of fatty acids as well as citrate cycle intermediates in mitochondria. Different uncouplers of oxidative phosphorylation increase the utilization of mitochondrial metabolites without utilisable energy production. In the previous paper we have demonstrated that sulphonylurea compounds can cause a partial uncoupling of oxidative phosphorylation [45]. This finding was supported by earlier observations, too [15, 32, 47]. Sulphonylureas can penetrate through plasma membrane and are detectable also in mitochondria [46]. It can be supposed that the mitochondrial inner membrane can bind the lipophyl sulphonylurea compounds and the non-specific interference with the lipid bilayer can cause disturbances in the function of the integral membrane proteins.

Although the therapeutic dose is less for glibenclamide than for gliquidone, we have not found a significant difference in their inhibitory capacity upon mitochondrial bioenergetic processes either *in vitro* or *in vivo*. The daily treatment with 5 mg glibenclamide or gliquidone/kg of the normal rats for 14 days elicited only a marginal

inhibition in the mitochondrial oxidation capacity. Only the supermaximal dose from these drugs (50 mg/kg) produced a significant inhibition of oxidative parameters. The concentrations of sulphonylureas investigated in this study are not comparable with the usual therapeutic doses, therefore the relevance of the mitochondrial effects to human clinical pharmacology remains to be clarified. Certainly, our results suggest that both gliquidone and glibenclamide do not interact with the mitochondrial bioenergetic processes in therapeutic dosages under *in vivo* conditions. However, in different liver or kidney damages we have no sufficient information whether these drugs can be accumulated, and therefore their local concentration may interfere with mitochondrial bioenergetics in these organs.

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REFERENCES

1. Bailey, J. C., Broadbent, M. E.: Chronic effect of glibenclamide, chlorpropamide and metformin on plasma glucose and insulin in non diabetic rats. Gen. Pharmacol., **12**, 323–326 (1981).
2. Beck-Nielsen, H., Hother-Nielsen, O., Pedersen, O.: Mechanism of action of sulphonylureas with special reference to the extrapancreatic effect: an overview. Diabetic. Med., **5**, 613–620 (1988).
3. Beck-Nielsen, H., Pedersen, O., Lindskov, H. O.: Increased insulin sensitivity and cellular insulin binding in obese diabetics following treatment with glibenclamide. Acta Endocrinol. (Copenh.), **90**, 451–462 (1979).
4. Belfiore, F., Rabuazzo, A. M., Iannello, S., Campione, R., Castorina, S. and Urzi, F.: Extrapancreatic action of glibenclamide in man: reduction in vitro of the inhibitory effect of glucagon and epinephrine on the hepatic key glycolytic enzymes phosphofructokinase (PFK) and pyruvate kinase (PK). Eur. J. Clin. Invest., **19**, 367–371 (1989).
5. Best, J. D., Judzewitsch, R. G., Pfeifer, M. A., Beard, J. C., Halter, J. B., Porte, D. Jr.: The effect of chronic sulphonylurea therapy on hepatic glucose production in non-insulin-dependent diabetes. Diabetes, **31**, 333–338 (1982).
6. Beyer, J., Haupt, E., Cordes, U., Sell, G., Schöffling, K.: Pharmakologische Gesichtspunkte bei vergleichenden Untersuchungen mit verschiedenen blutzuckersenkenden Sulphonylharnstoffen. Arzneim. Forsch., **23**, 1668–1673 (1973).
7. Beyer, J., Hopp, J., Schöffling, K.: Klinisch-experimentelle Ergebnisse mit GLURENORM im Vergleich zu anderen Sulphonylharnstoffen. in: Glurenorm, Pharmakokinetik und Pharmakodynamik, Dr. Karl Thomae GmbH, Biberach an der Riss, pp. 195–210 (1975).
8. Borthwick, L. J. and Wilson, S.: Diabetic control with gliquidone – a short acting sulphonylurea. European J. Clin. Pharmacol., **26**, 475–479 (1984).
9. Boyd, A. E. III., Aguilar-Bryan, L., Nelson, D. A.: Molecular mechanisms of action of glyburide on the beta cell. Am. J. Med., **89** (Suppl. 2A) 3S–10S (1990).
10. Campbell, D. B., Adriaenssens, P., Hopkins, Y. W. and Gordon, B.: Pharmacokinetics and metabolism of gliclazide: A Review. in: Gliclazide and the Treatment of Diabetes, eds H. Keen, A. D. S. Caldwell, Moira Murphy and C. Bowker, (Acad. Press London) pp. 142–187 (1980).
11. Cerasi, E.: The mode of actions of sulphonylureas: A comparative study of the acute effects of gliquidone and glibenclamide in normal man, subjects with low insulin response, and mildly

diabetic patients. in: Glurenorm, Pharmakokinetik und Pharmakodynamik, Dr. Karl Thomae GmbH, Biberach an der Riss, pp. 173–194 (1975).
12. Cerasi, E., Efendic, S., Thornqvist, D. and Luft, R.: Effect of two sulphonylureas on the dose kinetics of glucose-induced insulin release in normal and diabetic subjects. Acta Endocrinol., **91**, 282–293 (1979).
13. Christ, O. E., Heptner, W. and Rupp, W.: Investigations on absorption, excretion and metabolism in man after administration of ^{14}C-labelled HB 419. Metab. Res., **1** (Suppl.) 51–54 (1969).
14. Davidson, M. B., Sladen, G.: Effect of glyburide on glycogen metabolism in cultured rat hepatocytes. Metabolism, **36**, 925–930 (1987).
15. Debeer, L., Mannaerts, G., de Schepper, P. J.: Metabolic effects of hypoglycaemic sulphonylureas V. In vitro effects of sulphonylureas on mitochondrial adenosine triphosphatase activity. Biochem. Pharmacol., **23**, 251–258 (1974).
16. Del Prato, S., De Kreutzenberg, S. V.: Hepatic sensitivity to insulin: effects of sulphonylurea drugs. Amer. J. Med., **90**, 6A–29S–36S (1991).
17. Eleig, W. E., Noether-Fleig, G., Fussgaenger, R., Ditschuneit, H.: Modulation by a sulphonylurea of insulin-dependent glycogenesis, but not of insulin binding, in cultured rat hepatocytes. Diabetes, **33**, 285–290 (1984).
18. Feinglos, M. N., Lebovitz, H. E.: Sulphonylureas increase the number of insulin receptors. Nature, **276**, 184–185 (1978).
19. Garrino, M. G., Meissner, H-P. and Henquin, J-C.: The non-sulphonylurea moiety of gliquidone mimics the effects of the parent molecule on pancreatic B-cell. Eur. J. Pharmacol., **124**, 309–316 (1986).
20. Gerich, J. E.: Oral hypoglycaemic agents. New Engl. J. Med., **821**, 1231–1243 (1989).
21. Groop, L., Wahlin-Boll, E., Groop, P-H. et al.: Pharmacokinetics and metabolice effects of glibenclamide and glipizide in Type 2 diabetics. Eur. J. Clin. Pharmacol., **28**, 697–704 (1985).
22. Haupt, E., Köberich, W., Cordes, U., Beyer, J., Schöffling, K.: Untersuchungen zu Dosis-Wirkungs-Relationen verschiedener Sulphonylharnstoffderivate der alten und neuen Generation. Arzneim. Forsch., **22**, 2203–2208 (1972).
23. Hue, L., Sherratt, H. S. A.: Inhibition of gluconeogenesis in the rat. Evidence for inhibition of glucose-6-phosphatase. Biochem. J., **240**, 765–769 (1986).
24. Kaku, K., Matsuda, M., Matsutani, A., Kaneko, T.: Effect of tolbutamide on fructose-6-phosphate, 2-kinase and fructose-2,6-bisphosphatase in rat liver. Biochem. Biophys. Res. Commun., **139**, 687–692 (1986).
25. Kaneko, T., Kaku, K., Matsutani, A., Matsuda, M.: Pancreatic and exptrapancreatic action of sulphonylurea. in: Diabetes 1988, eds Larkins, R. G., Zimmet, P. Z., Chisholm, D. J., Excerpta Medica, Amsterdam, New York, Oxford, pp. 349–352 (1989).
26. Kolterman, O. G.: The impact of sulphonylureas on hepatic glucose metabolism in type II diabetes. Diabetes Metab. Rev., **3**, 399–414 (1987).
27. Kolterman, O. G., Gray, R. S., Shapiro, G., Scarlett, J. A., Griffin, J., Olefsky, J. M.: The acute and chronic effects of sulphonylurea therapy in type II diabetic subjects. Diabetes, **33**, 346–354 (1984).
28. Kopitar, Z., Koss, F. V.: Pharmakokinetisches Verhalten von ARDF 26, einem neuen Sulphonylharnstoff. in: Glurenorm, Pharmakokinetik und Pharmakodynamik, Dr. Karl Thomae GmbH, Biberach an der Riss, pp. 37–55 (1975).
29. Lebovitz, H. E.: Oral hypoglycaemic agents. in: The Diabetes Annual/1, eds Alberti, K. G. M. M., Krall, L. P., Elsevier, Amsterdam, New York, Oxford, pp. 93–110 (1985).
30. Lebovitz, H. E., Feinglos, M. N.: Sulphonylurea drugs: mechanism of antidiabetic action and therapeutic usefulness. Diabetes Care, **1**, 189–198 (1978).
31. Luft, R., Waingot, R., Efendic, S.: On the pathogenesis of maturity-onset diabetes. Diabetes Care, **4**, 58–63 (1981).
32. Mannaerts, G., Debeer, L., Ceuterick-Goethald, A. M., de Schepper, P. J.: Metabolic effects of hypoglycaemic sulphonylureas IV. Interference of sulphonylureas with mitochondrial oxidative phosphorylation. Biochem Pharmacol., **23**, 239–250 (1974).
33. Matsutani, A., Kaku, K., Kaneko, T.: Tolbutamide stimulates fructose-2,6-bisphosphate formation in perfused rat liver. Diabetes, **33**, 495–498 (1984).
34. McGuinness, D. P., Cherrington, A. D.: Effect of glyburide on hepatic glucose metabolism. Amer J. Med., **89** (Suppl. 24), 2A–26S–2A–37S (1990).

35. Monge, L., Mojena, M., Ortega, J. L. et al.: Chlorpropamide raises fructose-2,6-bis-phosphate concentration and inhibits gluconeogenesis in isolated rat hepatocytes. Diabetes, **35**, 89–96 (1986).
36. Osswald, H.: Renaler Ausscheidungmechanismus des GLURENORM-Wirkstoffes. in: Glurenorm, Pharmakokinetik und Pharmakodynamik, Dr. Karl Thomae GmbH, Biberach an der Riss. pp. 113–123 (1975).
37. Pedersen, O., Hother-Nielsen, O., Bak, J., Hjollund, E., Beck-Nielsen, H.: Effects of sulphonylureas on adipocyte and skeletal muscle insulin action in patients with non-insulin-dependent diabetes mellitus. Am. J. Med., **90**, 6A 22S–28S (1991).
38. Rinninger, F., Kemmler, W., Mehnert, H.: Extrapankreatische Wirkung des Sulphonylharnstoffpräparates Gliquidon: Verstärkung der insulinstimulierten Glykogensynthese an Hepatozyten der Ratte in Primärkultur über einen Postrezeptormechanismus. Verh. Dtsch. Ges. Inn. Med., **89**, 733–735 (1983).
39. Rinninger, F., Kirsch, D., Haring, H. U. and Kemmler, W.: Extrapancreatic action of the sulphonylurea gliquidone: post-receptor effect on insulin-stimulated glycogen synthesis in rat hepatocytes in primary culture. Diabetologia, **26**, 462–465 (1984).
40. Rogers, B. J., Standaert, M. L., Pollet, R. J.: Direct effect of sulphonylurea agents on glucose transport in the BC3H-1 myocyte. Diabetes, **36**, 1292–1296 (1987).
41. Salhanick, A. I., Konowitz, P., Amatruda, J. M.: Potentiation of insulin action by a sulphonylurea in primary cultures of hepatocytes from normal and diabetic rats. Diabetes, **32**, 206–212 (1983).
42. Schmid-Antomarchi, H., De Weille, J., Fosset, M., Lazdunski, M.: The Receptor for Antidiabetic Sulphonylureas Controls the Activity of the ATP-modulated K^+ Channel in Insulin-secreting Cells. J. Biol. Chem., **262**, 15840–15844 (1987).
43. Schmidt, F. H., Stork, H., Bander, A. and Pfaff, W.: Concerning the Pharmacodynamics of HB 419. Horm. Metab. Res., **1** (Suppl.), 25–33 (1969).
44. Schöffling, K., Haupt, E., Köberich, W., Beyer, J.: Neuere Erkenntnisse bei der Anwendung von blutzuckersenkenden Sulphonamiden der ersten und zweiten Generation. Klin. Wschr., **51**, 68–77 (1973).
45. Somogyi, J., Vér, Á., Trója, G., Végh, E., Bühler, C., Hatfaludi, F., Csermely, P., Popovic, S.: Interference of the sulphonylurea antidiabeticum gliquidone with mitochondrial bioenergetics in the rat under in vitro conditions. Acta Physiol. Hung., **83**, 299-312 (1995).
46. Somogyi, J., Vincze, I., Willig, F. and Schmidt, F. H.: The subcellular distribution of ^{14}C-glibenclamide in different tissues of rat. Horm. Metab. Res., **6**, 181–183 (1974).
47. Taylor, R., Agius, L.: The biochemistry of diabetes. Biochem. J., **250**, 625–640 (1988).
48. Zilker, Th., Ermler, R., Henderkott, U., Bottermann, P.: Untersuchungen zum Wirkungsmechanismus von Gliquidon. Dtsch. med. Wschr., **100**, 1733–1741 (1975).
49. Zuber, M. X., Wnag, S. M., Thammavaram, V., Reed, D. K., Reed, B. C.: Elevation of the number of cell-surface insulin receptors and the rate of 2-deoxyglucose uptake by exposure of 3T3-L1. J. Biol. Chem., **260**, 14045–14052 (1985).

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Effect of streptozotocin-induced diabetes on kidney Na^+/K^+-ATPase[1]

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The maximal capacity of low affinity ouabain binding sites in kidney medulla was found to be increased by 20 ± 3.8% after 2 weeks, and by 35 ± 4.5% in 4 weeks diabetes. However, in kidney cortex no similar changes could be detected. Western blot analysis of Na^+/K^+-ATPase subunits in kidney medulla indicated a significant enhancement of both the α_1 and β_1 subunit in two and four weeks diabetic rats (α_1: 35 ± 3.1, 51 ± 5.8% and β_1: 31.3 ± 5.2 and 43.2 ± 6.8%, respectively). However, kidney cortex showed no significant change in any condition tested. In diabetes we could detect a significant change only in the medulla in case of the b subunit mRNA transcript, which showed 1.69 ± 0.59 and 2.89 ± 0.81 times increased in two and four weeks diabetic state, respectively. There was no change in the α_1 subunit mRNA abundance. Insulin treatment of diabetic animals did not result in a complete reversal of diabetes-induced changes in ouabain binding capacity or in the amount of Na^+/K^+-ATPase α_1 and β_1 subunit protein and mRNA levels. Our data indicate a good correlation between changes in low affinity ouabain binding capacity and the level of α_1 isoform in diabetic rats, and suggest an important role of the b subunit in the regulation of enzyme quantity.

Keywords: streptozotocin-induced diabetes, Na^+/K^+-ATPase, α_1 and β_1 subunit, mRNA, kidney

Human and experimental diabetes is accompanied by an early, prominent and persistent renal hypertrophy [6, 22]. However, there are some evidence in experimental animal models of diabetes that early insulin treatment can prevent or reverse renal hypertrophy [15]. Nephromegaly has been associated with a renal hyperfunction (increased glomerular filtration rate of Na^+ and water reabsorption,

[1]This paper is dedicated to the memory of Professor Tibor Kovács (1929–1994)

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increased Na^+/H^+ exchange and Na^+/K^+-ATPase-activity) [6, 14, 31]. The increased activity of Na^+-pump can be considered as a biochemical sign of renal hypertrophy [15]. This phenomenon represents a paradoxical response of kidney to the lack of insulin compared to other tissues [13–15].

Na^+/K^+-ATPase is a transmembrane enzyme primarily responsible for the active transport of sodium and potassium in mammalian cells [25, 28]. The enzyme exists as a heterodimer, there are three isoforms of the catalytic alpha (α) subunit and at least two of the beta (β) chain [29]. The alpha isoforms differ from each other in several biochemical and physiological characteristics and display a cell and tissue specific distribution [19, 29]. The α_1 isoform is widely distributed in all tissues, while α_2 and α_3 expression is mostly limited to excitable cells [12, 21, 23]. The distribution of β subunit isoforms in different cells is less well characterized. β_1 has a broad distribution, while β_2 is localized mostly to glia [24]. The significance of the tissue and cell specific distribution of the subunit isoforms is not known, but evidence is slowly accumulating to suggest that α isoforms may be differentially regulated by physiological and pathological stimuli [2, 10, 19].

Some data show that Na^+/K^+-ATPase isoform ratio changes during development in brain and heart, furthermore certain hormones alter both isoform ratio and isoform abundance [8, 9, 20]. It has been demonstrated that insulin stimulates Na^+/K^+-ATPase activity in a variety of tissues [8, 9, 17, 32].

In rats with experimental and spontaneous diabetes mellitus Na^+/K^+-ATPase activity and maximal ouabain binding capacity have been reported to be decreased in heart, retina muscle and kidney glomeruli but to be increased in kidney tubules and intestines [3, 4, 13, 27, 30]. The aim of this study was to investigate whether the change in P_i facilitated ouabain binding is the consequence of changes in enzyme protein abundance in kidney cortex and medulla derived from normal and streptozotocin-induced diabetic rats with and without insulin treatment. We further examined whether the alteration in protein abundance correlates with the expression of Na^+/K^+-ATPase subunits' mRNAs.

Methods*

One group of non-diabetic male rats was used as control (C). 60 mg/kg i.v. streptozotocin (STZ) was given to an other group and diabetes was verified 24 h later by the appearance of hyperglycaemia and glucosuria. A group of the diabetic animals was sacrificed after two weeks (D_2), another group after 4 weeks (D_4) of STZ administration. In insulin replacement studies insulin was administered to diabetic rats in an individual dose to normalize blood glucose level 2 and 4 weeks after STZ injection for 2 weeks before the time of the assay (D_2R, D_4R) [30]. Kidneys were removed and the cortex and medulla layers were separated and stored at −80 °C until use for the assays. Crude microsomal fractions were prepared by

* **Abbreviations:** C, control rats; D_2, 2 weeks diabetic rats; D_4, 4 weeks diabetic rats; D_2R, 2 weeks diabetic + 2 weeks insulin reverted rats; D_4R, 4 weeks diabetic + 2 weeks insulin reverted rats; STZ, streptozotocin; Na^+/K^+-ATPase, Na^+ and K^+-dependent ATPase (EC3:6.1.37)

differential centrifugation [30]. Protein content was assayed by the method of Bradford using ovalbumin as standard [5]. (^{3}H)ouabain binding was measured as previously described [3, 18]. (^{3}H)ouabain (1.22 TBq/mmol) and unlabelled ouabain were applied in final concentrations of 2×10^{-8}–10^{-5} M (spec. act. 500–15 000 cpm/pmol). Nonspecific (^{3}H)ouabain binding was measured in the presence of 1 mM unlabelled ouabain. The samples were filtered through Whatman GFC filters. Filters were washed and radioactivity counted by liquid scintillation method. Maximal binding (B_{max}) expressed in picomol per milligram of protein, and the dissociation constant (K_d) expressed in nanomol or micromol per liter, were calculated according to the methods of Scatchard using a computer program ENZFITTER (version 1.05 EGA single ligand one binding site model, Elsevier-Biosoft, Cambridge, UK) [11]. Solubilized microsomal proteins were analyzed on 10% SDS polyacrylamide gels [16] and transferred to nitrocellulose sheets for Western blot analysis [23]. These membranes were incubated with isoform specific Na^+/K^+-ATPase antibodies (UBI, USA), and a peroxidase conjugated second antibody. The reaction was detected using an ECL Western blotting detection kit (Amersham), and the film negatives were analyzed by laser densitometry. Total cellular RNA was purified by the guanidium isothiocyanate method [7]. RNA electrophoresis, blotting and hybridization were performed according to general protocols [1]. Autoradiograms were analyzed by laser densitometry, and each peak of Na^+/K^+-ATPase gene transcript was related to the area of the ethidiumbromide stained 18S rRNA band. cDNA probes specific for α_1 and β isoforms were a generous gift of Dr. J. B. Lingrel (Univ. Cincinnati). For the statistical analysis a Student's *t*-test was used throughout this study to compare the differences in various parameters examined. The difference between the means was considered to be significant if $p < 0.05$. Data were analyzed by non-linear least squares procedure.

Results

All animals treated with STZ, developed hyperglycaemia on the following day (the blood glucose level of the animals was minimum 25 mM). Body weight of STZ-diabetic rats was significantly lower after 2 and 4 weeks diabetic state than that of the age-matched non-diabetic controls (Table I). The kidney shows a significant hyperplasia both in 2 and 4 weeks diabetes. The kidney/body weight ratio was also enhanced during diabetes and was not reversed on insulin treatment. The blood glucose levels of the STZ-diabetic groups were 3.7- and 4.2-fold higher than those of age-matched non-diabetic controls. Blood glucose was decreased close to the control level by insulin administration and a relative body weight gain was also observed during two weeks of insulin administration.

The basic characteristics of [^{3}H]ouabain binding to kidney cortex and medulla microsomes from control, diabetic and diabetic insulin-treated groups were assessed in a series of experiments. We defined relative [^{3}H]ouabain affinities over the concentration of 2×10^{-8} to 10^{-5} M to evaluate the binding sites and to determine the K_d values. In this concentration range a Scatchard type plot gives one population of binding sites with apparent dissociation constant in the control group ($K_d = 52.4 \pm 5.9$, 59.6 ± 4.8 μmol in the case of kidney cortex and medulla, respectively). Maximal [^{3}H]ouabain binding capacity of the kidney medulla layer was increased in STZ-induced diabetes compared to the control and to the insulin-treated diabetic animals. Changes in ouabain binding capacity proved to be significant in kidney medulla of both two- and four-week diabetic animals. The enhancement in

ouabain binding was partly reversed by insulin treatment. The K_d values did not change significantly (Table II). No significant changes were detected in kidney cortex between the control, two- and four-week diabetic as well as insulin-treated diabetic groups.

Na^+/K^+-ATPase isoforms. Immunoblotting with anti-α_1, α_2 and α_3 as well as β_1 and β_2 monospecific antibodies were performed on protein from kidney cortex and medulla microsomal membrane prepared from control and diabetic rats. We have found α_1 and β_1 subunits but not α_2, α_3 and β_2 isoforms in the cortex and medulla layers. The sensitivity of the antibody probes for α_2 and α_3 as well as β_2 was checked on immunoblots containing different amounts of partially purified brain (α_1, α_2, α_3, β_1 and β_2), heart (α_1 and α_2 β_1 and β_2), liver and kidney (α_1 and β_1) Na^+/K^+-ATPase preparations. Anti-α_2 and anti-α_3 antibodies did not cross react with the α_1 subunit in liver and kidney preparations (not shown). Isoenzyme specificity of the kidney did not change after streptozotocin and/or insulin treatment. Figure 1A shows a representative Western blot of α_1 and β_1 subunits of kidney medulla, while panel B contains the results of the densitometric analysis of all the data in kidney medulla and cortex.

Table I

Body and kidney weight and blood glucose levels in control, STZ-diabetic and insulin-treated STZ-diabetic rats

Groups (n)	C (17)	D_2 (13)	D_4 (16)	D_2R (14)	D_4R (14)
Body weight (g)					
Initial	190 ± 24	196 ± 18	218 ± 20	187 ± 19	198 ± 20
2 weeks	211 ± 24	176 ± 18*	196 ± 19*	167 ± 15*	178 ± 15*♦
4 weeks	227 ± 24	----	183 ± 19.8*♦	181 ± 20*♦	166 ± 18*♦
6 weeks	236 ± 24	----	----	----	188 ± 21*♦
Kidney weight (g)	1.25	1.52*	1.49*	1.42*	1.45*
Kidney/body	0.0053	0.0086*	0.0081*	0.0079*	0.0077*
Glucose (mM)	7.8 ± 1.5	28.7 ± 3.5*♦	32.8 ± 3.6*♦	8.8 ± 2.6♦	9.8 ± 2.5♦

Experimental diabetes was induced by streptozotocin injection (60 mg/kg body weight i.v.). Diabetic rats were killed either after 2 weeks (D_2 group) or after 4 weeks (D_4 group). Insulin treatment was started after 2 or 4 weeks of STZ administration (groups D_2R, D_4R, respectively) and continued for 2 weeks as described in Materials and Methods. Data are means ± SE, n = number of experimental animals. Statistically significant ($p < 0.05$) differences between the age-matched controls and either STZ-diabetic or insulin-treated STZ-diabetic rats are denoted by *. Statistically significant ($p < 0.05$) differences between STZ-diabetic (D_2 and D_4) and insulin-treated STZ diabetic (D_2R, D_4R) rats are denoted by ♦

Table II

Maximal ouabain binding capacity and K_d values of kidney cortex and medulla microsomes from control, STZ-diabetic and insulin-treated STZ-diabetic rats

Groups	Kidney cortex		Kidney medulla	
	K_d (μmol)	B_{max} (pmol/mg protein)	K_d (μmol)	B_{max} (pmol/mg protein)
C (n = 16)	52.4 ± 5.9	43.1 ± 4.3	59.3 ± 8.8	35.9 ± 4.3
D_2 (n = 6)	54.6 ± 5.9	39.1 ± 4.9	61.3 ± 5.3	43.3 ± 3.9*•
D_4 (n = 7)	50.6 ± 9.7	41.2 ± 3.9	57.7 ± 7.2	48.6 ± 3.6*••
D_2R (n = 5)	49.9 ± 8.3	43.8 ± 4.9	64.6 ± 7.8	37.7 ± 7.2•
D_4R (n = 5)	47.2 ± 8.3	44.8 ± 4.2	62.5 ± 8.3	41.9 ± 4.6*•

Maximal ouabain binding capacity (B_{max}) of kidney cortex and medulla microsomes was determined as described in Section 2. Values are means ± SE, n = number of experiment. The B_{max} and K_d values were the same in the two, four and six weeks control groups, therefore C denotes all the age-matched control groups. Statistically significant ($p < 0.05$) differences between control and STZ-diabetic (D_2, D_4) are denoted by *. Statistically significant ($p < 0.05$) differences between control and insulin-treated STZ-diabetic (D_2R, D_4R) rats are denoted by •. Statistically significant ($p < 0.05$) differences between STZ-diabetic (D) and insulin-treated STZ-diabetic (DR) rats are denoted by •

After two weeks of streptozotocin treatment there was a significant (35 ± 3.1%, $p < 0.05$) increase in the amount of the a1 subunit and a 31 ± 5.2% ($p < 0.05$) increase in the case of β_1 subunit in kidney medulla. The enhancement of the amount of α_1 and β_1 subunit proteins was more predominant in the four-week diabetic state (51 ± 5.8% and 3.2 ± 6.8%, respectively $p < 0.01$). Insulin treatment decreased the amount of both subunits in two-week diabetic animals and restored the relative abundance of both α_1 and β_1 subunits to the control level in case of four-week diabetes. The relative amount of α_1 and β_1 isoform did not change significantly in cortex in any conditions tested.

Northern blots. To determine whether the increase in ouabain binding capacity and α_1 and β_1 subunit abundances seen in diabetes are associated with increases in mRNA transcripts encoding the subunits, total cellular RNA was probed with ^{32}P labelled isoform specific cDNAs. As shown in Fig. 2 we found differences in the isoform specific mRNA transcription between cortex and medulla. In diabetes the only significant change could be detected in the medulla in case of the β isoform transcript, which showed a 169 ± 35.8% and 289 ± 43.1% ($p < 0.05$) increase in the two- and four-week diabetic state compared to the control, respectively. Insulin administration could totally (D_2) or partially (D_4) reverse the alterations observed. There was no difference in α_1 subunit mRNA abundance in any state of diabetes. The α_1 mRNA transcription was not significantly affected.

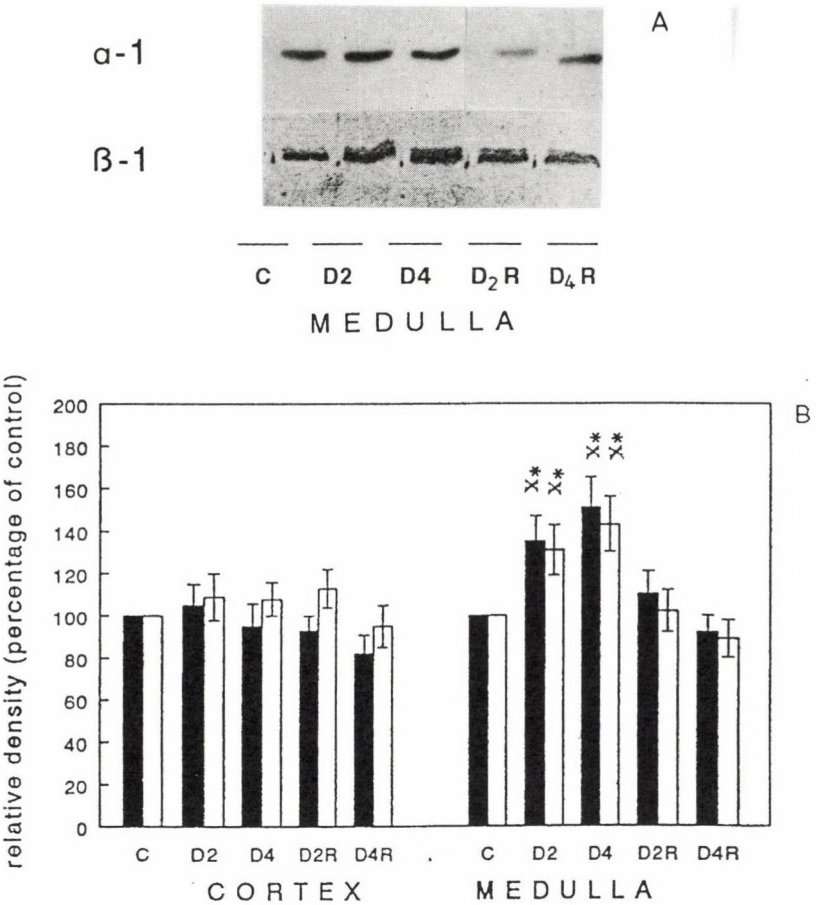

Fig. 1. Abundance of Na^+/K^+-ATPase α_1 β_1 subunit isoforms in kidney of control, STZ-diabetic insulin-treated rats. Microsomal membranes (50 μg) of kidney cortex and medulla from control (C), 2 weeks STZ-diabetic rats without (D_2) and with insulin treatment (D_2R), 4 weeks STZ-diabetic rats without (D_4) and with insulin treatment (D_4R) were subjected to SDS-PAGE. Relative abundance of α_1 and β_1 were quantitated by immunoblotting using isoform specific antisera and peroxidase conjugated antirabbit IgG as primary and secondary antibodies, respectively. A: a representative photo of an ECL developed Western blot of α_1 and β_1 subunit isoform detected in kidney medulla. B: relative densities of Na^+/K^+-ATPase α_1 and β_1 subunit isoform in kidney medulla and cortex. Closed bars: α_1, open bars: β_1. Data are means $\pm$ SE of 5 experiments. Statistically significant ($p < 0.05$) differences between controls and either STZ-diabetic or insulin-treated STZ-diabetic rats are denoted by *. Statistically significant ($p < 0.05$) differences between STZ-diabetic and insulin-treated STZ-diabetic rats are denoted by x

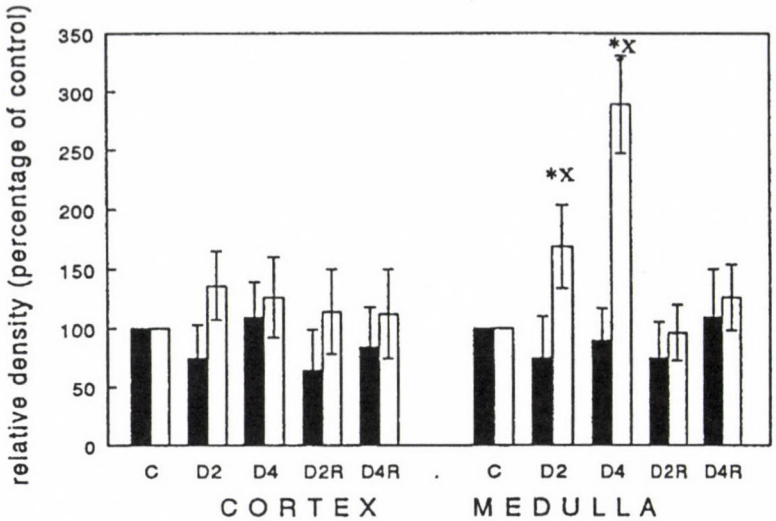

Fig. 2. Densitometric analysis of Northern blot of Na^+/K^+-ATPase α_1 and β subunit mRNAs prepared from kidney cortex and medulla of control, STZ-diabetic and STZ-diabetic insulin-treated rats. Relative densities of Na^+/K^+-ATPase α_1 and β subunit isoform. Closed bars: α_1, open bars: β_1. Data are means $\pm$ SE of 5 experiments. Statistically significant ($p < 0.05$) differences between controls and either STZ-diabetic or insulin-treated STZ-diabetic rats are denoted by *. Statistically significant ($p < 0.05$) differences between STZ-diabetic and insulin-treted STZ-diabetic rats are denoted by x

Discussion

Streptozotocin-induced diabetes in rats results in the increase in kidney mass. Kidney weight gain developes within the first two weeks of STZ-caused experimental diabetes. The main histological characteristic of this state is medullar hyperplasia [6]. The Na^+/K^+-ATPase activity is concomitantly increased [14] and as our experiments proved so is the maximal ouabain binding capacity. The α subunit of Na^+/K^+-ATPase is responsible for ouabain binding, and the different isoforms of α subunit show different ouabain binding affinities [29]. There are only one low affinity ouabain binding isoform (α_1) and two high affinity ouabain binding isoform (α_2 and α_3) [12, 29]. According to our results in agreement with the earlier data in kidney cortex and medulla only the low affinity binding sites (α_1 isoform) are present. In kidney cortex the number of ouabain binding sites did not change significantly in diabetes, however, there is a significant enhancement in kidney medulla.

The question arises whether the changes in ouabain binding capacity in diabetes is due to alterations of biochemical properties of the enzyme or derive from the quantitative changes of the transport molecule. To evaluate the expression of Na^+/K^+-ATPase isoforms, antisera specific for α_1, α_2, α_3, β_1, and β_2 subunits were used to probe Western blots of kidney cortex and medulla microsomes prepared from control,

STZ-treated diabetic and STZ-treated insulin injected rats. The major changes were observed in kidney medulla, while the cortex in accordance with the histological findings turned to be less affected. We could detect a concomitant increase in both α_1 and β_1 protein level in diabetic states while was could only prove a parallel change in β mRNA level. Ouabain binding data confirm our results concerning the α_1 protein level. There is other report showing that the synthesis and degradation rate of α_1 isoform are enhanced in the nervous system of spontaneously diabetic rats [27]. In contrast to other tissues, where $\alpha_2\beta_1$ isoform composition was regulated by insulin [17], our results show that $\alpha_1\beta_1$ isoform level changes in diabetic kidney medulla and is affected by insulin similarly as we reported on brain cortex [26, 30].

The question is whether the observed alterations in experimental diabetes are derived from the absence of insulin, from the relative overflow of glucagon and other hormones or from other metabolic disorders is not resolved in this study. From our data we concluded that the activity changes of Na^+/K^+-ATPase as well as the alteration of ouabain binding capacity in experimental diabetes and in case of insulin administration is a result of the change of protein amount. The mRNA transcription showed a good correlation with the β but not with the α_1 protein subunit. There are also data showing a prolonged latency of changes in the synthesis of Na^+/K^+-ATPase catalytic subunit after insulin administration [27], and these results suggest that the regulation of Na^+/K^+-ATPase activity in diabetes does not directly take place through insulin response elements in case of α subunit. However, these are the first data concerning the changes in expression of β subunit, which is supposed to have a regulatory function in the enzyme activity.

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REFERENCES

1. Asubel, F. M., Brent, R., Ekingston, R., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K.: Analysis of RNA by Northern hybridization. In: Ausubel, F. M., Brent, R., Ekingston, R., Moore, D. D., Seidman, J. G.: Current Protocols in Molecular Biology. Ausubel, F. M. et al. Wiley, I and Sons, Chichester, New York (1987), 4.9.1.–4.9.8.
2. Azuma, K. K., Hensley, C. B., Putnam, D. S., McDonough, A. A.: Hypokalemia decreases Na^+/K^+-ATPase α_2 but not α_1-isoform abundance in heart, muscle and brain. Am. J. Physiol., **260**, C958–964 (1991).
3. Bányász, T., Kovács, T., Somogyi, J.: Alteration in sarcolemmal Na^+/K^+-ATPase activity in heart ventricular muscle during diabetes. Eur. Heart J., **9**, Suppl. (1988), pp. 70.
4. Barada, K., Okolo, C., Field, M., Cortas, N.: Na^+/K^+-ATPase in diabetic rat small intestine. J. Clin. Invest., **93**, 2725–2731 (1994).

5. Bradford, M. M.: A rapid sensitive method for quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem., **72**, 248–254 (1976).
6. Brown, D. M., Andres, G. A., Hostetter, T. H., Mauer, S. M., Price, R., Venkatachalam, M. A.: Kidney complications. Diabetes (Suppl. 1), **31**, 71–76 (1982).
7. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., Rutter, W. J.: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, **18**, 5294–5299 (1979).
8. Geering, K.: Subunit assembly and posttranslational processing of Na^+-pump. Acta Physiol. Scand., **146**, 177–181 (1992).
9. Herrera, V. L. M., Emanuel, J. R., Ruiz-Opazo, N.: Isoform specific modulation of Na^+/K^+-ATPase alpha subunit gene expression in hypertension. Science, **241**, 221–223 (1988).
10. Ikeda, U., Hyman, R., Smith, T., Medford, R. M.: Aldosteron-mediated regulation of Na^+/K^+-ATPase gene expression in adult and neonatal cardiocytes. J. Biol. Chem., **266**, 12058–12066 (1991).
11. Jensen, J.: Heterogenity of pig kidney Na, K-ATPase as indicated by ADP- and ouabain-binding stoichiometry. Biochim. Biophys. Acta, **1110**, 81–87 (1992).
12. Jewell, E. A., Shamraj, O. I., Lingrel, J. B.: Isoform of the α subunit of Na, K-ATPase and their significance. Acta Physiol. Scand., **146**, 161–166 (1992).
13. Kjallsen, K., Braendgaard, H., Sidenius, P., Stenfatt, J., Larsen, S., Norgaard, A.: Diabetes decreases Na^+/K^+-pump concentration in skeletal muscles, heart ventricular muscles and peripheral nerves of rat. Diabetes, **36**, 842–848 (1987).
14. Ku, D. D., Sellers, B. M., Meezan, E.: Development of renal hypertrophy and increased renal Na^+/K^+-ATPase in streptozotocin-diabetic rats. Endocrinology, **119**, 672–679 (1986).
15. Ku, D. D., Roberts, R. B., Sellers, B. M., Meezan, E.: Regression of renal hypertrophy and elevated renal Na^+/K^+-ATPase activity after insulin treatment in streptozotocin-diabetic rats. Endocrinology, **120**, 2166–2173 (1987).
16. Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature, **227**, 680–686 (1970).
17. McGill, D. L., Guidotti, G.: Insulin stimulates both the α_1 and the α_2 isoform of the rat adipocyte Na^+/K^+-ATPase. J. Biol. Chem., **266**, 15824–15831 (1991).
18. Norgaard, A., Kjeldsen, K., Hansen, O., Clausen, T.: A simple rapid method for the determination of the number of ^3H-ouabain binding sites in biopsies of skeletal muscle. Biochem. Biophys. Res. Commun., **111**, 319–325 (1983).
19. Ng, Y. C., Yao, A. Z., Akera, T.: Tissue-specific isoform regulation of Na^+/K^+-ATPase by thyroid hormone in ferrets. Am. J. Physiol., **257**, H534–H539 ((1989).
20. O'Brien, M., Granner, D. K.: Regulation of gene expression by insulin. Biochem. J., **278**, 601–606 (1991).
21. Orlowski, J., Lingrel, J. B.: Tissue-specific and developmental regulation of rat Na^+/K^+-ATPase catalytic α isoform and β subunit mRNAs. J. Biol. Chem., **263**, 10436–10442 (1988).
22. Seyer-Hansen, K.: Renal hypertrophy in streptozotocin-diabetic rats. Clin. Sci. Mol. Med., **51**, 551–556 (1976).
23. Shyjan, A. W., Levenson, R.: Antisera specific for alpha1, alpha2, alpha3 and beta subunits of Na^+/K^+-ATPase: Differential expression of alpha and beta subunits in rat tissue membrane. Biochemistry, **28**, 4531–4535 (1989).
24. Shyjan, A. W., Gottardi, C., Levenson, R.: The Na^+/K^+-ATPase β_2 subunit is expressed in rat brain and copurified with Na^+/K^+-ATPase activity. J. Biol. Chem., **265**, 5166–5169 (1990).
25. Skou, J. C.: Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. Physiol. Rev., **45**, 596–617 (1965).
26. Somogyi, J., Vér, Á., Szántó, I., Csermely, P., Kalapos, I., Bányász, T., Kovács, T.: Alterations in Na^+/K^+-ATPase isoforms of different tissues in streptozotocin induced diabetes mellitus. in: The

Sodium Pump Structure, Mechanism, Hormonal Control and its Role of Disease (Eds: Bamberg, E., Schoner, W.) Darmstadt Steinkopff; New York: Springer (1994) pp. 876–879.

27. Specht, S. C., Martin, J., Gaud, R. E., and De Hoyos, J.: Molecular isoform of Na^+/K^+-ATPase in the nervous system and kidney of the spontaneously diabetic BB/Wor rat. Biochim. Biophys. Acta, **1118**, 77–82 (1991).
28. Sweadner, K. J.: Purification from brain of an intrinsic membrane protein fraction enriched in Na^+/K^+-ATPase. Biochim. Biophys. Acta, **508**, 486–499 (1978).
29. Sweadner, K. J.: Isoenzymes of the Na^+/K^+-ATPase. Biochim. Biophys. Acta, 185–220 (1989).
30. Vér, Á., Csermely, P., Bányász, T., Kovács, T., Somogyi, J.: Alterations in properties and isoform ratios of brain Na^+/K^+-ATPase in streptozotocin diabetic rats. Biochim. Biophys. Acta, **1237**, 143–150 (1995).
31. Wald, H., Popovtzer, M. M.: The effect of streptozotocin induced diabetes mellitus on urinary excretion of sodium and renal Na^+/K^+-ATPase activity. Pfluegers Arch., **401**, 97–107 (1984).
32. Yuk-Chow, N. G., Tolerico, P. H. and Book, C. B. S.: Alterations in the level of Na^+/K^+-ATPase isoform in heart, skeletal muscle, and kidney of diabetic rats. Am. J. Physiol., **265**, E243–E251 (1993).

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Changes of the 78 kDa glucose-regulated protein (grp78) in livers of diabetic rats#

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The 78 kDa glucose-regulated protein (grp78) is an abundant member of the 70 kDa molecular chaperone family in the lumen of the endoplasmic reticulum participating in the quality control of secretory proteins. In the present paper we have analysed the synthesis and level of grp78 in livers of control, streptozotocin-diabetic, and the spontaneously diabetic Zucker rats. The level of grp78 mRNA significantly decreased in streptozotocin-diabetic rats. The effect was reversed by insulin treatment. In case of Zucker rats we did not detect any significant change in grp78 mRNA, grp78 protein level showed opposite changes being essentially unchanged in streptozotocin-diabetes and significantly reduced in Zucker rats. Autoradiograms of Ca-dependent phosphorylation of postmitochondrial supernatants of control and streptozotocin-diabetic livers indicated no significant changes in the 70 kDa region. Decrease in the availability of grp78 may participate in the attenuation of hepatic protein secretion in diabetes.

Keywords: diabetes, glucose regulated proteins, chaperones, heat shock proteins, insulin, protein phosphorylation

This paper is dedicated to the memory of Professor Tibor Kovács (1929–1994), who actively participated in the initiation of this project, and whose talent and spirit helped a lot to those who were privileged to know him.

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The 78 kDa glucose-regulated protein (grp78, BiP)* is an abundant member of the 70 kDa molecular chaperone family in the lumen of the endoplasmic reticulum. grp78 synthesis is induced after either glucose starvation or treatment with calcium ionophores. The protein was shown to bind the immunoglobulin heavy chain and its possible involvement in the quality control of secretory proteins was also suggested [13–16, 19, 25, 44].

Diminished extracellular glucose level induces the synthesis of numerous glucose-regulated proteins such as grp78, grp94 and GLUT-1 [13, 19, 44, 45]. Parfett et al. [27] reported a transient increase of grp78 mRNA in NOD mice. GLUT-1 mRNA as well as GLUT-1 protein levels were also reported to increase in diabetic animal models [39, 40]. In spite of the intimate link between changes in extracellular glucose level and the regulation of the synthesis of glucose regulated proteins, our knowledge of their function in diabetes is rather limited. As an initial attempt to characterize the effect of diabetes on molecular chaperones we analyzed the mRNA, protein levels of grp78, as well as its autophosphorylation in the liver of control and diabetic rats. Our results suggest that a decreased availability of grp78 may participate in the impaired protein secretion of diabetic livers.

Materials and Methods

Chemicals

The chemicals used for polyacrylamide gel electrophoresis were from Bio-Rad (Richmond, CA). Streptozotocin was purchased from Boehringer Mannheim (Germany). Anti-KSEKDEL and anti-CTGEEDTSEKDEL [38] antibodies (recognizing the endoplasmic reticulum retention signal present in grp78) were obtained from Stressgen (Victoria, B.C., Canada) and from Affinity BioReagents (Neshanic Station, NJ), respectively. The grp78 (clone p3C5) and β-actin (clone RβA-1) cDNA probes were generous gifts of drs. Amy S. Lee [20] and Laurence H. Kedes (both from the Univ. of Southern California, Los Angeles), respectively. Chicken blood DNA, SalI was a GIBCO BRL (Berlin, Germany) product. The Multiprime random oligonucleotide priming system, ECL immunodetection kit, [alpha-^{32}P]-dCTP (110 TBq/mmol) and [gamma-^{32}P]-ATP (185 TBq/mmol) were from Amersham Life Science (Braunschweig, Germany). All the other chemicals used were from Sigma Chemicals Co. (St. Louis, MO).

Animals

Eighteen weeks old (200–220 g) male Sprague–Dawley rats (LATI, Gödöllő, Hungary) were treated with 50 mg/kg i.p. streptozotocin under ether anaesthesia. A group of six diabetic rats was sacrificed 2 weeks, another 4 weeks after streptozotocin treatment, respectively. Six animals received daily insulin injections of an individual dose to normalize their blood glucose level for two weeks prior sacrification. The weight and blood glucose levels of the animals were monitored and showed characteristic changes of

* The abbreviations used are: dnaK, *Escherichia Coli* 70 kDa heat shock protein homologue; GLUT-1, glucose transporter; grp78, 78 kDa glucose-regulated protein (BiP); Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate.

diabetic and "reversed" state, respectively [43]. Twelve weeks old spontaneously diabetic male Zucker (fa/fa) and lean (fa/0) rats (IFFA-CREDO, l'Albresle, France) were also examined.

Measurement of grp78 mRNA levels

The recombinant pBR322 plasmid (clone p3C5) containing the grp78 specific 2.5 kbase long insert [20] was opened by SalI digestion for 60 minutes at 37 °C. The opened plasmid was labelled with [alpha-^{32}P]-dCTP by the random oligonucleotide priming system (Multiprime) of Amersham overnight at room temperature. The radiolabelled probe was separated on a Sephadex G-50 column. Total cellular RNA was isolated from rat livers by the method of Chirgwin et al. [7]. RNA was quantitated by measuring its absorbance at 260 nm. 20 μg of RNA was subjected to electrophoresis on a 1.2% agarose-formaldehyde gel and the gel was blotted onto Amersham Hybond-N nylon filters by capillary transfer. RNA blots were stabilized by heating in vacuum at 80 °C for 2 hours and by UV light transillumination for 15 min. The blots were prehybridized at 42 °C for 60 min in a mixture containing 5 × SSC (0.75 M NaCl, 75 mM sodium-citrate, pH 7.0), 5 × Denhardt solution (1 mg/ml each of bovine serum albumin, Ficoll 400 and poly-vinyl-pirrolidone), 50% deionized formamide and 1% SDS. After the addition of approximately 10^6 cpm of alpha-^{32}P-dCTP labelled grp78 specific cDNA probe and 200 μl/ml chicken blood DNA, hybridization was performed at 42 °C for 16 h. The filters were washed for 2 × 10 min at room temperature with 2 × SSC and 0.1% SDS, and for 2 × 5 min at 65 °C with 0.2 × SSC and 0.1% SDS. mRNA levels were quantitated by autoradiography and densitometry on an LKB-laserdensitometer. Results were normalized to the amount of β-actin mRNA. Full length rat β-actin cDNA probe was excised by BglI digestion from clone pRβA-1 and was used for hybridization utilizing a procedure identical to that of grp78.

Quantitation of grp78 and Ca-dependent phosphorylation

Livers of control and diabetic rats were homogenized in two volumes of a buffer containing 20 mM Hepes, pH 7.4, 30 mM KCl, 2 mM PMSF, 100 μg/ml aprotinin, 2 mM dithio-threitol and 1 mM EDTA. Postmitochondrial supernatants were obtained by sequential centrifugation of the samples at 2,000 × g, 10 min, 4 °C and 15,000 × g, 20 min, 4 °C, respectively. Protein concentration was determined by the Bradford method [3] using bovine gamma-globuline as a standard. 200 μg of proteins were subjected to SDS-PAGE [18] and transferred to nitrocellulose filters [42]. grp78 levels were analysed by probing the Western-blots with anti-grp 78 antibodies [38] recognizing the endoplasmic reticulum retention signal of grp78. Immunocomplexes with peroxidase-conjugated anti-rabbit antibodies were quantitated using the ECL immunodetection kit and by densitometry with an LKB laser-densitometer.

In phosphorylation assays 200 μg protein of hepatic postmitochondrial supernatant was incubated in 50 mM Hepes buffer, pH 7.4, at 37 °C for 20 minutes in the presence of 5 mM $CaCl_2$ and 400 kBq of 0.1 mM [gamma-^{32}P]-ATP. Reaction was stopped with boiling for 5 minutes in Laemmli sample buffer and samples were subjected to SDS-PAGE [18] and autoradiography.

Statistics

Data represent means ± SDs. Levels of significance were determined using the Student's *t*-test.

Results

Changes of grp78 mRNA in diabetic rats

Rat hepatic grp78 mRNA levels were significantly decreased both 2 weeks and 4 weeks after streptozotocin injection (to 47 and 38% of control level, respectively). Normalization of blood glucose level by 2 weeks insulin treatment completely reversed the effects observed. In contrast to the results in streptozotocin-diabetes, the spontaneously diabetic Zucker (fa/fa) rats did not show any significant decrease of hepatic grp78 mRNA (Fig. 1).

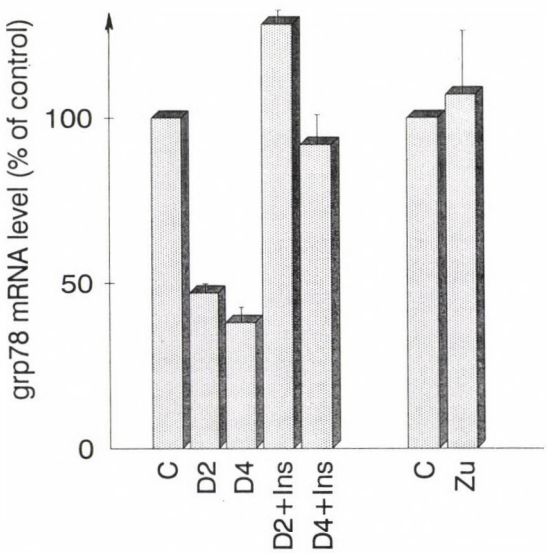

Fig. 1. Changes of grp78 mRNA in diabetic rats Hepatic grp78 mRNA levels were determined as described in Materials and Methods. Experimental groups: C, control; D2, two weeks of streptozotocin-diabetes; D4, four weeks of streptozotocin-diabetes; D2+Ins, two weeks of streptozotocin-diabetes + 2 weeks of insulin treatment; D4+Ins, four weeks of streptozotocin-diabetes + 2 weeks of insulin treatment; Zu, spontaneously diabetic Zucker (fa/fa) rats. Data are means ± SDs of the densitometric analysis of three autoradiograms of mRNA-s from two animals per each group compared to their respective controls

Changes of grp78 level in diabetic rats

Levels of hepatic immunodetectable grp78 were essentially unchanged in streptozotocin-diabetic rats and significantly ($p < 0.025$) diminished in spontaneously diabetic Zucker (fa/fa) rats (Fig. 2). In some samples from livers of Zucker rats more than one anti-KSEKDEL immunopositive lanes could be detected in the 70–80 kDa region, which may reflect an increased posttranslational modification (limited

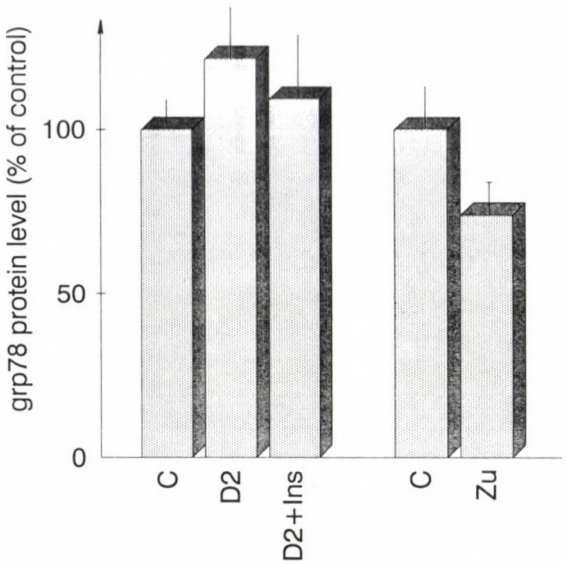

Fig. 2. Changes of grp78 level in diabetic rats grp78 levels were determined by subjecting 200 μg protein or rat liver postmitochondrial supernatant from control and diabetic animals to SDS-PAGE and Western-blot analysis as described in Materials and Methods. Western blots were probed with an anti-KSEKDEL antibody recognizing grp78. Experimental groups: C, control; D2, two weeks of streptozotocin-diabetes; D2+Ins, two weeks of streptozotocin-diabetes + 2 weeks of insulin treatment; Zu, spontaneously diabetic Zucker (fa/fa) rats. Data are means $\pm$ SDs of the densitometric analysis of three Western blots from five animals per each group

proteolysis?) of grp78 in diabetes. Similar grp78 levels could be detected using two antipeptide antibodies (anti-KSEKDEL and anti-CTGEEDTSEKDEL) against the C-terminus of grp78 (data not shown). In Zucker diabetic rats a 57 kDa anti-KSEKDEL immunopositive lane (detecting possibly protein-disulphide isomerase) was also diminished compared to the respective controls (to $31 \pm 18\%$ $p < 0.005$). The observed decrease noes not reflect an overall attenuation of protein levels of all molecular chaperones, since the levels of immunodetectable 27, 70 and 90 kDa heat shock proteins (hsp27, hsc70 and hsp90) did not change significantly in diabetic liver (data not shown).

Ca-dependent phosphorylation of postmitochondrial supernatant in control and diabetic rats

Ca-ATP-dependent phosphorylation is a sensitive tool to discriminate between the general (Mg-ATP-dependent) phosphorylation of proteins and the Ca-ATP-dependent (auto)phosphorylation of various molecular chaperones including hsp90 [10], grp94 [11] and grp78 [22]. Phosphorylation of postmitochondrial supernatants

from livers of control, streptozotocin-diabetic and insulin-treated diabetic rats in the presence of Ca-ATP did not reveal any major differences in the 70–80 kDa region (data not shown) which indicates no major changes in the autophosphorylation rate of hepatic grp78 in streptozotocin-diabetes.

Discussion

Exposure of cells to glucose starvation and calcium ionophores stimulates the synthesis of a specific set of proteins including grp78 [19]. However, the situation seems to be more complex, since dietary glucose restriction leads to a decrease in the mRNA of hepatic grp78 [36] and Parfett et al. [27] reported a transient increase of grp78 mRNA in spontaneously diabetic NOD mice. Our results show a significant decrease in grp78 mRNA in rat livers after streptozotocin treatment which was reversible after insulin treatment. Two and four week streptozotocin-diabetic rats may better correspond to the overt diabetic NOD mice, where the increase in hepatic grp78 mRNA has been already levelled off [27]. As a further example for the complex regulation of grp-genes in various models of diabetes, in case of Zucker (fa/fa) rats we found no significant change in grp78 mRNA compared to control rats. Heat shock is known to increase the stability of grp78 mRNA [32]. Different changes of grp78 mRNA stability may also contribute to the differences of grp78 mRNA levels in various models of diabetes.

Synthesis of grp78 mRNA is known to be mediated by an ER-resident protein kinase [9, 26]. The fact that protein kinase inhibitors were reported to prevent, and the phosphorprotein phosphatase inhibitor ocadaic acid promoted the induction of glucose regulated proteins [29, 30] also indicates the involvement of protein phosphorylation in the process. Protein kinases, and phosphatases such as the insulin receptor tyrosine kinase itself, are differently affected in various models of diabetes [17] which may indicate the involvement of a phosphorylation-dependent signalling step in changes of grp78 induction in diabetes.

grp94 mRNA was also decreased in livers of streptozotocin-diabetic rats.* The similar regulation of grp78 and grp94 mRNA in diabetes may reflect the high similarity in their promoter region [19, 23, 37].

grp78 protein levels showed opposite changes in spontaneously diabetic Zucker (fa/fa) and streptozotocin-diabetic rats than the corresponding mRNA levels. Decrease of grp78 in spontaneously diabetic Zucker (fa/fa) rats cannot be explained by a transcriptional defect, since the corresponding mRNA level was unchanged. Both increase [4] and decrease [35] of hepatic translation rates of various proteins have been reported in diabetes. Thus the observed changes may result from the impaired translation of the protein but may also reflect a shortening of the extraordinarily long

*P. Csermely, P. Gergely, I. Szántó, Z. Marcsek, T. Bányász and J. Somogyi, manuscript in preparation.

half-life (> 48 hours [14]) of grp78 in diabetes. Similar findings were reported in the case of hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase where the diabetic enzyme level has been decreased parallel with an unchanged mRNA level [8]. An increased susceptibility for proteolytic degradation may reflect a defect in the structure of the protein and may account for the decrease of grp78 protein levels in spontaneously diabetic Zucker (fa/fa) rats all the more, since grp78 mRNA uses both cap-dependent scanning and internal ribosome binding mechanisms for translation [24, 31] which makes a translational defect in this case less likely. On the contrary, the extraordinarily stable translational mechanism of grp78 (together with the possible lack of accelerated proteolysis) may account for the preservation of grp78 protein levels in spite of reduced mRNA in streptozotocin-induced diabetes.

An increase of malfolded proteins in the lumen of endoplasmic reticulum (which is supposed to occur in diabetes) should provoke an increase in the synthesis and level of glucose regulated proteins. However, grp synthesis – and glycosylation – may itself be influenced by diabetes, worsening the adaptation of hepatocytes to the presumably increased demand of the "quality control" of secretory proteins.

grp78 is known to phosphorylate itself [22]. The autophosphorylation of grp78 plays a role in the dissociation of grp78 from other proteins [5, 12, 41] in the analogy of similar effects on hsp60 [33] and hsp90 [21].* On the other hand, heat-induced phosphorylation of the hsp70 homologue, dnaK is reported to increase its binding to polypeptides [34]. Phosphorylation may impair the activity of grp78 [37]. In spite of these intimate links between the phosphorylation and activity of grp78, we did not observe any change in Ca-dependent (auto)phosphorylation in the 70 kDa region. This might reflect a true lack of detectable grp78 phosphorylation but fluctuations in overall grp78 levels may also obscure some minor changes in the phosphorylation of the protein in diabetes.

We have no direct information on the possible alterations of hepatic protein secretion in diabetes. Though the secretion of numerous proteins, such as albumin [28] and the VLDL constituent apolipoproteins B and E [35] are markedly decreased, these effects are largely attributed to defects of mRNA synthesis and translation, respectively. Glycoproteins seem to be less sensitive to diabetes-induced attenuation of protein secretion [2]. Studies on pancreatic protein secretion of streptozotocin-induced diabetic rats indicate an inhibition of amylase secretion and a higher amount of immunodetectable pancreatic lipase along the secretory pathway [1, 6].

These findings may indicate an increased retention of abnormal proteins by the endoplasmic reticulum. Reduced availability of grp78 may contribute to impaired functioning of the "quality control" mechanisms of the endoplasmic reticulum resulting in the accumulation of abnormal proteins in the luminal compartimentum.

* M.S.Z. Kellermayer and P. Csermely, BBRC, **211**, 166-174 (1995).

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REFERENCES

1. Bendayan, M. and Levy, E.: Immunocytochemical and biochemical evaluation of pancreatic lipase in acinar cells of control and streptozotocin-induced diabetic rats. Pancreas, **3**, 269–273 (1988).
2. Berry, E. M., Ziv, E. and Bar On, H.: Protein and glycoprotein synthesis and secretion by the diabetic liver. Diabetologia, **19**, 535–540 (1980).
3. Bradford, M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem., **72**, 248–254 (1976).
4. Brady, P. S. and Brady, L. J.: Hepatic carnitine palmitoyltransferase turnover and translation rates in fed, starved, streptozotocin-diabetic and diethylhexyl phthalate-treated rats. Biochem. J., **246**, 641–649 (1987).
5. Carlino, A., Toledo, H., Skaleris, D., DeLisio, R., Weissbach, H. and Brot, N.: Interactions of liver grp78 and *Escherichia coli* recombinant grp78 with ATP: multiple species and disaggregation. Proc. Natl. Acad. Sci. USA, **89**, 2081–2085 (1992).
6. Chey, W. Y., Shay, H. and Shuman, C. R.: External pancreatic secretion in diabetes mellitus. Ann. of Internal. Med., **59**, 812–821 (1963).
7. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J.: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, **18**, 5294–5299 (1979).
8. Colosia, A. D., Marker, A. J., Lange, A. J., el Maghrabi, M. R., Granner, D. K., Tauler, A., Pilkis, J. and Pilkis, S. J.: Induction of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase mRNA by refeeding and insulin. J. Biol. Chem., **263**, 18669–18677 (1988).
9. Cox, J. S., Shamu, C. E. and Walter, P.: Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. Cell, **73**, 1197–1206 (1993).
10. Csermely, P. and Kahn, C. R.: The 90 kDa heat shock protein (hsp-90) possesses an ATP-binding site and autophosphorylating activity. J. Biol. Chem., **266**, 4943–4950 (1991).
11. Csermely, P., Miyata, Y., Schnaider, T. and Yahara, I.: Autophosphorylation of grp94 (endoplasmin). J. Biol. Chem., **270**, 6381–6388 (1995).
12. Dorner, A. J., Wasley, L. C. and Kaufman, R. J.: Protein dissociation from grp78 and secretion are blocked by depletion of cellular ATP levels. Proc. Natl. Acad. Sci. USA, **87**, 7429–7432 (1990).
13. Gething, M. J. and Shambrook, J.: Protein folding in the cell. Nature, **355**, 33–45 (1992).
14. Haas, I. G.: BiP (grp78), an essential hsp70 resident protein in the endoplasmic rehculum. Experientia, **50**, 1012–1020 (1994).

15. Hammond, C. and Helenius, A.: Folding of VSV G protein: sequential interaction with BiP and calnexin. Science, **266**, 456–458 (1994).
16. Hendrick, J. P. and Hartl, F-U.: Molecular chaperone functions of heat shock proteins. Ann. Rev. Biochem., **62**, 349–384 (1993).
17. Kahn, C. R., White, M. F., Shoelson, S. E., Backer, J. M., Araki, E., Cheatham, B., Siddle, K., Sun, X., Wilden, P. A., Yamada, K., Csermely, P., Folli, F., Goldstein, B. J., Huertas, P., Rothenberg, P. L. and Saad, M. J. A.: The insulin receptor and its substrate: molecular determinants of early events in insulin action. Recent Prog. in Horm. Res., **48**, 291–339 (1993).
18. Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**, 680–685 (1970).
19. Lee, A. S.: Coordinated regulation of a set of genes by glucose and calcium ionophores in mammalian cells. Trends in Biochem. Sci., **12**, 20–23 (1987).
20. Lee, A. S., Delegeane, A. and Scharff, D.: Highly conserved glucose-regulated protein in hamster and chicken cells: preliminary characterization of its cDNA clone. Proc. Natl. Acad. Sci. USA, **78**, 4922–4925 (1981).
21. Legagneux, V., Morange, M. and Bensaude, O.: Heat shock increases turnover of 90 kDa heat shock protein phosphate groups in HeLa cells. FEBS Letters, **291**, 359–362 (1991).
22. Leustek, T., Toledo, H., Brot, N. and Weissbach, H.: Calcium-dependent autophosphorylation of the glucose-regulated protein, grp78. Arch. Biochem. Biophys., **289**, 256–261 (1991).
23. Liu, E. S. and Lee, A. S.: Common sets of nuclear factors bind to the conserved promoter sequence motif of two coordinately regulated ER protein genes, grp78 and grp94. Nucl. Acids Res., **19**, 5425–5431 (1991).
24. Macejak, D. G. and Sarnow, P.: Internal initiation of translation mediated by the 5'leader of a cellular mRNA. Nature, **353**, 90–94 (1991).
25. Melnick, J., Aviel, S. and Argon, Y.: The endoplasmic reticulum stress protein grp94, in addition to BiP, associates with unassembled immunoglobulin chains. J. Biol. Chem., **267**, 21303–21306 (1992).
26. Mori, K., Ma, W., Gething, M. J. and Shambrook, J.: A transmembrane protein with a cdc2+/CDC28-related kinase activity is required for signalling from the ER to the nucleus. Cell, **74**, 743–756 (1993).
27. Parfett, C. L. J., Brudzynski, C. and Stiller, C.: Enhanced accumulation of mRNA for 78-kilodalton glucose-regulated protein (grp78) in tissues of nonobese diabetic mice. Biochem. Cell Biol., **68**, 1428–1432 (1990).
28. Peavy, D. E., Taylor, J. M. and Jefferson, L. S.: Correlation of albumin production rates and albumin mRNA levels in livers of normal, diabetic, and insulin-treated diabetic rats. Proc. Natl. Acad. Sci. USA, **75**, 5879–5883 (1978).
29. Price, B. D., Mannheim, R. L. A. and Calderwood, S. K.: Brefeldin A, thapsigargin and AlF4- stimulate the accumulation of GRP78 mRNA in a cycloheximide dependent manner, whilst induction by hypoxia is independent of protein synthesis. J. Cell Physiol., **152**, 545–552 (1992).
30. Resendez, E. J., Ting, J., Kim, K. S., Wooden, S. K. and Lee, A. S.: Calcium ionophore A23187 as a regulator of gene expression in mammalian cells. J. Cell Biol., **103**, 2145–2152 (1986).
31. Sarnow, P.: Translation of glucose-regulated protein 78/immunoglobulin heavy-chain binding protein mRNA is increased in poliovirus-infected cells at a time when cap-dependent translation of cellular mRNAs is inhibited. Proc. Natl. Acad. Sci. USA, **86**, 5795–5799 (1989).
32. Schiaffonati, L., Tacchini, L. and Pappalardo, C.: Heat shock response in the liver: expression and regulation of the HSP70 gene family and early response genes after *in vivo* hyperthermia. Hepatology, **20**, 975–983 (1994).
33. Sherman, M. Y. and Goldberg, A. L.: Heat shock in *Escherichia coli* alters the protein-binding properties of the chaperonin groEL by inducing its phosphorylation. Nature, **357**, 167–169 (1992).

34. Sherman, M. Y. and Goldberg, A. L.: Heat shock of *Escherichia coli* increases binding of dnaK (the hsp70 homolog) to polypeptides by promoting its phosphorylation. Proc. Natl. Acad. Sci. USA, **90**, 8648–8652 (1993).
35. Sparks, J. D., Zolfaghari, R., Sparks, C. E., Smith, H. C. and Fisher, E. A.: Impaired hepatic apolipoprotein B and E translation in streptozotocin diabetic rats. J. Clin. Invest., **89**, 1418–1430 (1992).
36. Spindler, S. R., Crew, M. D., Mote, P. L., Grizzle, J. M. and Walford, R. L.: Dietary energy restriction in mice reduces hepatic expression of glucose-regulated protein 78 (BiP) and grp94 mRNA. J. Nutr., **120**, 1412–1417 (1990).
37. Sugawara, S., Takeda, K., Lee, A. and Dennert, G.: Suppression of stress protein grp78 induction in tumor B/C10ME eliminates resistance to cell mediated cytotoxicity. Cancer Res., **53**, 6001–6005 (1993).
38. Takemoto, H., Yoshimori, T., Yamamoto, A., Miyata, Y., Yahara, I., Inoue, K. and Tashiro, Y.: Heavy chain binding protein (BiP/grp78) and endoplasmin are exported from the endoplasmic reticulum in rat exocrine pancreatic cells, similar to protein disulfide-isomerase. Arch. Biochem. Biophys., **296**, 129–136 (1992).
39. Tal, M., Schneider, D. L., Thorens, B. and Lodish, H. F.: Restricted expression of the erythroid/brain glucose transporter isoforms to perivenous hepatocytes in rats. Modulation by glucose. J. Clin. Invest., **86**, 982–992 (1990).
40. Tal., M., Kahn, B. and Lodish, H. F.: Expression of the low Km GLUT-1 glucose transporter is turned on in perivenous hepatocytes of insulin-deficient diabetic rats. Endocrinology, **129**, 1933–1941 (1991).
41. Toledo, H., Carlino, A., Vidal, V., Redfield, B., Nettleton, M. Y., Kochan, J. P., Brot, N. and Weissbach, H.: Dissociation of glucose-regulated protein grp78 and grp78-IgE Fc complexes by ATP. Proc. Natl. Acad. Sci. USA, **90**, 2505–2508 (1993).
42. Towbin, H., Staehelin, T. and Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**, 4350–4354 (1979).
43. Vér, Á., Csermely, P., Bányász, T., Kovács, T. and Somogyi, J.: Alterations in the properties and isoform ratio of brain Na^+/K^+ATPase in streptozotocin diabetic rats. Biochem. Biophys. Acta, **1237**, 143-150 (1995).
44. Welch, W. J.: Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. Physiological Reviews, **72**, 1063–1081 (1992).
45. Wertheimer, E., Sasson, S., Cerasi, E. and Ben-Neriah, Y.: The ubiquitous glucose transporter GLUT-1 belongs to the glucose-regulated protein family of stress-inducible proteins. Proc. Natl. Acad. Sci. USA, **88**, 2525–2529 (1991).

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Effect of irradiation on the GTP binding kinetics of chicken embryo brain plasma membranes[1]

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The membrane bound form of the catalytic subunit of adenylate cyclase of chicken embryo brain has been found earlier to be rather radioresistant [28].

The radiation induced changes of G proteins of membrane preparations of 19 day old chicken embryo brains were investigated in this work. The activation of catalytic subunit of adenylate cyclase by G protein dependent activators (Gpp/NH/p and NaF) was found elevated at lower radiation doses (0–400 Gy), while both basal enzyme activity (measured without any activator) and the activity measured in the presence of activators decreased at higher doses (above 800 Gy).

Heterogeneity of GTP binding sites measured with 3-H-Gpp/NH/p a GTP-ase resistant GTP analogue was observed in the case of control (with Kd1 = 0.0663 $\pm$ 0.034 μmol/l, Bmax = 0.0079 $\pm$ 0.0022 nmol/ml and Kd2 = 2.038 $\pm$ 0.4779 μmol/l, $Bmax_2$ = 0.0291 $\pm$ 0.0017 nmol/ml). The lower affinity high capacity binding sites seemed to be more radiosensitive, than the higher affinity sites. A marked decrease was observed in the number of low affinity binding sites above 200 Gy and these low affinity binding sites practically disappeared after irradiation with 400 Gy. At high doses (above 1600 Gy) the catalytic subunit was damaged, too.

On the basis of the decrease of low affinity binding sites together with an increase in activation of the catalytic subunit via G proteins one can conclude that it is caused by radiation induced damage of Gi protein that can be more radiosensitive, than Gs protein.

Keywords: G proteins, Gpp/NH/p binding kinetics, irradiation, chicken embryo brain

The effect of ionizing radiation on the cellular membranes have been widely reported in the last decades [8, 14]. The radiosensitivity of different membrane functions (fluidity, ionic channels 8, 16) and components (lipids 18, 26; enzymes e.g.

[1] This paper is dedicated to the memory of Professor Tibor Kovács (1929–1994)

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Ca^{2+} ATPase 9, 25) has been investigated. Many papers have been published about effects of gamma or X irradiation on the adenylate cyclase activity both *in vivo* and *in vitro* [6, 7, 23, 24, 27, 28, 30, 31].

Adenylate cyclase system is a membrane associated complex of catalytic subunit and heterotrimers of regulatory GTP binding proteins (Gs and Gi) and plays an important role in signal transduction [1, 11]. Changes in the adenylate cyclase activity, or levels of cyclic AMP can be good markers of radiation-induced alteration of cell function. In an earlier work we have found a low dose x-irradiation-induced adenylate cyclase activation, and an increase in cyclic AMP level of fibroblasts [24]. Both the catalytic activity and SH groups of chicken embryo brain adenylate cyclase proved to be rather radioresistant *in vitro* [27, 28]. Similarly, other investigators found differences in the radiosensitivity after *in vivo* and *in vitro* irradiation [2]. Elevation of catalytic activity was observed at low doses and an inhibition at higher doses [27, 28]. In contrast a well-known SH reagent N-ethylmaleimide (NEM) caused a parallel decrease in the number of SH groups as well as in the enzyme activity. Recently, we have found a relationship between the radiosensitivity of adenylate cyclase and its membrane association i.e. the membrane associated adenylate cyclase was less radiosensitive, than the free catalytic subunit [28]. Adenylate cyclase as a multimeric enzyme complex could be a good model for investigation of radiation induced alterations in the membrane functions, or radiation-inactivation of enzymes [31]. Some papers have been published about the effects of different agents on G proteins, and therefore on the activation of adenylate cyclase [3, 12, 13, 19, 27, 28, 31].

In the present work the potential radiosensitivity of G protein complexes of chicken embryo brain membrane preparations was investigated including either the effect of gamma irradiation on the G protein-involved activation of the catalytic subunit (Gpp/NH/p, NaF activation) and on the binding kinetics of GTP (using a GTP-ase insensitive GTP analogue, the 3-H-Gpp/NH/p as marker).

Methods

Preparation of plasma membranes

Partially purified membrane fraction was prepared from 19 day old chicken embryo brain homogenate. The supernatant following a centrifugation at 3000 *g* for 15 min was overlayered on a discontinuous sucrose density gradient and was ultracentrifuged at 88,000 *g* for 90 min a 4 °C. The interphase of 24 and 40 percentage sucrose was used as partially purified membrane fraction. The protein content was determined by the method of Bradford [4].

Irradiation of membranes

Gamma irradiation of purified membrane fraction was carried out at 0 °C, with a dose of 0.36 Gy/sec in NORATOM Gamma 350 C.

Enzyme assay

Adenylate cyclase activity was measured by the method of Brooker et al. with some modifications [5, 29]. The reaction mixture contained 50 mmol/l Tris-HCl (pH 7.5), 5 mmol/l $MgCl_2$, 0.4 mmol/l Na-EDTA, 1 mg/ml BSA (bovine serum albumin), 12 mmol/l creatine phosphate, 1 mg/ml creatine phosphokinase, 0.5 mmol/l 3-isobutyl-1-methyl-xantine, 8–10 μg membrane protein (depending on the preparation) in a final volume of 100 μl. The reaction was started by the addition of membrane fraction and was incubated at 30 °C for 20 min with constant shaking. The reaction was stopped by addition of 50 mmol/l Na acetate (pH 4.7). The amount of other adenine nucleotides was decreased by addition of Al_2O_3. The amount of cyclic AMP produced was measured by modified cAMP radioimmunoassay method of Brooker et al. [5, 28]. The ^{125}I cAMP was labelled in our laboratory (Succinyl-cAMP-tyrosyl methylester was purchased form Sigma St. Louis MO USA and ^{125}I from Amersham, England). The bound and free radioactivities were separated by 30% PEG (polyethyleneglycol) in the presence of 0.5% BSA. The radioactivity was measured in a gamma counter (Gamma Co., Budapest).

^{3}H-Gpp/NH/p binding assay

The procedure was carried out according Salomon et al. with some modifications [20, 29]. The assay solution contained Tris-HCl buffer (50 mmol/l, pH 7.5), 5 mmol/l $MgCl_2$, 0.4 mmol/l Na-EDTA and varying concentrations (10^{-9}–10^{-5} mmol/l) of ^{3}H-Gpp/NH/p. The reaction was started by the addition of 20 μl membrane preparation (20–30 μg protein), and was carried out in a final volume of 200 μl at 30 °C for 60 minutes. Equilibrium was obtained after 5–6 minutes. The assay was stopped by adding 1 ml of cold 0.9% NaCl solution, and the plasma membrane bound radioactivity was separated from the unbound by rapid filtration through Sartorius filters (Sartorius No. 113 07). The filters were washed twice with 1 ml cold 0.9% NaCl solution, and dissolved in 0.5 ml methylcellosolve. The radioactivity bound on the filter was measured in 5 ml toluene-dioxane scintillation cocktail in LKB Rackbeta liquid scintillation spectrometer. The non-specific binding was measured in the presence of 2×10^{-4} mol/l unlabelled GTP, and it did not exceed the 30% of the total binding at near saturation (10^{-6} mol/l Gpp/NH/p) and was less at lower concentrations. All results were prepared with non-specific bindings values subtracted from the data.

The results of saturation were represented on Scatchard plot [21] and analysed by the Gra-Fit program of Leatherborrow (Sigma St. Louis, USA).

Results

In an earlier work we found that gamma irradiation of plasma membrane of chicken embryo brain enhanced the G protein dependent activation of adenylate cyclase at lower doses (200–400 Gy, 27). The basal enzyme activity (measured without any activator) proved to be rather radioresistant (Fig. 1) and it was inhibited at higher doses (above 800 Gy) where the activated form of adenylate cyclase was also inhibited. As it can be seen in the Fig. 1, the activation ratio caused by 5 mmol/l NaF (maximal activation of adenylate cyclase of chicken embryo brain was 2.3–2.5-fold found at 10 mmol/l NaF – higher concentration of NaF can inhibit the enzyme) was 1.6 fold in the case of control, and was further elevated to 4–5-fold by radiation. The $MnCl_2$ activation (direct activation of catalytic subunit) did not change after irradiation, and a radiation-caused inhibition was observed in the presence of $MgCl_2$. Irradiation of membrane fraction had less effect on the forskolin (which activates directly of the catalytic subunit) activation of adenylate cyclase (data not shown). These results

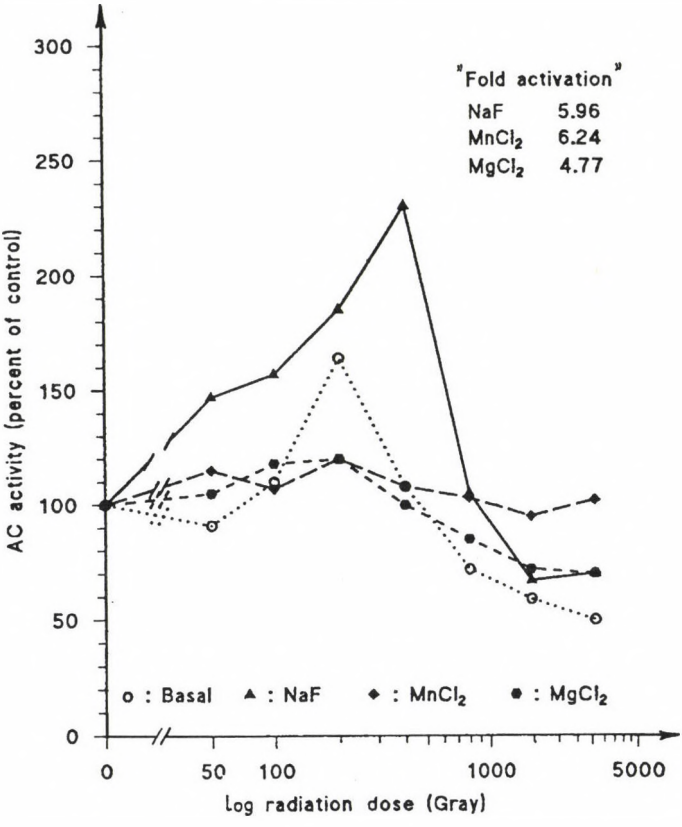

Fig. 1. The radiosensitivity of the adenylate cyclase activity of chicken embryo brain membrane measured without any activator (basal activity, o -- o) and measured in the presence of activators (5 mmol/l NaF: ▼--▼, 5 mmol/l $MnCl_2$: ◆--◆, 10 mmol/l $MgCl_2$: •--•, respectively). The enzyme assay was carried out by the modified method of Brooker et al. [5, 28], see Methods), and the amount of cyclic AMP was determined by radioimmunoassay [5, 25, 27]. The points represent a mean value of three experiments, two parallels in each. The inlet contains "the fold activation" as the ratio of adenylate cyclase activity in the presence of activators and the basal activity in controls (Control = nonirradiated sample)

suggested the possible role of G proteins in the radiosensitivity of adenylate cyclase. Therefore one function, the GTP binding activity of G proteins was used to follow the effect of irradiation on G proteins. ^{3}H-Gpp/NH/p a GTP-ase resistant GTP analogue was used as substrate. The saturation binding kinetics was detected on control membrane preparation and on membrane preparations subjected to various doses (100–3200 Gy) of gamma irradiation.

Two types of GTP binding sites were found in control (see Fig. 3) with Kd1 = 0.0663 ± 0.034 μmol/l, $Bmax_1$ = 0.0079 ± 0.0022 nmol/ml and Kd2 = 2.038 ± 0.4779 μmol/l, $Bmax_2$ = 0.0291 ± 0.0017 nmol/ml (Table I). High

affinity binding sites proved to be radioresistant. Neither they binding capacity Bmax, nor their GTP affinity (Kd) changed after gamma irradiation doses of 100, 200, 400, 800, 1600 and 3200 Gy, respectively (Figs 2, 3/A–F and Table I). However, the low affinity binding sites seemed to be radiosensitive. The Kd values of low affinity binding sites increased markedly showing the decrease in the binding affinity, while their binding capacity decreased (Figs 2, 3A–F, and Table I). Virtual Kd values with high standard error were detected at 800 Gy and at higher doses indicating the disappearance of the low affinity binding sites, i.e. losing their GTP binding capacity.

Table I

Changes in the ^{3}H-Gpp(NH)p binding capacity (Bmax) and affinity (Kd) of chicken embryo brain membrane after ^{60}Co-gamma irradiation

Irradiation dose	$Bmax_1$		Kd_1		$Bmax_2$		Kd_2	
	(nmol/ml)	%	(μmol/l)	%	(nmol/ml)	%	(μmol/l)	%
Control	0.0079 ± 0.0022	100	0.0663 ± 0.034	100	0.0291 ± 0.0017	100	2.038 ± 0.4779	100
100 Gy	0.0098 ± 0.0018	125	0.0579 ± 0.028	87	0.0216 ± 0.0018	74	2.145 ± 0.9734	106
200 Gy	0.0082 ± 0.0017	104	0.0685 ± 0.0259	103	0.0085 ± 0.0013	29	2.1592 ± 1.5751	107
400 Gy	0.0062 ± 0.0006	76	0.0663 ± 0.018	100	0.0090 ± 0.0025	31	7.9743 ± 5.7571	394
800 Gy	0.0117 ± 0.0010	148	0.0701 ± 0.0128	106	0.0090 ± 0.0018	31	5.2471 ± 3.9314	259
1600 Gy	0.0100 ± 0.0009	127	0.0583 ± 0.0119	88	0.0084 ± 0.0017	29	5.3776 ± 3.8063	266
3200 Gy	0.0100 ± 0.0007	127	0.0727 ± 0.0119	110	0.0103 ± 0.0031	35	8.4253 ± 6.0962	416

There is the characterization of binding sites of control and irradiated membrane preparations of 19-day old chicken embryo brain based on binding experiments which appear in Figs 2–3. Kd ± S.D. and Bmax ± S.D. values are presented together with percentual changes. The high affinity low capacity binding sites (Kd_1, $Bmax_1$) seem to be radioresistant, while low affinity high capacity binding sites (Kd_2, $Bmax_2$) were damaged by gamma irradiation above 100 Gy. The binding assay was carried out as it is indicated in Fig. 2 and in Methods

A significant decrease in the total GTP binding capacity (sum of $Bmax_1$ and $Bmax_2$) was observed even after irradiation with 100, 200 and 400 Gy doses (Fig. 2). In spite of the complete disappearance of low affinity binding sites no further decrease in the total binding capacity was observed at higher doses (800 Gy or more). However, some remainder binding capacity was detectable at this high dose range ($Bmax_2$), but with very high standard deviation.

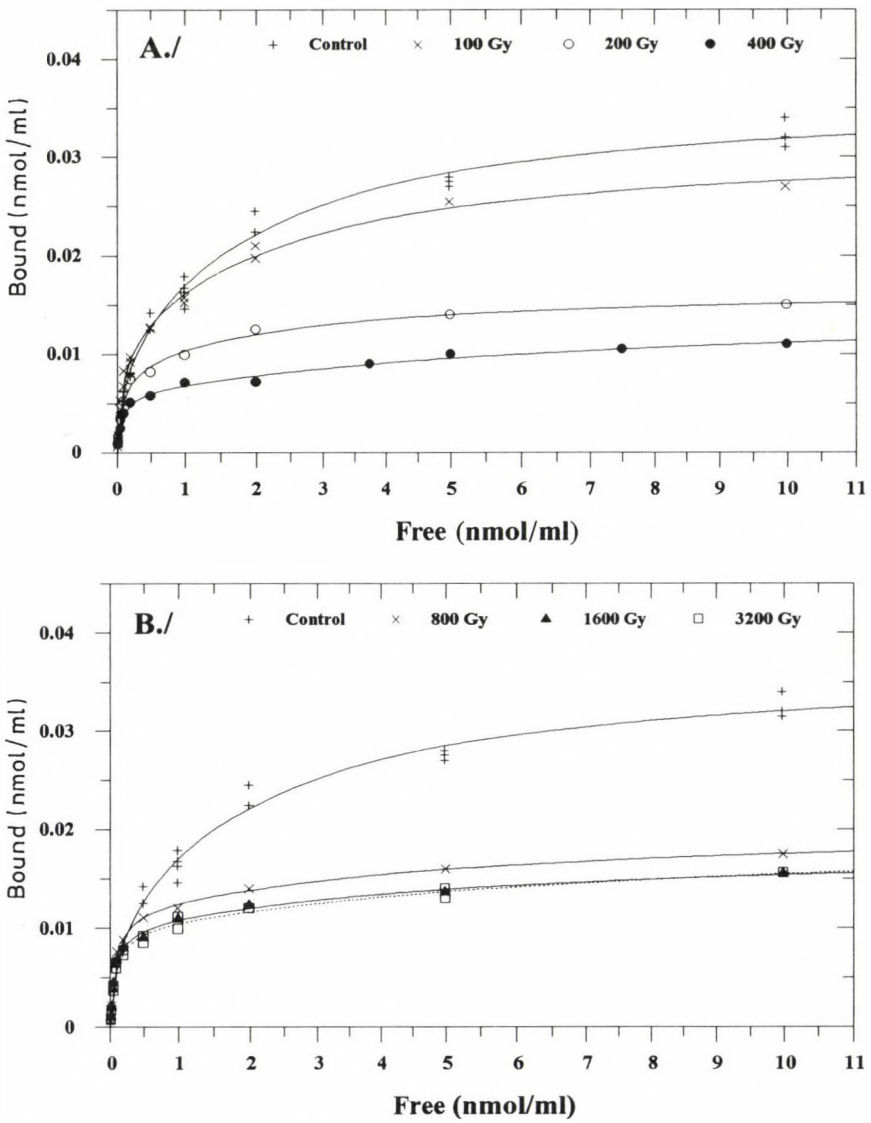

Fig. 2. The effect of gamma irradiation on ^{3}H-Gpp/NH/p saturation of membrane preparations of 19-day old chicken embryo brain. **A**: Control (+--+), 100 Gy (x--x), 200 Gy (o--o) and 400 Gy (•--•) irradiated, respectively. **B**: Control (+--+) 800 Gy (x--x), 1600 Gy (▼--▼) and 3200 Gy (□--□) irradiated, respectively. 3H-Gpp/NH/p binding assay was carried out modified method of Salomon et al. [20, 29]. The non-specific binding (measured in the presence of 10^{-4} mol/l GTP). The points represent a mean value of three different experiments, two parallels in each

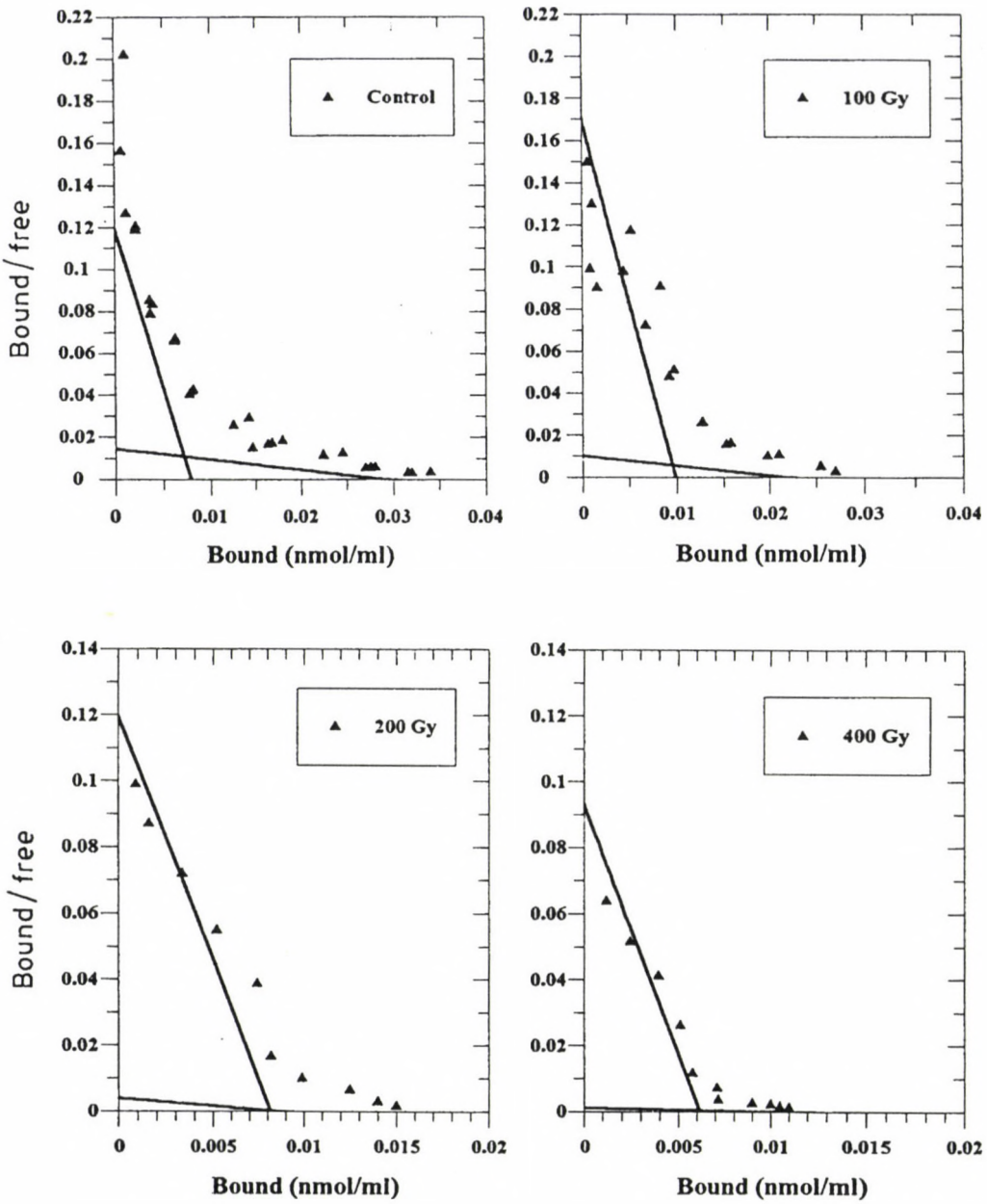

Fig. 3a

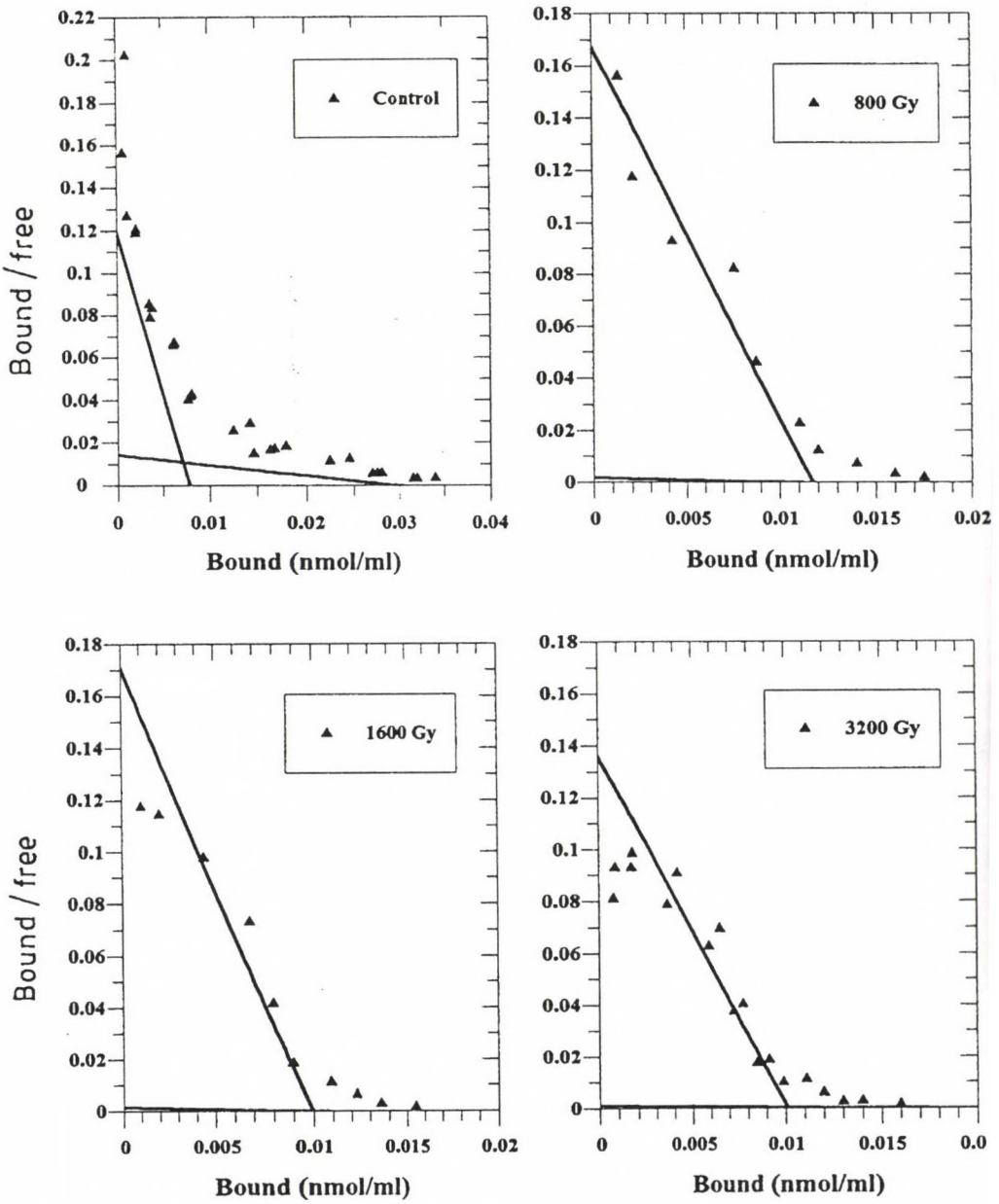

Fig. 3. Scatchard plot of ^{3}H-Gpp/NH/p saturation kinetics of membrane preparation of 19-day old chicken embryo brain. GraFit method (SIGMA Co.; Leatherborrow) was applied for analysis. **A**: Control, 100, 200, 400 Gy irradiated samples. **B**: Control, 800, 1600 and 3200 Gy irradiated samples. The binding experiments were carried out as it is indicated in the legend of Fig. 2

Discussion

High radioresistance of the membrane-bound catalytic subunit of adenylate cyclase was observed earlier [28]. However, gamma irradiation of membrane preparation of chicken embryo brain resulted in a mixed picture in an increase of catalytic activity at lower doses (under 800 Gy), while higher doses (800–3200 Gy) inhibited both the basal activity (measured without any activator) and enzyme activities measured in the presence of activators.

Activation of catalytic subunit of adenylate cyclase may occur either directly (forskolin, $MnCl_2$), or via G proteins (hormones, GTP, GTP analogues, NaF etc.). G protein dependent activation and inactivation have different ways. The GTP activated Gsα binds directly to the adenylate cyclase, while Gi protein inhibits the enzyme by deliberation of β and γ subunits. These common subunits of Gs and Gi complexes bind to Gsα. However, both activation and inactivation of adenylate cyclase need GTP binding [1, 11]. During activation/inactivation of adenylate cyclase and GTP/GDP binding "circle" many connections may occur between the subunits themselves, too. There are also some movements in the membrane depending on the membrane fluidity, which is also radiosensitive [16]. The GTP binding affinity of alpha subunits of Gs and Gi proteins may have different radiosensitivity. On the basis of the activation of adenylate cyclase by lower doses of gamma irradiation the Gi protein seems to be more radiosensitive [28], and its inhibition can cause the activation of the enzyme [19]. The increase in the G protein dependent activation potentiated by irradiation [28], can also be caused either by higher radiosensitivity of beta and gamma subunits or by the decrease in the affinity of alpha subunit of Gs protein to beta and gamma subunits.

The appearance of the radioactivation of adenylate cyclase and decrease in the low affinity high capacity binding sites seem to occur at the same radiation doses (100–400 Gy). It can be postulated that there is some kind of connection between the damage of low affinity binding sites and increase in activating susceptibility of the catalytic subunit of adenylate cyclase. A part of radioresistant high affinity low capacity binding sites can be other GTP binding proteins [17, 22].

The radiosensitivity of GTP binding activity of plasma membranes may depend on many factors, including the multimeric enzyme complex of adenylate cyclase [31], the presence of other GTP binding proteins [17, 22], the anchoring fatty acids [17], membrane fluidity [16], and other factors, too.

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REFERENCES

1. Birnbaumer, L., Codina, J., Mattera, R., Yatani, A., Scherer, N., Toro, M-J., Brown, A. M.: Signal transduction by G proteins. Kidney International, **32**, Suppl. 23, S14–S37 (1987).
2. Bonincontro, A., Camett, C., Rosi, A., Sportelli, L.: Alteration of membrane conductivity in erythrocyte ghosts after gamma irradiation. Int. J. Radiat. Biol., **52**, 447–457 (1987).
3. Böhm, M., Giershik, P., Knorr, A., Larish, K., Weismann, K., Erdmann, E.: Role of altered G-protein expression in the regulation of myocardial adenylate cyclase activity and force of contraction in spontaneous hypertensive cardiomyopathy in rats. J. Hypertension, **10**, 115–1128 (1992).
4. Bradford, A.: Rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. Anal. Biochem., **72**, 248–254 (1976).
5. Brooker, G., Harper, J. F., Terasaki, W. L., Moylen, R. D.: Radioimmunoassay of cyclic AMP and cyclic GMP. in: Advances in Cyclic Nucleotide Research Vol. 10. eds Brooker, G., Grengard, P. and Robinson, G. A. pp. 1-33 Raven Press, New York, 1979, pp. 1-33.
6. Catravas, G. N. Jr., Wright, S. J., Trocha, P. J., Takenaga, J.: Radiation effects on cyclic AMP, cyclic GMP and amino acid level in CSF of the primate. Radiat. Res., **87**, 198–203 (1981).
7. Chatterjee, A., Bhattacharya A. K.: In vivo adenylate cyclase activity in ultraviolet and gamma-irradiated *Escherichia coli*. Internat. J. of Radiat. Biol., **53**, 977–982 (1988).
8. Cramp, W. A., Yatvin, M. B., Harms-Ringdahl, M.: Recent developments in the radiobiology of cellular membranes. Acta Oncologica, **33**, 945–952 (1994).
9. Dreval, V. I.: The effect of ionizing radiation on the Ca^{2+}, Mg^{2+} ATPase activity of plasma membranes. Radiobiologiia, **32**, 222–224 (1992).
11. Gilman, A. G.: G proteins and regulation of adenylyl cyclase. J. Am. Med. Association, **262**, 1819–1825 (1989).
12. Herdon, H.: N-ethylmaleimide inactivates adenylate cyclase coupled but not presynaptic striatal D-2 receptor system. Eur. J. Pharmacol., **154**, 115–116 (1988).
13. Horváth, Gy., Takáts, A.: Effect of radioprotectors on the aggregability, cyclic adenosine monophosphate and thiol content of blood platelets. Acta Physiol. Hung. (submitted).
14. Köteles, G. J.: New aspects of cell membrane radiobiology and their impact on radiation protection. Atomic Energy Rev., **17**, 3–30 (1979).
15. Kuzin, A. M., Shozhenikina, L. V., Usakova, T. E.: The effect of gamma-irradiation on the adenylate cyclase activity of isolated biomembrane of embryonic liver cells. Dokl. Akad. Nauk. SSSR, **233**, 978–983 (1977).
16. McClain, D. E., Trypus, C. A., May, L.: Effect of gamma radiation on membrane fluidity of MOLT-4 nuclei. Radiat. Res., **123**, 263–267 (1990).
17. Miller, A. C., Kariko, K., Myers, C. E., Clark, E. P., Samid, D.: Increased radioresistance of EJras-transformed human osteosarcoma cells and its modulation by lovastatin, an inhibitor of $p21^{ras}$isoprenylation. Int. J. Cancer, **53**, 302–307 (1993).
18. Parasassi, T., Sapora, O., Destasio, G., Ravagnan, G.: Alterations in erythrocyte membrane lipids induced by low doses ionizing radiation as revealed by 1,6-diphenyl-1,3,5-hexatriene fluorescence lifetime. Int. J. Radiat. Biol., **59**, 59–69 (1991).
19. Parsons, W. J., Rankumar, V., Stile, G. L.: Isobutyl-methylxantine stimulates adenylate cyclase by blocking the inhibitory regulatory protein, Gi. Molec. Pharmacol., **34**, 37–41 (1988).
20. Salomon, Y., Lin, M. C., Londos, C., Rendell, M., Rodbell, M.: The hepatic adenylate cyclase system. J. Biol. Chemistry, **250**, 4239–4245 (1975).
21. Scatchard, G.: The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci., **51**, 660–672 (1949).

22. Sklar, M. D.: The ras oncogenes increase the resistance of NIH 3T3 cells to ionizing radiation. Science, **239**, 645–647 (1988).
23. Sobolev, S. A., Rosenkranz, A. A., Kazarov, A. R.: A study of mechanism of post-irradiation changes in adenylate cyclase activity. Internat. J. Radiat. Biol., **44**, 31–39 (1983).
24. Somosy, Z., Takáts, A., Kubasova, T., Sudár, F., Köteles, G. J.: Effect of X-irradiation on adenylate cyclase activity and cyclic AMP content of primary human fibroblasts. Scanning Microscopy, **2**, 2165–2172 (1988).
25. Somosy, Z., Thuróczy, G., Köteles, G. J., Kovács, J.: Effects of modulated microwave and X-ray irradiation on the activity and distribution of Ca^{2+} ATPase in small intestine epithelial cells. Scanning Microscopy, **8**, 613–620 (1994).
26. Stark, G.: The effect of ionizing radiation on lipid membranes Biochim. Biophys. Acta, **1071**, 103–122 (1991).
27. Takáts, A., Binh, V.-H., Bertók, L.: Potential role of SH groups in the radiosensitivity of adenylate cyclase. Acta Physiol. Hung., **76**, 265–272 (1990).
28. Takáts, A., Binh, V.-H., Offenmüller, K., Bodó, K., Bertók, L., Köteles, G. J.: Adenylate cyclase – the more membrane associated, the less radiosensitive. J. Radiat. Res., **34**, 141–147 (1993).
29. Takáts, A., Borsos, B., Bertók, L.: A possible mechanism of the inhibitory effect of endotoxin on adenylate cyclase. Acta Biochim. Biophys. Hung., **24**, 191–202 (1989).
30. Trocha, P. J., Catravas, G. N.: Variation in cyclic nucleotide levels and lysosomal enzyme activities in the irradiated rat. Radiat. Res., **83**, 658–667 (1980).
31. Verkman, A. S., Skorecki, K. S., Ausiello, D. A.: Radiation inactivation of multimeric enzymes: application to subunit interactions of adenylate cyclase. Am. J. Physiol., **250**, C103–C114 (1986).

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Intestinal elimination of p-nitrophenol in the rat*

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The intestinal metabolism and metabolite transport of p-nitrophenol (PNP), as a model compound have been investigated in an *in vivo* isolated intestinal loop preparation in the rat. Different PNP concentrations (20 μM, 100 μM and 500 μM) were recirculated to determine the formation and transport of PNP-metabolites (PNP-glucuronide: PNP-G and PNP-sulphate: PNP-S) in the jejunal loop. It was found that the jejunum of the rat was able to metabolize PNP rapidly and to transport the metabolites efficiently back into the luminal solution. About 21, 16 and 6% of recirculated amount of PNP could be detected in 90 minutes as PNP-G in the lumen of jejunal loop when 20, 100 or 500 μM PNP was perfused. These results show that the luminal appearance of PNP-G tended to saturability. Sulphate conjugate of PNP was undetectable in the intestinal lumen at 20 and 100 μM PNP concentrations and PNP-S amounted to 0.07% of recirculated amount of PNP when it was used in a concentration of 500 μM. These results indicate that the intestinal metabolism and the excretion of metabolites may play a role in the elimination of xenobiotics containing phenolic hydroxyl groups and that the small intestine of the rat forms predominantly PNP-G after luminal administration of PNP.

Keywords: p-nitrophenol, small intestine, biotransformation, intestinal drug elimination

The role of hepatic biotransformation of drugs has been already studied extensively and it was only recently recognized that extrahepatic, including intestinal metabolism may also significantly influence the disposition of xenobiotics [2, 5-9, 11, 13]. A very important aspect of intestinal conjugation is its location at the site of entry of exogenous compounds, because the metabolites of drugs formed in the small

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This paper is dedicated to the memory of Professor Ferenc Varga (1929–1994).

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intestine can be excreted by enterocytes back into the intestinal lumen [10, 12, 14, 15], which means a defence against xenobiotics or a presystemic elimination of drugs.

It is known that p-nitrophenol (PNP) is conjugated almost exclusively with glucuronic acid and sulphate to be produced to PNP-glucuronide (PNP-G) and PNP-sulphate (PNP-S), therefore PNP has often been used as a model compound in conjugation studies [1, 3].

The present investigations were designed to study the intestinal metabolism of PNP and the transport of PNP-G and PNP-S in the jejunum in the rat *in vivo*.

Materials and Methods

Chemicals

p-nitrophenol, its glucuronide, the monopotassium salt of p-nitrophenol sulphate were obtained from the Sigma Chemical Co., St. Louis, MO. All other chemicals and reagents were analytical or HPLC grade. The standard isotonic perfusion medium had the following composition (mmol/l): NaCl 96.4, KCl 7.0, $CaCl_2$ 3.0, $MgSO_4$ 1.0, sodium phosphate buffer (pH 7.4) 0.9, TRIS buffer (pH 7.4) 29.5, glucose 14.0, mannitol 14.0.

Animals and experimental procedure

Male Wistar rats weighing 220–260 g, were used. The animals were unaesthetized with urethane (1.2 g/kg i.p.). The abdomen was opened by a mid-line incision, a jejunal loop (distance from the duodeno-jejunal flexure about 15 cm; length of jejunal loop about 10 cm) was cannulated. The lumen of the jejunal loop was gently flushed with warmed isotonic perfusion medium to remove digesta and food residues and then blown empty with 4–5 ml air. Perfusion through the lumen of intestine with isotonic medium containing PNP was carried out at a rate of 13 ml/min in a recirculation mode. The volume of samples obtained from the perfusion medium coming out from the jejunal loop was 250 μl, the initial perfusion volume was 15 ml. The temperature of perfusion medium was maintained constant at 37 °C. When the biliary excretion was investigated the bile duct was cannulated with a PE-10 tubing and the bile collected in 15 min periods.

Sample analysis

PNP and its metabolites were separated and quantitated by reverse-phase HPLC with absorbance detection at 280 and 305 nm. The analytical system was comprised of: a Gynkotek gradient HPLC pump (model M 480 G), a Gynkotek variable wavelength detector (model UVD 160 S) and a Nucleosil^R 5 C_{18}-5 μm (Macherey-Nagel GmbH, Düren, Germany) column. Samples were eluted with water: acetonitrile: ortho-phosphoric acid 85% (730:250:2) at a flow rate 1.1 ml/min. Quantitation of PNP, PNP-G and PNP-S was based upon integration of absorbance peak areas using a Gynkosoft PC-integration-software PC 1 and a Gynkosoft PC. The concentration of PNP and its metabolites was determined from the standard calibration curve using external standards.

Statistics

Data are expressed as the mean ± SEM. Significant differences were calculated by Student's *t*-test.

Results

The luminal disappearance of PNP and the cumulative luminal appearance of PNP-G are shown in Figs 1–3 at luminal perfusion of PNP in a concentration of 20 μM, 100 μM or 500 μM. A continuous and relatively rapid fall was observed in the amount of PNP of perfusion medium, at the end of experiments (at 90 min) these values amounted to 30.5, 301 and 1098 nmol which represent 10.2, 13.2 and 14.6 percentages of PNP administered to luminal perfusion solution at the beginning of experiments. A continuous elevation of luminal appearance of PNP-G was found, 21.4, 16.1 and 5.66% of the recirculated amount could be detected in 90 min in the lumen of jejunal loop when 20, 100 and 500 μM PNP was administered, respectively. Peak of the luminal appearance of PNP-G was reached at 36 minutes after the luminal administration of 20 μM PNP. Maximum values of PNP-G were detected at 60 min when higher concentrations of PNP (100 and 500 μM) were perfused, no significant increases were found in the luminal appearance of PNP-G in 60 and 90 min periods. PNP-S was not detected in the intestinal lumen when it was recirculated with perfusion medium containing 20 or 100 μM PNP concentration, however, a small amount (5.29 nmol) appeared in 90 min when 500 μM PNP was used (this value represents 0.07% of the originally administered PNP in these experiments, PNP-S values are not shown in Fig. 3).

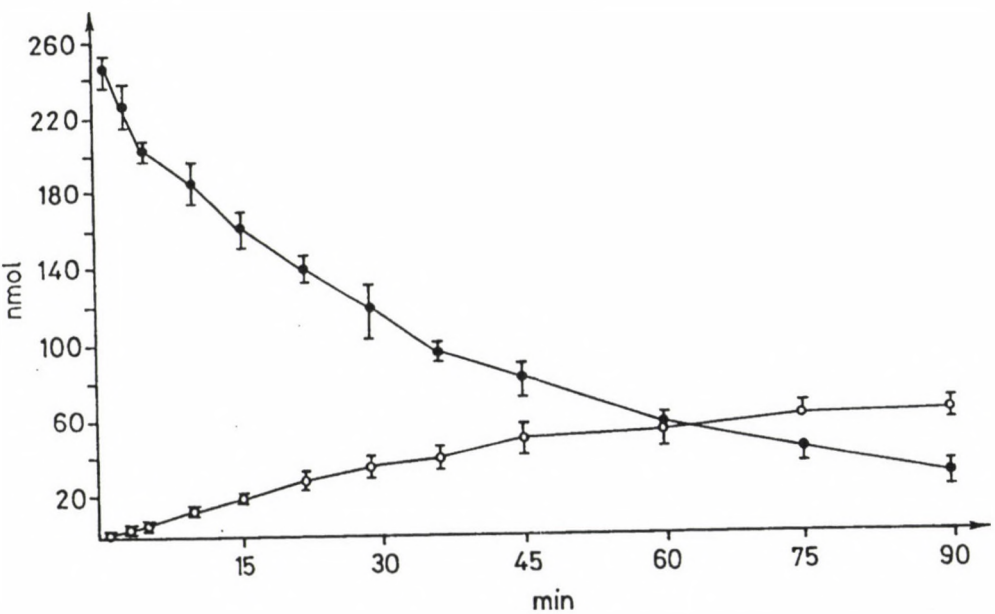

Fig. 1. Cumulative luminal appearance of PNP-G (o) and luminal disappearance of PNP (•) at luminal perfusion of the jejunal loop with isotonic medium containing 20 μM PNP. Values represent the mean ± SEM of five rats

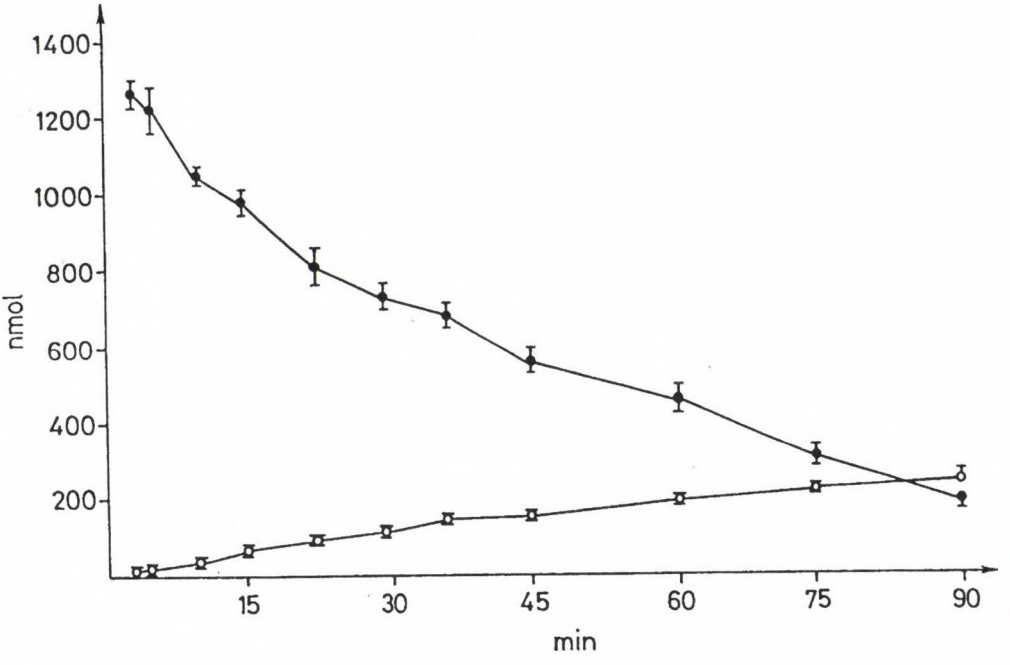

Fig. 2. Cumulative luminal appearance of PNP-G (o) and luminal disappearance of PNP (•) at luminal perfusion of the jejunal loop with isotonic medium containing 100 μM PNP. Values represent the mean ± SEM of seven rats

The dose-dependent changes of the cumulative luminal appearance of PNP-G are demonstrated in Fig. 4. These results indicate that the luminal appearance of PNP-G was elevated by increase of the dose of PNP, however, it tended to saturability: 5-fold higher PNP concentration (100 μM) produced 3.8-fold increase compared to the values measured at 20 μM PNP concentration in 90 min and it was found only 76% difference between the values of luminal appearance of PNP-G measured in 90 min at 100 and 500 μM PNP concentration, respectively.

In a series of experiments the biliary excretion of PNP and its metabolites was investigated during the luminal perfusion of PNP in a concentration of 100 μM, these results are shown in Fig. 5. It can be seen that the highest biliary excretion rate was measured at PNP-G (96.3 ± 11.3 nmol in 90 min) and a small fraction of PNP (9.11 ± 1.87 nmol) appeared in the bile in 90 min. In contrast to the undetectable amount of PNP-S in the lumen of jejunal loop, PNP appeared in sulphoconjugate form in the bile (18.2 ± 6.82 nmol in 90 min) when PNP was luminally perfused in a concentration of 100 μM. However, the total elimination capacity of the intestinal loop for PNP was significantly higher than that of the liver measured at these experimental conditions.

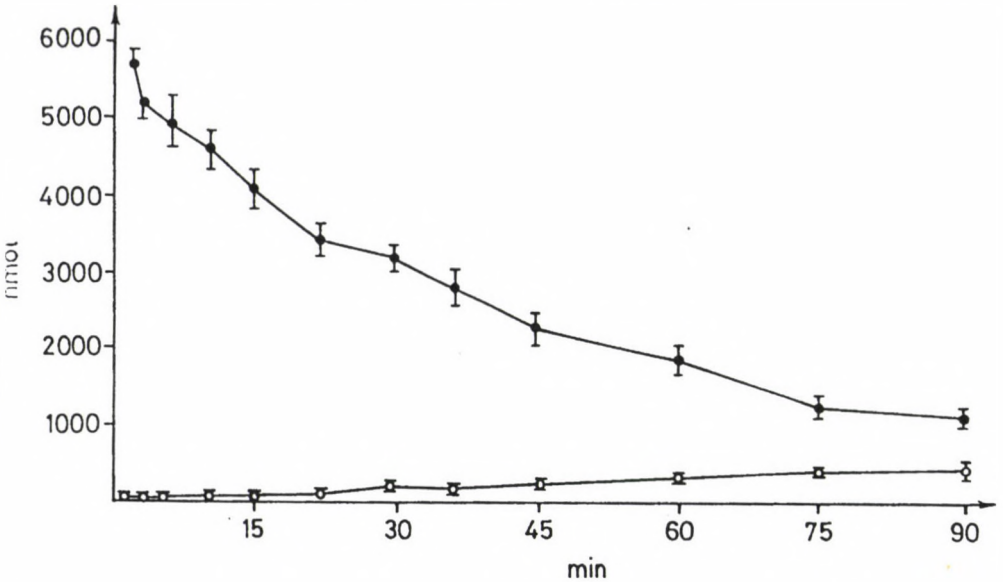

Fig. 3. Cumulative luminal appearance of PNP-G (○) and luminal disappearance of PNP (•) at luminal perfusion of the jejunal loop with isotonic medium containing 500 μM PNP. Values represent the mean ± SEM of six rats

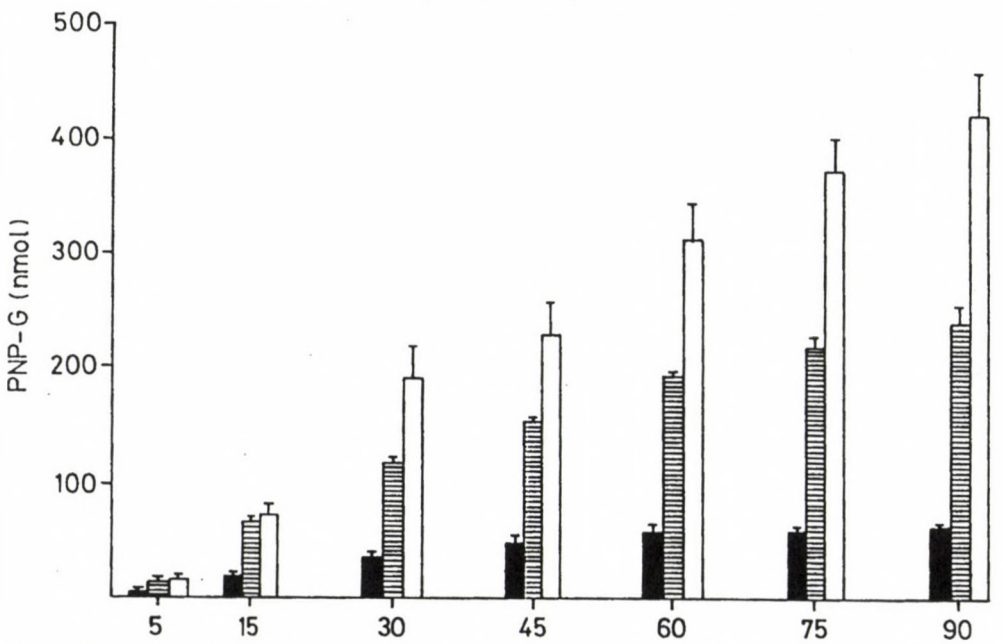

Fig. 4. Dose-dependent luminal appearance of PNP-G at luminal perfusion of the jejunal loop with isotonic medium containing 20 (▪), 100 (▤) and 500 (□) μM PNP. Values represent the mean ± SEM of five to seven rats

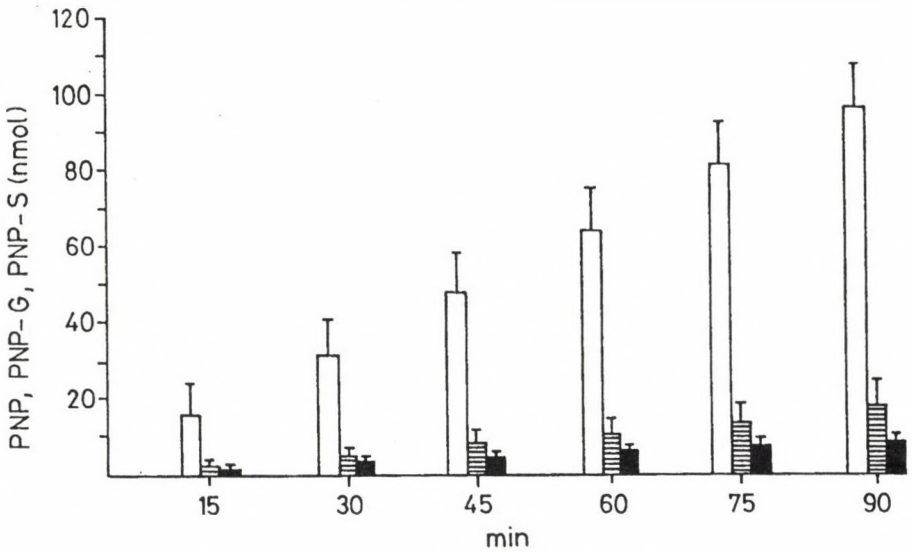

Fig. 5. Biliary excretion of PNP-G (□), PNP-S (▤) and PNP (■) at luminal perfusion of the jejunal loop with isotonic medium containing 100 μM PNP. Values represent the mean ± SEM of seven rats

Discussion

It was found that the small intestine of the rat metabolized the luminally administered PNP with a significant rate and the predominant metabolite (PNP-G) was rapidly transported back into the lumen of jejunum, e.g. 21% of the recirculated amount of PNP could be detected as PNP-G in the jejunal loop in 90 min when 20 μM PNP was perfused. The cumulative luminal appearance rate of PNP-G reached its peak in 36 min at 20 μM PNP and in 60 min when PNP was luminally perfused at higher concentrations (100 and 500 μM). The appearance of PNP-G in the intestinal lumen depended on the substrate (PNP) concentration of the luminal perfusion medium and tended to saturability: 5-fold higher PNP-concentration (100 μM) produced 3.8-fold increase compared to the value measured at 20 μM PNP concentration in 90 min and the luminal appearance of PNP-G using 500 μM PNP was only 1.8-fold greater than the corresponding value found at 100 μM PNP concentration perfused in 90 min. It is interesting to note that no sulphoconjugate of PNP was detected in the luminal perfusion medium when PNP was administered in 20 and 100 μM concentrations, however, a small amount of PNP-S was detected in the lumen of jejunal loop after the perfusion of PNP in a concentration of 500 μM. This finding may indicate an alternative metabolic appearance of PNP which means a compensatory pathway in the intestinal elimination of PNP when the glucuronidation tends to saturability. Similar compensatory rise of intestinal sulphatation of acetaminophen has been previously found with increment of the dose of this drug [6]. However, in the guinea pig a

significantly greater luminal appearance of PNP-S was detected than that of PNP-G and interestingly a compensatory increase in the luminal appearance of PNP-G was observed in these experiments when the luminal appearance of PNP-S tended to saturability with elevation of luminal PNP concentration [4].

The biliary excretion of PNP-G was significantly lower (96.3 $\pm$ 11.3 nmol) than the luminal appearance of this metabolite (241.4 $\pm$ 18.0 nmol) when 100 μM PNP was luminally perfused in 90 min, on the other hand, the biliary excretion of PNP-S (18.2 $\pm$ 6.82 nmol) exceeded the luminal appearance of sulphoconjugate of PNP which was undetectable in the jejunal lumen in these experiments. PNP was excreted at a relatively low rate in unconjugated form into the bile (9.11 $\pm$ 1.87 nmol) at luminal perfusion of 100 μM PNP in 90 min.

Theoretically it is possible that the metabolites of PNP formed by extraintestinal biotransformation can also appear in the intestinal lumen. However, a very low (about 1%) permeation of PNP-G in the jejunal mucosa was found when 1 μM PNP-G was administered to the blood side [10]. In the present experiments we have found PNP-S in the bile which was produced by the liver after luminal administration of 100 μM, however, PNP-S did not appear in the intestinal lumen under these experimental conditions. These findings indicate that metabolites of PNP found in the intestinal lumen in our experiments have been really formed by the jejunal loop which was luminally perfused with isotonic medium containing PNP.

These results show significant qualitative and quantitative differences between the intestinal and hepatic elimination of PNP after luminal perfusion of a jejunal loop. The total amount of PNP metabolites appeared in the intestinal lumen exceeded the sum of PNP-G and PNP-S which was excreted by the liver into the bile which indicates that the small intestine may play an important role in the overall elimination of phenolic compounds after oral or luminal administration.

REFERENCES

1. Baldwin, M. K., Selby, M. A., Bloomberg, H.: Measurement of phenol in urine by the method of Van Haaften and Sie: a critical appraisal. Analyst, **106**, 763–767 (1981).
2. Cassidy, M. K., Huston, J. B.: Phenol conjugation by lung in vivo. Biochem Pharmacol., **29**, 471–474 (1980).
3. Eadsforth, C. V., Coveney, D. C.: Measurement of phenol in urine using a high-performance liquid chromatographic method. Analyst, **109**, 175–176 (1984).
4. Fischer, E., Lauterbach, F.: Intestinal metabolism and metabolite transport of p-nitrophenol in the guinea pig in vivo after luminal and blood side administration. Z. Gastroenterol., **30**, 224 (1992).
5. George, C. F.: Drug metabolism by the gastrointestinal mucosa. Clin. Pharmacokinet., **6**, 259–274 (1981).
6. Goon, D., Klaassen, C. D.: Dose-dependent intestinal glucuronidation and sulphatation of acetaminophen in the rat in situ. J. Pharm. Exp. Ther., **252**, 201–207 (1990).
7. Hartiala, K. J. W.: Metabolism of hormones, drugs and other substances by the gut. Physiol. Rev., **53**, 496–534 (1973).

8. Hänninen, O., Lindström-Seppä, P., Pelkonen, K.: Role of gut in xenobiotic metabolism. Arch. Toxicol., **60**, 34–36 (1987).
9. Laitinen, M., Watkins, J. B.: Mucosal biotransformations. In: Gastointestinal Toxicology, eds Rozman, K., Hänninen, O., Elsevier Press, Amsterdam 1986, pp. 169–192.
10. Lauterbach, F., Schorn, M., Sprakties, G., Sund, R. B.: Compartmentalization of intestinal conjugation reactions and conjugate transport: studies with phenols and p-aminobenzoic acid. Progr. in Pharmacol. and Clin. Pharmacol., **7/2**, 231–242 (1982).
11. Machida, M., Morita, Y., Hazashi, M., Awazu, S.: Pharmakokinetic evidence for the occurrence of extrahepatic conjugative metabolism of p-nitrophenol in rats. Biochem. Pharmacol., **31**, 787–791 (1982).
12. Schwenk, M.: Directed efflux of glucuronides and sulphate esthers form the intestinal mucosa. Naunyn-Schmiedeberg's Arch. Pharmacol. Suppl., **311**, R15 (1980).
13. Schwenk, M.: Glucuronidation and sulphatation in the gastrointestinal tract. Progr. in Pharmacol. and Clin. Pharmacol., **7/2**, 155–169 (1989).
14. Sund, R. B., Hillestad, B.: Uptake, conjugation and transport of laxative diphenols by everted sacs of the rat jejunum and stripped colon. Acta Pharmacol. Toxicol., **51**, 377–387 (1982).
15. Sund, R. B., Lauterbach, F.: Drug metabolism and metabolite transport in the small and large intestine: Experiments with 1-naphthol and phenolphthalein by luminal and contraluminal administration in the isolated guinea pig mucosa. Acta Pharmacol. Toxicol., **58**, 74–83 (1986).
16. Wollenberg, P., Rummel, W.: Vectorial release of sulfoconjugates in the vascularly perfused mouse small intestine. Biochem. Pharmacol., **33**, 205–208 (1984).

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Effect of hyperglycaemia on the hepatic metabolism and excretion of xenobiotics*

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The biliary excretion of drugs has been investigated in hyperglycaemic male Wistar rats. Hyperglycaemia was produced by a continuous infusion of glucose in different concentrations. To examine the effect of hyperglycaemia on the biliary excretion rate of xenobiotics, one representative of non-metabolizing exogenous organic anions (rose bengal) and metabolizing phenolic drugs (p-nitrophenol) as model compounds were used. A dose-dependent increase of blood and bile glucose level was found after the continuous infusion of glucose in a concentration of 10, 20 or 30%. Biliary flow and biliary excretion of rose bengal were significantly depressed in hyperglycaemic rats. Hepatic conjugation of p-nitrophenol with glucuronic acid appeared to be decreased, however, a compensatory increase of sulphoconjugation of p-nitrophenol was observed in hyperglycaemic rats. Biliary excretion of unconjugated p-nitrophenol was negatively influenced by high blood glucose level and the overall hepatic elimination of p-nitrophenol was also diminished by hyperglycaemia. Insulin (1 U/kg i.v.) compensated partly the depressive effect of high blood sugar level on the biliary excretion rate of rose bengal, however, it was unable to abolish completely the negative effects of hyperglycaemia. These results indicate that hyperglycaemia does alter the hepatic elimination of xenobiotics.

Keywords: p-nitrophenol, rose bengal, hepatic metabolism, biliary excretion, hyperglycaemia

Although diabetes is known to cause alterations in transport function of small intestine, in hepatic structure and function and in intracellular metabolism in most tissues including the liver, little definitive data are available concerning the effects of diabetes, hyperglycaemia and insulin on the hepatic drug metabolism and excretion [4, 9, 11, 13, 16, 18, 19].

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This paper is dedicated to the memory of Professor Ferenc Varga (1929–1994)

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The present experiments were designed to study the effect of hyperglycaemia on the hepatic elimination of xenobiotics. In order to eliminate the complications caused by biotransformation, in a series of experiments the biliary excretion of rose bengal was investigated which is a model compound of exogenous organic anions excreted by the liver into to bile without metabolism [12]. On the other hand, to examine the alterations of hepatic biotransformation and the biliary excretion of metabolites, p-nitrophenol (PNP) was administered, because it is well known, that this drug is conjugated with glucuronic acid and sulphate producing PNP-glucuronide (PNP-G) and PNP-sulphate (PNP-S) and therefore PNP has often been used as a model compound in conjugation studies [2, 8].

Biliary and fecal concentrations of diazepam were reduced in streptozotocin-induced diabetes and these changes were reversible after insulin administration [1], therefore the effect of insulin on the biliary excretion of hyperglycaemic rats was also studied in the present experiments.

Materials and Methods

Chemicals

Rose bengal was obtained form Fluka A.G., Buchs G, p-nitrophenol, its glucuronide, the monopotassium salt of p-nitrophenol sulphate from the Sigma Chemical Co., St. Louis, MO., insulin from Richter Gedeon Rt., Budapest. All other chemicals and reagents were analytical or HPLC grade. The standard isotonic perfusion medium for luminal drug administration had the following composition (mmol/l): NaCl 96.4, KCl 7.0, $CaCl_2$ 3.0, $MgSO_4$ 1.0, sodium phosphate buffer (pH 7.4) 0.9, TRIS buffer (pH 7.4) 29.5, glucose 14.0, mannitol 14.0.

Animals and experimental procedure

Male Wistar rats weighing 240–260 g were used. The animals were unaesthetized with urethane (1.2 g/kg i.p.). The abdomen was opened by a mid-line incision, the bile duct was cannulated with a polyethylene tubing (PE–10), and the bile collected in 20-min periods after the i.v. (femoral vein) administration of rose bengal. When the hepatic metabolism and excretion of PNP were studied, a jejunal loop (distance from the duodeno-jejunal flexure about 15 cm; length of jejunal loop about 10 cm) was cannulated. The lumen of the jejunal loop was gently flushed with warmed isotonic perfusion medium to remove digesta and food residues and then blown empty with 4–5 ml air. Perfusion through the lumen of intestine with isotonic medium containing PNP (100 μM) was carried out at a rate of 13 ml/min in a recirculation mode. The volume of samples obtained from the perfusion medium coming out from the jejunal loop was 250 μl, the initial perfusion volume was 15 ml. Bile was collected in these experiments in 15-min periods. Body temperature of rats was maintained a 37 °C. The animals were fasted 16–20 hours prior to the experiment, water was given *ad libitum*. Hyperglycaemia was maintained by a continuous i.v. infusion of glucose in different concentrations (10, 20 or 30%) after the administration of a priming dose of glucose infusion solution (about 1.0 ml/100 g body wt) enabling a high blood glucose level (15–50 mM) to be reached right at the start of glucose infusion.

Sample analysis

Concentration of rose bengal was measured spectrophotometrically after an appropriate dilution of bile samples. PNP and its metabolites were separated and quantitated by reverse-phase HPLC with

absorbance detection at 280 and 305 nm. The analytical system was comprised of: a Gynkotek gradient HPLC pump (model M 480 G), a Gynkotek variable wavelength detector (model UVD 160 S) and a Nucleosil^R 5 C_{18}-5 μm (Macherey-Nagel GmbH, Düren, Germany) column. Samples were eluted with water: acetonitrile: ortho-phosphoric acid 85% (730:250:2) at a flow rate 1.1 ml/min. Quantitation of PNP, PNP-G and PNP-S was based upon integration of adsorbance peak areas using a Gynkosoft PC-integration-software PC 1 and Gynkotek PC. The concentration of PNP and its metabolites was determined from the standard calibration curve using external standards. Glucose was determined enzymatically using glucose oxidase (Boehringer Mannheim GmbH).

Calculations and statistics

Biliary excretion rate of rose bengal, PNP and its metabolites was calculated as a product of biliary concentration and bile volume. Data are expressed as the mean ± SEM. Significant differences were calculated by Student's *t*-test.

Results

Blood glucose level is shown in Fig. 1 after a continuous glucose infusion in different concentrations. Infusion of glucose in a concentration of 10% produced a significant elevation of blood glucose level which reached a steady state in 2 hours and amounted to about 300 mg/dl (17 mM). Blood glucose concentration was further elevated when 20 or 30% glucose was infused, this increase seemed to be roughly a dose-dependent change in blood sugar level. The range of blood glucose concentration measured at 20% glucose infusion was similar to the blood glucose level of experimental diabetic rats (between 300 and 600 mg/dl which means 17–34 mM).

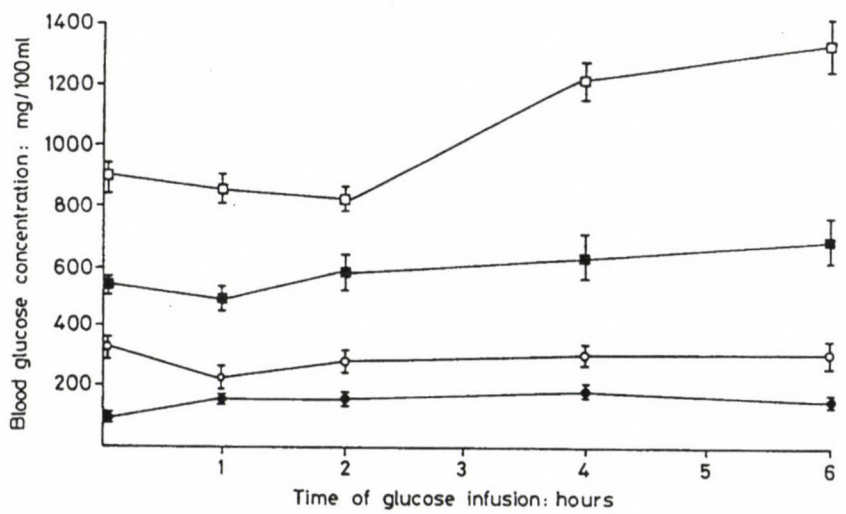

Fig. 1. Blood glucose level after a continuous glucose infusion in a concentration of 10% (○), 20% (▪) and 30% (□), control:•. Values represent the mean ±SEM of six to eight rats

Bile glucose concentration is demonstrated in Fig. 2. Glucose was infused for 2, 4 or 6 hours in separated groups of rats and bile was collected in 120 min in the first, second and third-two hours of infusion periods. A low glucose level was measured in control rats and a dose-dependent elevation was found in the bile glucose concentration after the infusion of 10, 20 or 30% glucose solution. The tendency of changes was similar to that of blood glucose, however, a longer period of time was needed to reach a steady state.

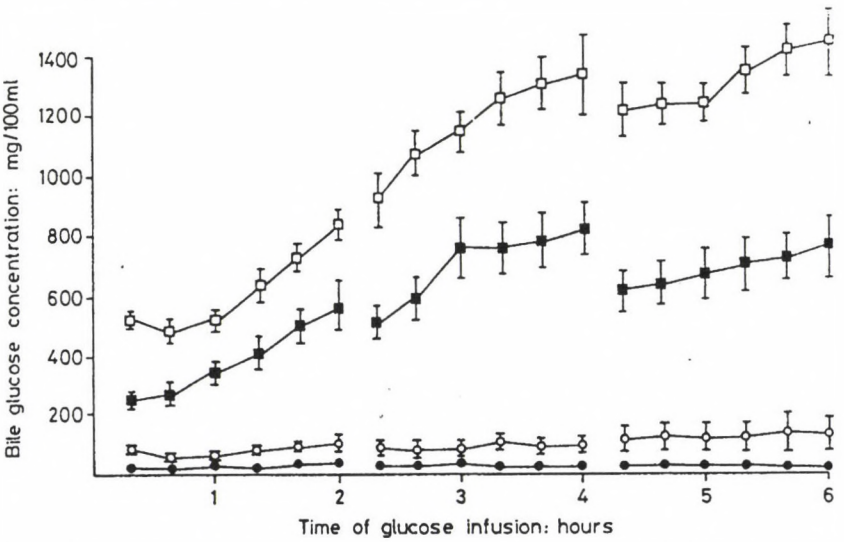

Fig. 2. Glucose level in the bile after a continuous glucose infusion in a concentration of 10% (○), 20% (■) and 30% (□), control: •. Glucose was continuously infused for 2, 4 or 6 hours in separate groups of rats, bile was collected in the last two hours of infusion periods. Values represent the mean ± SEM of six to eight rats

Changes in the biliary flow produced by hyperglycaemia are summarized in Fig. 3, experimental conditions were the same as it was described at Fig. 2, data are expressed in percentages of control. It can be seen that only a moderate decrease of biliary flow was detected after the infusion of 10% glucose solution. Significant depression of biliary flow was observed when higher glucose concentrations were administered, however, no considerable differences were found among the values of bile volume after the infusion of 20 and 30% glucose solution, respectively. A longer duration of hyperglycaemia provoked more pronounced decrease in the biliary flow.

Biliary flow and biliary excretion of rose bengal are shown in Fig. 4 expressed in percentages of control. Considerable depression could be seen both in the biliary excretion rate of rose bengal and in the biliary flow measured during the excretion of this drug. Changes in biliary flow seemed to be parallel with those of biliary excretion of rose bengal, the most remarkable alterations were detected during the second and third 2-h infusion periods.

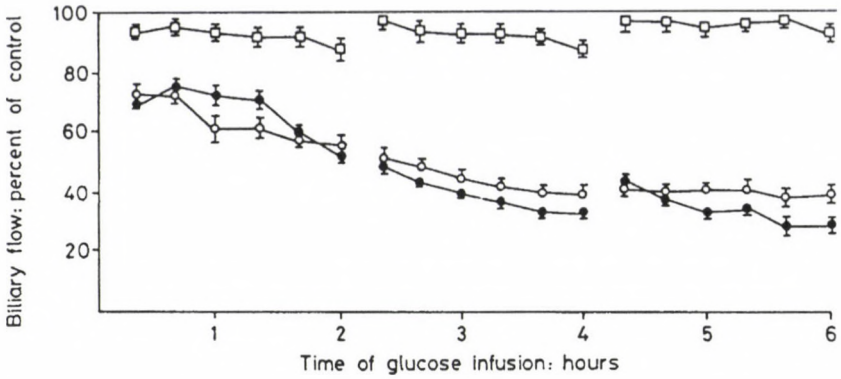

Fig. 3. Biliary flow after a continuous glucose infusion in a concentration of 10% (□), 20% (○) and 30% (•) expressed in percentages of control (biliary flow in control rats: 53.7 ± 1.86 μl/kg/min = 100%). Glucose was continuously infused for 2, 4 or 6 hours in separate groups of rats, bile was collected in the last 2-h periods of glucose infusion. Values represent the mean ± SEM of six to eight rats

Figure 5 shows the biliary appearance of PNP, PNP-G and PNP-S in control and hyperglycaemic rats. Hyperglycaemia was maintained by glucose (30%) infusion for 4 hours, PNP was luminally perfused in the jejunal loop in a concentration of 100 μM during the last 90-min period, bile was collected when PNP was perfused. PNP appeared in the bile in unchanged form at a significantly lower rate in hyperglycaemic rats than in controls. Similar tendency was seen at the biliary excretion of PNP-G, hyperglycaemic rats excreted less amount of PNP-glucuronide than controls did. In

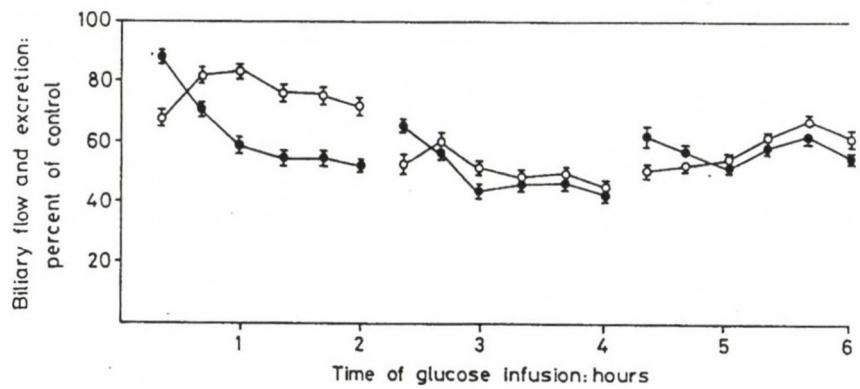

Fig. 4. Biliary flow (•) and biliary excretion of rose bengal (○) in hyperglycaemic rats expressed in percentages of control (biliary flow in control rats: 53.7 ± 1.86 μl/kg/min = 100%; biliary excretion of rose bengal in control rats: 152.3 ± 10.2 μg/kg/min = 100%). Glucose (30%) was continuously infused for 2, 4 or 6 hours in separate groups of rats, bile was collected in the last 2-h periods of glucose infusion. Rose bengal (100 mg/kg i.v.) was administered before the bile collection. Data represent the mean ± SEM of seven rats

contrast to the changes found in the biliary excretion of PNP and PNP-G, a definitely higher biliary excretion rate was produced by hyperglycaemia compared to the appropriate values of control rats. The total biliary excretion of PNP and its metabolites is demonstrated in Fig. 6, these results showed a decrease of overall hepatic elimination in hyperglycaemic rats when PNP was luminally perfused.

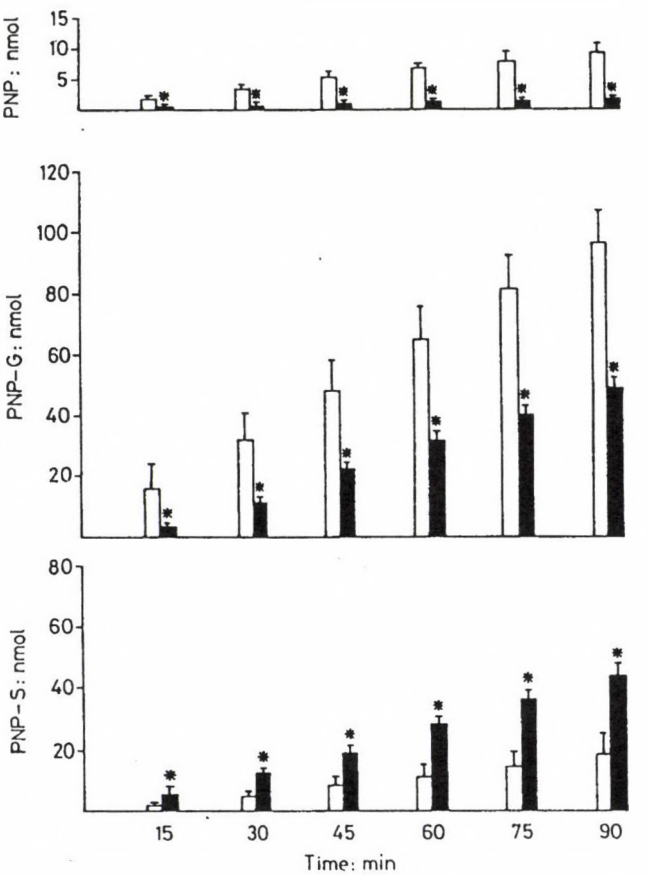

Fig. 5. Biliary excretion of PNP, its glucuronide (PNP-G) and sulphoconjugate (PNP-S) in control (□) and hyperglycaemic (■) rats. Glucose (30%) was continuously infused for 4 hours, bile was collected in the last 90-min period of glucose infusion. PNP was luminally perfused in a concentration of 100 μM during the time of bile collection. Bile was collected in these experiments in 15-min periods. Values represent the mean ± SEM of five to seven rats

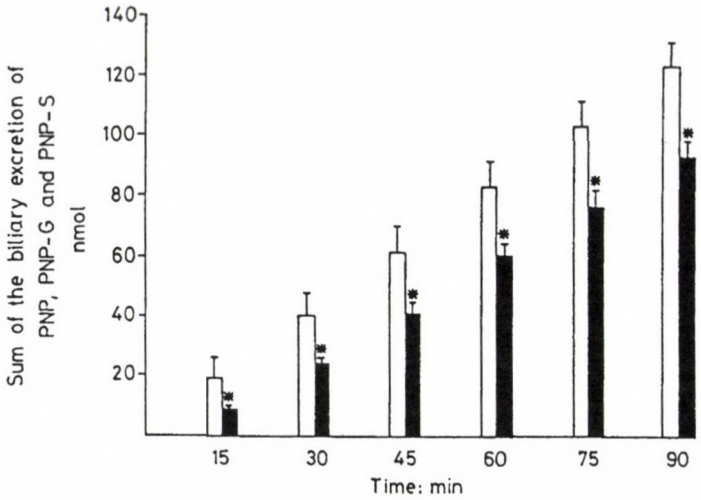

Fig. 6. Comparison of the total biliary excretion of PNP, PNP-G and PNP-S in control (□) and hyperglycaemic (▪) rats. Experimental conditions were the same as it was described at Fig. 5. Sum of the biliary excretion of PNP and its metabolites were calculated from the data presented in Fig. 5. Values represent the mean n SEM of five to seven rats

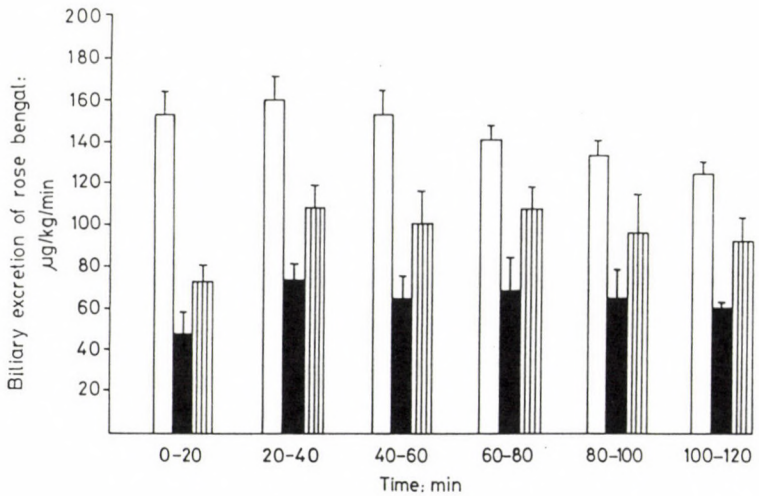

Fig. 7. Effect of insulin on the biliary excretion of rose bengal in hyperglycaemic rats (□: control; ▪: hyperglycaemic; ▥: hyperglycaemic + U/kg insulin i.v.). Glucose (30%) was continuously infused for 6 hours, bile was collected in the last two hours, insulin and rose bengal (100 mg/kg i.v.) were administered before the bile collection. Values represent the mean ± SEM of six to eight rats

Effects on insulin on the biliary excretion of rose bengal in hyperglycaemic rats are summarized in Fig. 7. Insulin was administered in a bolus injection (1 U/kg i.v.) simultaneously with rose bengal at start of bile collection. These data show that insulin elevated the depressed biliary excretion of rose bengal produced by hyperglycaemia, however it was unable to abolish completely the negative effects of high blood glucose level in the biliary excretion rate of this exogenous organic anion.

Discussion

The effect of hyperglycaemia on the hepatic elimination of drugs has been investigated in rats. Hyperglycaemia was produced by a continuous glucose infusion, different levels of hyperglycaemia could be reached and maintained by the change of the concentration of glucose solution. Hyperglycaemia is one of the most important leading symptoms of diabetes and it can be easily reproduced by glucose infusion without insulin deficiency which occurs obligatory in experimental diabetes provoked by streptozotocin [4, 14, 21].

Biliary flow was considerably reduced by hyperglycaemia in these experiments in spite of the significant elevation of glucose level in the bile. It is interesting to note that a definite increase (40–60%) was found in bile flow after long-term diabetes produced by streptozotocin [21] and one of the possible mechanisms that could be involved in explaining this phenomenon is the osmotic effect of glucose in the bile. The reason and exact mechanism of these discrepancies are not known, however, the pathophysiological changes are also different: streptozotocin produces insulin deficiency, whereas hyperglycaemic rats are able to release insulin. In addition, the effects of experimental diabetes are equivocal, e.g. a negative influence on the bile flow was detected after short time diabetes produced by streptozotocin [3].

A significant decrease was found in the biliary excretion rate of the non-metabolizing anionic rose bengal in hyperglycaemic rats. Similar results were observed in the biliary excretion of diazepam [1] and sulphobromophthalein [3] at 1 and 7 days after streptozotocin diabetes. However, the biliary excretion of phenol red was unchanged, procainamide ethobromide and ouabain excreted at a slightly elevated rate in the first 15 min and at a lower rate after 1 h in rats treated 28 days earlier with streptozotocin [21]. Results obtained in our acute experiments in hyperglycaemic rats resemble to changes observed in short term diabetes, in long term diabetes several other factors (e.g. change in steady state volume of distribution and in pharmacokinetic parameters) may influence the elimination of xenobiotics [5, 6].

Biliary excretion of PNP in unchanged form was depressed by hyperglycaemia, this change is similar to the action of high blood glucose level on the biliary excretion of rose bengal. However, PNP is metabolized in the liver and the metabolites appeared also in the bile. It was found that hyperglycaemia decreased the biliary excretion of PNP-G and increased that of PNP-S. Cumulative excretion of PNP and its metabolites over 90 min was decreased by hyperglycaemia. Stimulation of sulphoconjugation of

PNP and the higher biliary excretion of this metabolite mean probably a concomitant reaction with compensation of depressive effects of hyperglycaemia on the hepatic elimination of PNP and PNP-G. Similar compensatory increase in the excretion of a drug or its metabolites was observed in other experiments, as well [1, 10]. Increased conjugation of phenol red with glucuronic acid was detected in diabetic rats, however, the biliary excretion of phenol red-glucuronide remained unchanged [21]. Discrepancies between this finding and the decreased biliary excretion of PNP-glucuronide in our experiments can be explained probably by differences in the insulin level. Glucose flux in diabetic rats (in insulin deficiency) is shunted to insulin-insensitive pathway when insulin-dependent routes are inhibited, thereby elevating the activity of UDP-glucose dehydrogenase, which is the rate-limiting step in UDP-glucuronic acid synthesis [15]. It is known that change of UDP-glucuronic acid level may influence the rate of glucuronidation of drugs and the biliary excretion of glucuronides [7, 17].

Insulin elevated the biliary excretion of rose bengal in hyperglycaemic rats, however, it was unable to eliminate the negative effects of high blood sugar level completely under these experimental conditions. Insulin has been reported to stimulate bile flow [11, 13, 18] and biliary excretion of drugs [20] in control animals. Mechanism of action of insulin is not known, it is possible that insulin is involved in the synthesis of carrier proteins or the enzymes that catalyse biotransformation of drugs. Further investigations are needed to clarify the role of insulin under normal and hyperglycaemic of diabetic conditions, furthermore to study the importance of changes in insulin level and dose-dependent actions of insulin.

REFERENCES

1. Andrews, S. M., Griffiths, L. A.: The metabolism and disposition of [2^{14}C]diazepam in the streptozotocin-diabetic rat. Xenobiotica, **14**, 751–760 (1984).
2. Baldwin, M. K., Selby, M. A., Bloomberg, H.: Measurement of phenol in urine by the method of Van Haaften and Sie: a critical appraisal. Analyst, **106**, 763–767 (1981).
3. Carnovale, C. E., Rodriquez-Garay, E. A.: Reversible impairment of hepatobiliary function induced by streptozotocin in the rat. Experientia, **40**, 248–250 (1984).
4. Csáky, T. Z., Fischer, E.: Intestinal sugar transport in experimental diabetes. Diabetes, **30**, 568–574 (1981).
5. Daintith, H., Stevenson, J. H., O'Malley, K.: Influence of diabetes mellitus on drug metabolism in man. Int. J. Clin. Pharmacol. Biopharm., **13**, 55–58 (1976).
6. Dajani, R. M., Kayyali, S., Saheb, D. E., Birbary, G.: A study on the physiological disposition of acetophenetidin by the diabetic man. Comp. Gen. Pharmacol., **5**, 1–9 (1974).
7. Eacho, P. I., Sweeny, D., Weiner, M.: Conjugation of p-nitroanisol and p-nitrophenol in hepatocytes isolated from streptozotocin diabetic rats. J. Pharmacol. Exp. Ther., **218**, 34–40 (1981).
8. Eadsforth, C. V., Coveney, D. C.: Measurement of phenol in urine using a high-performance liquid chromatographic method. Analyst, **109**, 175–176 (1984).
9. Fischer, E., Lauterbach, F.: Effects of hyperglycaemia on sugar transport in the isolated mucosa of guinea pig small intestine. J. Physiol. (Lond.), **355**, 567–586 (1984).

10. Fischer, E., Lauterbach, F.: Compartmentalization of the intestinal metabolism and metabolite transport of p-nitrophenol in the guinea pig in vivo. Naunyn-Schmiedeberg's Arch. Pharmacol., **345**, R66 (1992).
11. Garberoglio, C. A., Richter, H. M., Henarejos, A., Moossa, A. R., Baker, A. L.: Pharmacological and physiological doses of insulin and determinants of bile flow in dogs. Am. J. Physiol., **245**, G 157–163 (1983).
12. Jirsa, M., Raban, P.: Metabolism of rose bengal. Nature (Lond.), **196**, 1100–1101 (1962).
13. Jones, R. S.: Effect of insulin on canalicular bile formation. Am. J. Physiol., **231**, 40–43 (1976).
14. Rerup, C. C.: Drugs producing diabetes through damage of the insulin secreting cells. Pharmacol. Rev., **22**, 485–520 (1970).
15. Sochor, M., Bacquer, N. Z., McLean, P.: Regulation of pathways of glucose metabolism in kidney. Effect of experimental diabetes on the activity of pentose phosphate pathway and glucuronate-xylulose pathway. Arch. Biochem. Biophys., **189**, 632–646 (1979).
16. Pain, V. M., Lanoix, J., Pergeron, J. J. M., Michael, J. C.: Effect of diabetes on the ultrastructure of the hepatocyte and on the distribution and activity of ribosomes in the free and on the bound populations. Biochem. Biophys. Acta, **353**, 487–498 (1974).
17. Price, V. F., Jollow, D. J.: Increased resistance of diabetic rats to acetaminophen-induced hepatotoxicity. J. Pharmacol. Exp. Ther., **220**, 504–513 (1982).
18. Thomsen, O. O., Larsen, J. A.: Interaction of insulin, glucagon, and DBcAMP on bile acid-dependent bile production in the rat. Scand. J. Gastroenterol., **17**, 687–693 (1982).
19. Topping, D. L., Targ, M. E.: Time-course of changes in blood glucose and ketone bodies, plasma lipids and liver fatty acid composition in streptozotocin-diabetic rats. Hormone Res., **6**, 129–137 (1975).
20. Varga, F., Málly, J., Fischer, E.: Effect of insulin on the biliary excretion of some organic anions in rats. Arch. Toxicol. Suppl., **4**, 391–393 (1980).
21. Watkins, J. B., Dyktsra, T. P.: Alterations in biliary excretory function by streptozotocin-induced diabetes. Drug Metabol. Disp., **15**, 177–183 (1987).

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Segmental flexibility of cardiac myosins[1]

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Conventional and saturation transfer electron paramagnetic resonance spectroscopy (EPR and ST EPR) and differential scanning calorimetry (DSC) were used to study the motional dynamics and structural stability of cardiac myosins.

Cardiac myosins isolated from bovine and human heart muscle were spin-labelled with a maleimide- or an iodoacetamide-based probe molecule at the reactive sulhydryl sites (Cys-697 and Cys-707). The probe molecules rotated with an effective rotational correlation time of 0.04 μs which was at least six times shorter than the rotational correlation time of the same label on skeletal myosin. In the presence of MgADP, flexibility changes in the multisubunit structure of myosins were detected, but this did not lead to changes of the overall rotational property of the myosin heads.

Temperature dependence of the EPR spectra of maleimide spin-labelled myosin showed continuous decrease of the spectral parameters (amplitude ratio of the diagnostic peak heights in the ST EPR time domain and hyperfine splitting constant) at increasing temperature. In contrast, marked changes were obtained at about 17 °C in light chain-2 deficient myosin. DSC measurements supported the view that the removal of the light chain-2 produced additional loosening in the multisubunit structure of cardiac myosin. It is postulated that the light chain-2 is an integral part of myosin, and there is an intersite communication between light chain-2 and the 20 kDa subunit.

Keywords: cardiac myosin, spin-labelling, DSC-measurement, flexibility of myosin heads, effect of nucleotides

Current models of muscle contraction assume that force is generated by myosin-actin interaction coupled to the ATPase cycle. The head of myosin molecule (subfragment one, S 1) is rigidly attached to actin and forms a complex in the absence of nucleotides. In the presence of MgATP, the chemical energy liberated from ATP

[1] This paper is dedicated to the memory of Professor Tibor Kovács (1929–1994)

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hydrolysis produces conformational changes in myosin. The structural change might induce rotation of myosin head while bound to actin, and it makes the muscle to shorten [7, 19]. This mechanism of the energy transduction is a crucial question of the muscle contraction.

It is well-known that the biochemical characteristics of cardiac muscle is different from skeletal muscle. The myosin of cardiac muscle have greater susceptibility for alkaline denaturation [25], and exhibit different amino acid compositions [20, 31] and/or amino acid sequence [1, 18]. Little is known about the molecular dynamical background of these different characteristics. In this study an attempt is made to gather data about the different molecular behaviour of cardiac- and skeletal muscle myosin using EPR technique and paramagnetic probes which report upon molecular motions, and applying DSC which provides information about the structural stability. From earlier studies it is known that (i) maleimide (MSL) and iodoacetamide (IASL) spin labels attach to the Cys-707 and Cys-697 residues which are located in the 20 kDa segment of the myosin motor domain [1, 9, 23]; (ii) light chain-2 (LC-2) can be easily separated from skeletal muscle myosin by DTNB treatment; in the case of cardiac myosin it can be removed only by proteolytic digestion [31]; (iii) the greatest difference between the two kinds of myosin is suggested in the 50 kDa domain which contains the actin binding site [29].

It was also known from hydrodynamic measurements [16] that there were differences in the sedimentation of LC-2 deficient and intact cardiac muscle myosins; intact cardiac myosin showed a conformational change at 18 °C which could not be detected after removal of the LC-2 light chain. The main functional difference was assigned to the 50 kDa domain and/or to the interaction of heavy chain with the LC-2 light chain. It seems to be disadvantageous that the paramagnetic probe molecules attach to the largely conserved region of the 20 kDa domain that involves the sulfhydryl sites. However, it was also reported that the 20 kDa domain is extended and flexible enough, therefore marked effects in mobility of the attached labels are expected after modification [11, 23, 37].

Using IASL and MSL paramagnetic probes attached to the myosin thiol groups, the globular head portion of cardiac muscle myosin containing the catalytic and transmission domains proved to be less rigid than that of the skeletal muscle; the MSL probes reflected the rotational motion of the entire globular subunit, whereas IASL was a sensitive detector of the local conformational changes in interaction with ADP. The removal of light chain-2 resulted in the destabilization of the structural rigidity. The internal molecular arrangement of actin filaments was influenced by attachment of myosin to actin.

Materials and Methods

Preparation of myosin

Bovine and human heart myosin was prepared by the methods described by Shiverick [28] and Léger and co-workers [6]. After washing of the tissue, myosin was extracted in buffer consisting of 0.6 M KCl, 20 mM imidazol, 1 mM dithiothreitol (DTT), 1 mM EDTA, pH 7.0. After centrifugation for 4 hours at 0 °C with 100 000 × *g*, the crude myosin was purified using Sepharose 4B chromatography.

Preparation of LC-2 deficient myosin

Cardiac myosin was cleaved with α-chymotrypsin (400:1) for 1.5 minutes at 25 °C in buffer containing 0.12 M NaCl, 20 mM phosphate, 1 mM EDTA, 1 mM DTT, at pH 7.0. The product was purified by column chromatography on Sepharose 4B. Protein concentrations were determined either by the method of Lowry [21] or by reading the absorption at 280 nm using absorption coefficients 0.55 $mg.ml^{-1}cm^{-1}$ for myosin and 0.56 $mg.ml^{-1}cm^{-1}$ for LC-2 deficient myosin.

Spin-labelling

The isolated proteins were labelled either with 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (4-maleimido-TEMPO) or with 4-iodoacetamido-2,2,6,6-tetramethylpiperidinooxyl (4-iodoacetamido-TEMPO). Myosin suspended in 0.5 M KCl, 50 mM TRIS, 1 mM EDTA at pH 8.0 was reacted for 60–90 minutes with 2–4 moles of MSL or with 3.5 moles of IASL per mole of myosin for 10–14 hours over ice. The reaction was terminated by precipitation of myosin with ice cold water, and thereafter the protein was collected by centrifugation and dissolved in 0.5 M KCl, 25 mM HEPES, 1 mM EDTA at pH 7.0. In some cases myosin was treated with 5 mM $K_3(Fe(CN)_6)$ to reduce the signal intensity arising from weakly immobilized labels [15]. The protein was clarified by centrifugation at 50 000 × g for 1 hour and used at a final concentration of 9.6 mg/ml (20 μM).

EPR measurements

The EPR measurements were taken with ERS 220 (Centre of Scientific Instruments, Germany) or ESP 300 E (Bruker, Germany) X-band spectrometers. For conventional EPR technique 100 kHz field modulation (0.1–0.25 mT amplitude) and 2–20 mW microwave power were used. Second harmonic adsorption, 90° out-of-phase spectra were recorded with 50 kHz field modulation (0.5 mT amplitude) and detection at 100 kHz out-of-phase. The microwave power was 85 mW (ERS 220) or 63 mW (ESP 300 E), which corresponds to an average microwave field amplitude of 0.025 mT in the central region of the flat cell of Zeiss (Germany). The microwave magnetic field intensity was determined with peroxylamine disulphate ion radicals in the same sample cell as for the myosin samples following Fajer and Marsh [10].

Signals due to EPR absorption were detected by microcomputer system interfaced to the ERS 220 spectrometer. The spectra were recorded on a digital transient recorded and stored on diskettes. Usually, 200 scans (scan time: 2.0 s) were done on samples and, in some cases, the signal to noise ratio was improved by the Fourier self-deconvolution procedure. Recording the spectra with the ESP 300 E spectrometer, the standard WIN EPR software was used for evaluation. The double integrals of the spectra were normalized to unity for spectrum manipulation.

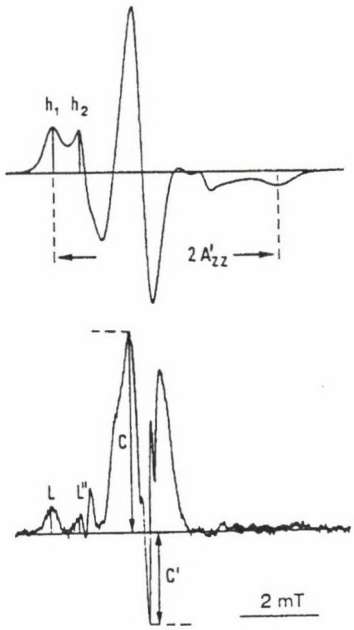

Fig. 1. Conventional (upper) and ST EPR spectrum (bottom) of human cardiac myosin labelled with MSL. The spectra were recorded at 20 °C. The concentration of myosin was 20 μM. The spectral parameters (the low-field peaks h_1 and h_2, the hyperfine splitting constant $2A'_{zz}$ and the diagnostic peaks of the ST EPR spectrum L, L", C and C') used to characterize the EPR spectra are also shown

Results and Discussion

Characterization of the labelled sites

The method used to label the reactive thiol sites of cardiac myosins was essentially the same as described earlier by Thomas and co-workers [33] and Belágyi et al. [2] for skeletal muscle myosin. The degree of labelling was 0.1–0.15 mol label/mol protein for IASL and 0.5–0.75 mol label/mol protein for MSL. The EPR spectra of both MSL- and IASL-cardiac myosins showed the superposition of spectra from strongly and weakly immobilized labels (Figs 1 and 2). The fractions of labels attached to weakly immobilizing sites varied a little from batch to batch, but never exceeded 10% of the total EPR absorption. The conventional EPR spectra were characterized by the distance between the outermost hyperfine extreme $2A'_{zz}$ and the rotational correlation time of the labels. For determination of the rotational correlation time the method of Goldman et al. [14] was used. The EPR spectra were recorded with increasing concentration of glycerol or sugar (Fig. 3). The rigid limit value for $2A'_{zz}$ at $\eta \to \infty$ was obtained by a least square fit procedure, and was (6.674 $\pm$ 0.03) mT for MSL.

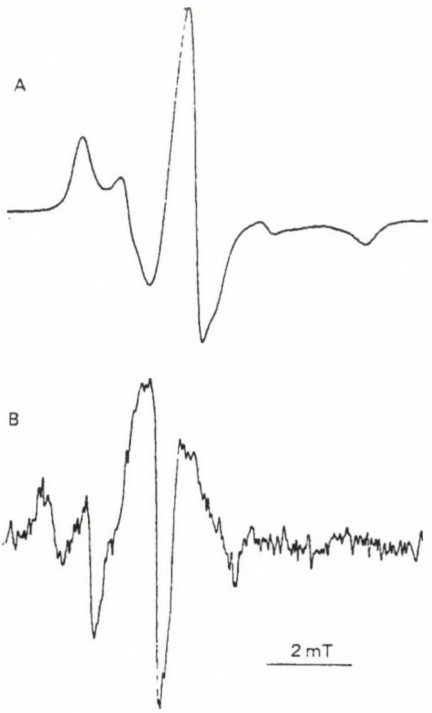

Fig. 2. Conventional (A) and ST EPR spectrum (B) of bovine cardiac myosin labelled with MSL. Spectra were taken at 20 °C. The concentration of myosin was 20 μM

This value was significantly larger than the $2A'_{zz}$ value which was measured on myosin minifilaments ($2A'_{zz}$ = 6.484 mT) or on myosin precipitate ($2A'_{zz}$ = 6.510 mT). Since the labels were strongly immobilized at $\eta \rightarrow \infty$, but not at side-by-side aggregate of myosin molecules, therefore the former value (6.674 mT) was used as rigid limit. In contrast, $2A_{zz}$ = 6.875 mT was obtained for maleimide labelled skeletal myosin at $\eta \rightarrow \infty$ and 6.800 $\pm$ 0.025 mT at 1 cP and 20 °C. The rotational correlation times of MSL myosins at room temperature and 1 cP were 42 ns (cardiac myosin) and 260 ns (skeletal myosin), respectively. Addition of glycerol to myosin precipitate resulted in a large increase of the hyperfine splitting indicating a strong effect of glycerol on the rate of rotational motion of labels in the head region of cardiac myosin, or at least in the environment of the attached labels. It should be noted that we did not observe remarkable change of the rotational correlation time for intact myosin and LC-2 deficient myosin evidencing that the removal of the LC-2 light chain from cardiac myosin did not affect the rotational motion of the attached MSL label [22]. The results suggest that (i) significant rotational mobility of the attached labels exists even in minifilament form and in the side-by-side aggregate of myosin molecules; (ii) there is a substantial difference in the flexibility of cardiac and skeletal

myosins; the effective rotational correlation time for cardiac myosin was 6 times shorter than that of for skeletal muscle myosin assuming that the labels were located on the same sites of myosin.

At low ionic strengths, where cardiac myosin was in filament form, the hyperfine splitting constant increased significantly and the value attained to $2A'_{zz} = 6.484 \pm 0.03$ mT at room temperature ($\tau_2 = 70$ ns). From ST EPR spectra of myosin filaments we calculated the spectral parameters L"/L and C'/C, they were

$$L"/L = 0.24 \pm 0.1 \ (n = 4) \text{ and}$$

$$C'/C = 0.041 \pm 0.15 \ (n = 4)$$

which correspond to rotational correlation times of 6 μs and 2 μs, respectively (Figs 1 and 2). These values indicate that the self-organization of myosin into filaments is accompanied with a strong immobilization of the labels in the head region of the myosin. It suggests that a significant portion of the mobility originates from the segmental flexibility of the protein.

The low-field spectral parameter, L"/L, in the ST EPR spectrum of glycerinated muscle fibres took up a value of

$$L"/L = 1.22 \pm 0.06 \ (n = 10) \text{ for skeletal muscle whereas}$$

$$L"/L = 0.83 \pm 0.1 \ (n = 4)$$

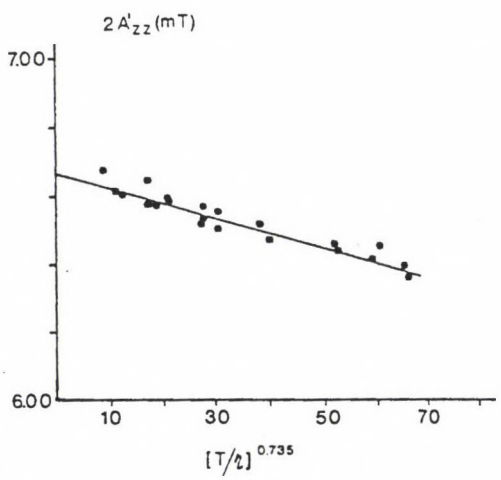

Fig. 3. Variation of spectral parameter $2A'_{zz}$ as function of viscosity $(T/\eta)^{0.735}$, T is the absolute temperature. Intact bovine cardiac myosin was spin-labelled with MSL, and the conventional spectra of myosin samples were taken at different viscosities. Viscosity was increased by addition of different amount of sucrose to the buffer solution. The extrapolated value of the hyperfine splitting at $\eta \to \infty$ gives the rigid limit value

was calculated for cardiac muscle fibres in rigor [4]. The values of τ_2 showed that the rate of the reorientation of the label in cardiac muscle fibres was faster approximately with one order of magnitude in rigor, evidencing some internal flexibility of cross-bridges even in their attached state.

Interaction of spin labelled myosin with ADP

The binding of ATP or ADP to IASL skeletal myosin resulted in a significant decrease in the proportion of the strongly immobilized label. The experimental data could be fitted by curves predicted for either one or two independent ADP binding sites and association constant in the range of $10–12.4 \times 10^2\ M^{-1}$. It was reported that the changes in the ratio of the first-two peak heights depended on whether the myosin was skeletal or cardiac myosin, the ratio at cardiac myosin was significantly lower indicating different local conformational changes in the two classes of myosins [29]. Since the reactive sulfhydryl sites are near the nucleotide binding pocket in the crystal structure of myosin [23], changes are expected in the environment of the probe molecules.

Our results on cardiac myosins showed that the conformational changes obtained after MgADP binding depended strongly on the probe molecules (Fig. 4). In these experiments we used the hyperfine splitting parameter as a measure of the rotational mobility. The increase of the mobility induced by MgADP varied with the concentration of nucleotide and it attained to a saturating level independently of the chemical structure of the spin label. The conformational change induced by ADP was significantly higher for IASL labelled myosin. Calculations gave evidence that cardiac myosins bound two moles of MgADP per mol of myosin. It is known that iodoacetamide probe molecules exhibit larger flexibility of the attaching linkage in comparison with maleimide molecules, therefore we can state that the myosin (MSL)-MgADP complex behaves like a rigid moiety on the time scale of the conventional EPR, whereas the IASL probe molecule is a sensitive monitor of the nucleotide binding, and its binding is accompanied with a local conformational change in the environment of the reactive sulfhydryl sites. Experiments performed on glycerol-extracted muscle fibres showed that the addition of MgADP to maleimide spin labelled fibres in rigor buffer did not result in significant axial rotation of the cross-bridges [34], but in the case of an isothiocyanate-based spin label, the nucleotide binding produced a remarkable change of the distribution of the attached labels with respect to the longer axis of the filaments, but no measurable change of the rate of rotational motion was observed in the ST EPR time domain after addition of MgADP [2]. We came to the conclusion that MgADP produced an intrinsic change in the multisubunit structure of the myosin head region, but this did not lead to the changes of the global rotational properties of the myosin heads.

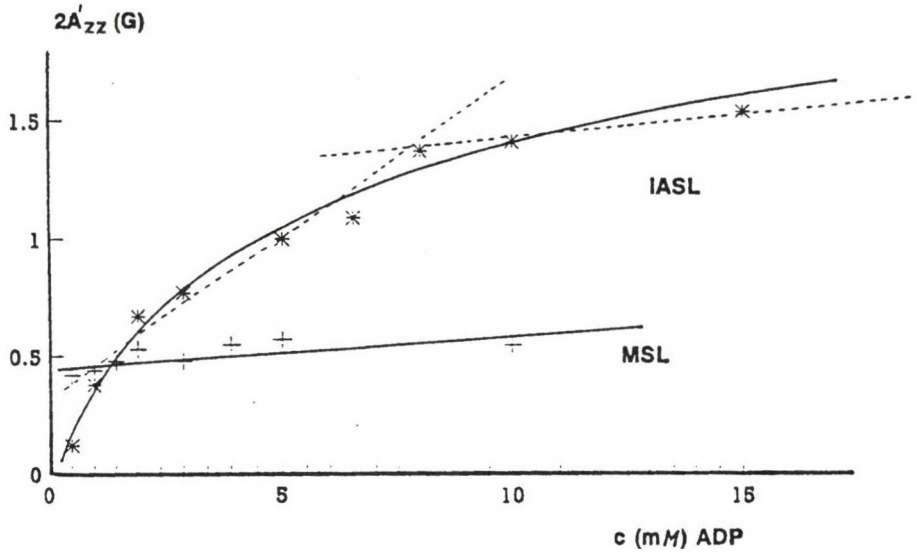

Fig. 4. The change of spectral parameter $2A'_{zz}$ as function of the MgADP concentration. Conventional EPR spectra of cardiac myosin labelled with either MSL or IASL spin labels were recorded after addition of MgADP at 20 °C. The incubation of samples lasted for 5 min. Symbols: * IASL bovine cardiac myosin, + MSL bovine cardiac myosin. From the experimental data the molar ratio of ADP bound to myosin was calculated. The intersection point of the dotted lines corresponds to about 2 moles of ADP to 1 mol of myosin

Orientational dependence of EPR spectra in macroscopically aligned actin filament system

It is known from earlier results that spin labels (MSL or IASL) attached to the fast reacting thiol sites of myosin in glycerinated muscle fibres in rigor exhibited large orientational order with respect to the fibre axis [32]. It is also known that the same orientational distribution of spin labels was detected when spin-labelled HMM penetrated into the muscle fibres and bound to actin [3]. The results indicated the particular attachment of myosin heads to actin in the absence of ligands as ATP, ADP or PP_i. It is expected that in macroscopically aligned actin filament system an orientation dependence of spin labels can be measured when spin labelled myosin or its fragments are allowed to bind to the filament system.

Our experiments on maleimide spin labelled myosin bound to actin have shown that $2A'_{zz}$ depended on the orientation of the aligned actin filaments with respect to the laboratory magnetic field. The measurements were performed in two orientations $\underline{H} \parallel \underline{k}$ and $\underline{H} \perp \underline{k}$, where $\underline{k}$ is the filament long axis. The orientational order was small, but differed significantly from zero. The ordering of spin labels did not depend on the

molar ratio of myosin to actin which showed that the orientational distribution of the of myosin heads/labels was determined entirely by the binding process on aligned actin filaments. In contrast, the binding of myosin or HMM to aligned actin filaments affected the distribution of labels located on actin.

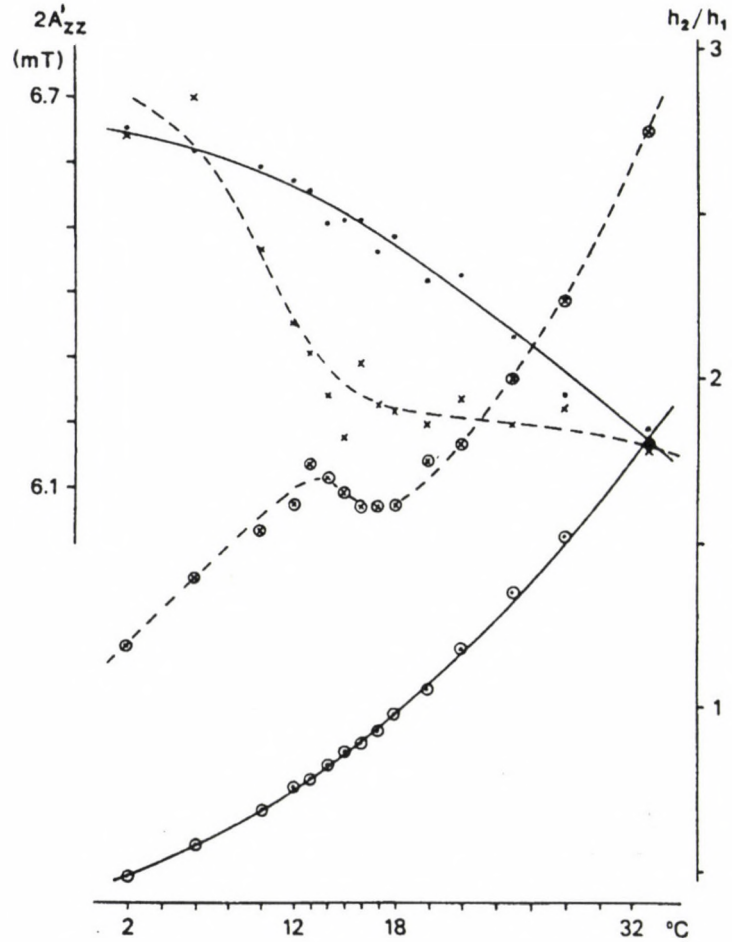

Fig. 5. Temperature dependence of $2A'_{zz}$ and h_2/h_1. Intact and LC-2 deficient myosins were labelled with MSL, and conventional EPR spectra were measured at different temperatures. At about 17 °C, abrupt changes in both spectral parameters were obtained on LC-2 deficient myosin. Symbols: $2A'_{zz}$: • intact myosin, × LC-2 deficient myosin; h_2/h_1: o intact myosin, ⊕ LC-2 deficient myosin

Temperature dependence of spectral parameters

The results on intact myosin and LC-2 deficient myosin labelled either with iodoacetamide or maleimide spin labels are presented in Fig. 5. Both the hyperfine splitting constant ($2A'_{zz}$) and the ratio of the peak heights (h_2/h_1) were used to characterize the temperature induced changes in the environment of the bound labels. It is assumed that the probe molecules bind to the same sites which can be controlled by measuring the ATPase activity of myosin in high ionic strength medium [9, 24]. However, the EPR spectra at different temperatures reported two resolved populations differing in rotational mobility. This conformational heterogeneity suggests two different populations of myosin heads which are in thermal equilibrium at a particular temperature, and transitions are allowed between the two forms of myosin. The structural change is the consequence of the intrinsic flexibility of the protein structure [12, 36]. At increasing temperature, a redistribution of the conformational substates can be observed: the population of myosin heads with smaller restriction of the rate of reorientation increases at the expense of the other population.

On myosin samples labelled with MSL we observed a continuous decrease of the hyperfine splitting together with a similarly continuous increase of the peak height ratio at rising temperature, evidencing the slowly loosening of the protein structure which was produced by the continuous heat absorption. In contrast, we could detect a rapid change of the peak height ratio at about 17 °C on LC-2 deficient myosin labelled with MSL, and simultaneously at the same temperature we obtained a large alteration in the shape of the curve for $2A'_{zz}$. The measurements in the ST EPR time range support the view that no sudden change in the domain motion (submillisecond time range) exists (Fig. 6). The results show that only the internal motion of the myosin heads is affected by the temperature.

Removal of the LC-2 light chain influenced greatly the motional dynamics of myosin heads labelled with MSL. Both $2A'_{zz}$ and h_2/h_1 spectral parameters showed significant changes at about 17 °C. This finding supports the conclusion that the

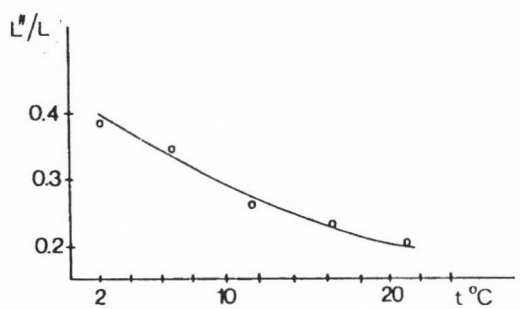

Fig. 6. ST EPR spectral parameter L"/L as a function of temperature. LC-2 deficient cardiac myosin labelled with MSL measured at different temperatures. The calculated spectral parameter decreases continuously with rising temperature

predominant change of myosin structure induced by temperature was a local conformational change in the neighbourhood of the thiol sites. Recent experiments showed that the segment containing the essential thiols, SH1 and SH2, is highly flexible [17]. Very likely, the LC-2 light chain modulates the flexibility of the 20 kDa segment of cardiac myosin that holds the label, and its removal affects the attaching linkage or the environment of the probe molecule. This suggests an intersite communication between LC-2 light chain and the 20 kDa segment of the motor domain.

DSC measurements

To gain further insight into the structural properties of cardiac myosins, DSC measurements were performed on intact and LC-2 deficient myosin in the temperature range of 5–60 °C. It is known that myosin is a multisubunit protein consisting of several domains, therefore a rather complex thermogram comprising at least four endothermic transitions are expected, corresponding to the thermal transition of the myosin rod with α-helical structure, and to those of the structural domains in the head region of myosin originally reported by Bálint and co-workers [1].

The complex heat capacity profile of intact myosin is shown in Fig. 7. Three major transitions could be observed with T_m = 17.5, 45 and 54.5 °C, as transition temperatures. Removal of the LC-2 light chain was accompanied with the disappearance of the 17.5 °C transition. The total enthalpy of the transitions was 1738 kcal/mol, similar results were obtained on skeletal myosin [5]. Studying the

Table I

Transition temperatures and enthalpies of myosin unfolding

Structural subunit	Transition temperature (°C)	Transition enthalpy (kcal/mol)
HMM 20 kD or LC-2	17.0	150*
HMM S-2	41.5	163
HMM 50 kD domain	45.0	377
HMM 27 kD + 20 kD	48.0	301
LMM	54.5	747

Intact and LC 2-deficient cardiac myosin was measured in buffer solution containing 500 mM KCl, 25 mM HEPES and 1 mM EDTA, pH 7.0. The concentration of the samples was 20 μM. The thermograms were taken with DASZM-4 Privalov-type differential scanning calorimeter with a scan rate of 0.25 °C/min.

Total enthalpy: 1738 kcal/mol

* The transition at 17 °C could not be detected on LC 2-deficient myosin.

melting profile of cardiac myosin we could identify five endothermic peaks at peak maximum of 17.5, 41.5, 45, 48 and 54.5 °C, the transition enthalpies were 150 kcal/mol, 163 kcal/mol, 277 kcal/mol, 301 kcal/mol and 747 kcal/mol. Our suggestion for the assignment of the transitions to the substructure of myosin are given in Fig. 7 and Table I. An accurate analysis by deconvolution allowed the identification of six transitions with total enthalphy of 1884 kcal/mol calculated from the part areas of the melting profile. The contribution of the LC-2 light chain was 151 kcal/mol.

According to the recent model of myosin, it is believed that it comprises structural units that fold up independently into a stable domain structure [1, 23, 35]. The measurements performed on skeletal myosin showed that the highest transition temperature could be assigned to the unfolding of the coiled-coil α-helix rod portion of the protein moiety [13]. More recent experiments provided evidence that the melting profile of myosin rod could be resolved into six quasi-two state transitions [5].

By examining the effect of mild heat treatment on ATPase and proteolytic sensitivity of myosin S 1, it was concluded that the 50 kDa segment of the myosin head could be preferentially unfold during heat treatment at 35 °C, while the 20 kDa and 27 kDa fragments remained unchanged [26]. It was shown by limited tryptic digestion that – in term of thermal stability – the head region of myosin comprised of two domains, the unstable 50 kDa domain which unfolded independently of the remainder structure, and the more stable 20 kDa and 27 kDa segments of the heavy chain [8]. It was proposed that the latter two segments and the light chain could melt

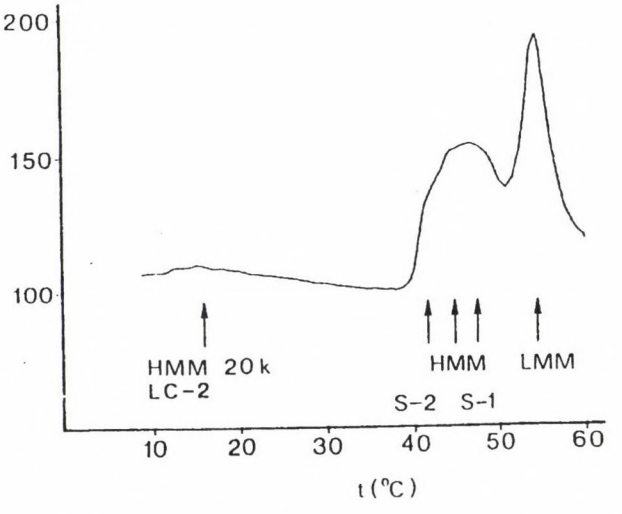

Fig. 7. DSC profile of intact cardial myosin. The heating current (I) in arbitrary units is plotted against temperature. Five transitions were identified, the assignment of the different peaks to the subunits of myosin is designationed by arrows

together as a cooperative unit. Recent measurements on the thermal unfolding of skeletal myosin gave evidence that the first transition of the profile 41 °C was very likely due to the thermal transition of the S 2 region [27]. Considering all the experimental data it seems to be not unreasonable to assign the thermal transitions to the following structural subunits: HMM S2 at T_m = 41.5 °C, HMM 50 kDa at T_m = 45 °C, HMM 20 kDa + 27 kDa at T_m = 48 °C and LMM at T_m = 54.5 °C (Table I). The higher T_m values calculated for the domains of HMM in our experiments can be explained by the difference of the conditions of the measurements (sensitivity of the DSC apparatus, different buffer solution, protein concentration). Observations performed on subfragment 2 region of myosin showed an endotherm peak at 55 °C as well, arising from the melting of helical regions of S 2 [30]. The peak of this transition might interfere with that of LMM.

For the interpretation of the lowest endothermic transition we have two possibilities: (i) conformational change in the LC-2 light chain of the myosin; (ii) more likely it could be related to the inherent instability of the 20 kDa segment. This region is stabilized by the interaction of the LC-2 light chain and the 20 kDa segment of the myosin, the stabilization energy might correspond to the transition enthalpy. This assumption might correlate with the EPR observation on the conformational transition at 17 °C. The removal of the LC-2 light chain increases the instability of the 20 kDa domain which is accompanied with the increase of the flexibility and with the enhancement of the rate of motion. The larger mobility is quenched in the presence of the LC-2 light chain. We can only speculate that the flexibility changes observed here are of importance to the transmission of signals from regulatory domain to the catalytic domain allowing the switching of the myosin motor. The mechanism by which the conformational changes are directly induced in the large myosin head remains to be established.

Acknowledgements

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REFERENCES

1. Bálint, N., Sréter, F. A., Gergely, J.: Fragmentation of myosin by papain-studies on myosin from adult fast and slow skeletal and cardiac, and embryonic muscle. Arch. Biochem. Biophys., **168**, 557–566 (1975).
2. Belágyi, J., Frey, I., Pótó, L.: ADP-induced changes in ordering of spin-labelled myosin heads in muscle fibres. Eur. J. Biochem., **224**, 215–222 (1994).
3. Belágyi, J., Pallai, G., Gróf, P., Trombitás, K.: Interaction of heavy meromyosin and actin in myofibrils. A spin label study. Studia Biophysica, **73**, 159–164 (1978).

4. Belágyi, J., Röth, E.: Orientation dependence and motional properties of spin labels in cardiac and skeletal muscle fibres. Gen. Physiol. Biophs., **6**, 571–581 (1987).
5. Bertazzon, A., Tian, G. H., Tsong, T. Y.: Differential scanning calorimetry (DSC) study of thermal unfolding of myosin and its subfragments in several forms of assemblies. Biophys. J., **53**, 236a (1988).
6. Bouvagnet, P., Léger, J. O. C., Dechesne, C., Léger, J. J.: Fiber types and myosin types in human atrial and ventricular myocardium. Circ. Res., **55**, 794–804 (1984).
7. Brenner, B.: Muscle mechanics and biochemical kinetics. In Molecular Mechanism in Muscular Contraction, ed. Squire, J., Mcmillan Press, London 1990, pp. 77–149.
8. Burke, M., Zaager, S., Bliss, J.: Substructure of skeletal myosin subfragment 1 revealed by thermal denaturation. Biochemistry, **26**, 1492–1496 (1987).
9. Crowder, M. S., Cooke, R.: The effect of myosin sulphydryl modification on the mechanics of fibre contraction. J. Mus. Res. Cell Motil., **5**, 131–146 (1984).
10. Fajer, P., Marsh, D.: Microwave and modulation field inhomogenities and effect of cavity Q in saturation transfer EPR spectra. Dependence of sample size. J. Mag. Res., **49**, 212–224 (1982).
11. Flicker, P. F., Wallimann, T., Viber, P.: Electron microscopy of scallop myosin: location of regulatory light chains. J. Mol. Biol., **169**, 723–741 (1983).
12. Fraunfelder, H., Sligar, S. G., Wolynes, P. G.: The energy landscapes and motions of proteins. Science, **254**, 1598–1603 (1991).
13. Gerasimov, V. V., Megrelisvili, I. S., Zalisvili, M. M., Gasseliadze, H. A., Nadirasvili, N. S., Sraibman, F. O.: Heat denaturation of muscle proteins. In Biophysics and Biochemistry of Muscle Contraction (Russian) ed. G. M. Frank, Nauka, Moscow 1976, pp. 29–33.
14. Goldman, S. A., Bruno, G. V., Freed, J. H.: Estimating slow-motional rotational correlation times for nitroxides by electron spin resonance. J. Phys. Chem., **76**, 1858–1860 (1972).
15. Graceffa, P., Seidel, J. C.: A reaction involving protein sulfhydryl groups, a bound spin-label, and $K_3Fe(CN)_6$ as a probe of sulfhydryl proximity in myosin. Biochemistry, **19**, 33–39 (1980).
16. Hoffmann, U., Belágyi, J.: Conformational changes of intact and LC-2 deficient myosin from bovine heart as revealed by EPR, DSC, and hydrodynamic studies. Hoppe-Seyler's Z. Physiol. Chem., **372**, 679 (1991).
17. Huston, E. E., Grammer, J. C., Yount, R. C.: Flexibility of the myosin heavy chain: direct evidence that the region containing SH1 and SH2 can move 10 A° under the influence of nucleotide binding. Biochemistry, **27**, 8945–8952 (1988).
18. Huszár, G., Elzinga, M.: Homologous methylated and nonmethylated histidine peptides in skeletal and cardiac myosins. J. Biol. Chem., **247**, 745–753 (1972).
19. Huxley, H. E.: The mechanism of force generation in striated muscle. Science, **202**, 1356–1366 (1969).
20. Kuehl, W. M., Adelstein, R. S.: The absence of 3-methylhistidine in red, cardiac and fetal myosin. Biochem. Biophys. Res. Commun., **39**, 956–964 (1970).
21. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, P. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193**, 265–275 (1951).
22. Lőrinczy, D., Hoffmann, U., Pótó, L., Belágyi, J., Laggner, P.: Conformational changes in bovine heart myosin as studied by EPR and DSC techniques. Gen. Physiol. Biophys., **9**, 589–603 (1990).
23. Rayment, I., Rypniewski, W. R., Schmidt-Bäse, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G., Holden, H. M.: Three-dimensional structure of myosin subfragment-1: a molecular motor. Science, **261**, 50–58 (1993).
24. Schaub, M. C., Watterson, J. G., Waser, P. G.: Radioactive labeling of specific thiol groups as influenced by ligand binding. Hoppe-Seyler's Z. Physiol. Chem., **356**, 325–339 (1975).
25. Seidel, J.: Similar effect on enzymatic activity due to chemical modification of either of two sulfhydryl groups of myosin. Biochim. Biophys. Acta, **180**, 216–219 (1969).

26. Setton, A., Mühlrad, A.: Effect of mild heat treatment on the ATPase activity and proteolytic sensitivity of myosin subfragment 1. Arch. Biochem. Biophys., **235**, 411–417 (1984).
27. Shriver, J. W., Zegar, I., Kamath, U.: Differential scanning calorimetry of the unfolding of myosin fragments. Biophys. J., **53**, 236a (1988).
28. Shiverick, K. T., Thomas, L. L., Alpert, N. R.: Purification of cardiac myosin. Application to hypertrophied myocardium. Biochim. Biophys. Acta, **393**, 124–133 (1975).
29. Stone, D. B., Mendelson, R. A., Botts, J., Cheung, P. H.: EPR and fluorescence depolarization studies of bovine cardiac myosin. Circ. Res., **49**, 677–684 (1981).
30. Swenson, C. A., Ritchie, P. A.: Conformation transitions in the subfragment-2-region of myosin. Biochemistry, **19**, 5371–5375 (1980).
31. Tada, M., Bailin, G., Bárány, K.: Proteolityc fragmentation of bovine heart heavy meromyosin. Biochemistry, **8**, 4842–4850 (1969).
32. Thomas, D. D., Cooke, R.: Orientation of spin-labeled myosin heads in glycerinated muscle. Biophys. J., **32**, 891–906 (1980).
33. Thomas, D. D., Seidel, J. C., Gergely, J., Hyde, J. S.: The quantitative measurement of rotational motion of the subfragment-1 of myosin by saturation transfer EPR spectroscopy. J. Supramol. Struct., **3**, 376–390 (1975).
34. Thomas, D. D., Svenson, E. C., Polnaszek, C. F.: ADP does not induce rigid axial rotation of myosin heads in rigor muscle fibres. Biophys. J., **37**, 380a (1985).
35. Vibert, P., Cohen, C.: Domains, motions and regulation in the myosin head. J. Musc. Res. Cell Motil., **9**, 296–305 (1988).
36. Wells, C., Bagshaw, C. R.: Segmental flexibility and head-head interaction in scallop myosin. J. Mol. Biol., **164**, 137–157 (1983).
37. Winkelmann, D. A., Almeda, S., Vibert, P. J., Cohen, C.: A new myosin fragment: visualization of the regulatory domain. Nature, **307**, 758–760 (1984).

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Effect of radioprotectors on the aggregability, cAMP and thiol content of blood platelets[1]

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Aminothiol radioprotectors (MEA, AET, WR-1065) were found to be inhibitors of platelet aggregation. The aim of this study has been to clarify the potential involvement of the adenylate cyclase-cAMP system in this effect.

In vitro aminothiol compounds (> 1 mmol/l) inhibited platelet function as well as AC-activity, i.e. significantly reduced their cAMP content. In less than 1 mmol/l concentration WR-1065 elevated the cAMP content of PGI_2-stimulated platelets, while basal cAMP level mainly remained unchanged. On the effect of WR-1065 the level of non-protein thiols (NP-SH) markedly elevated in the platelets, while their protein-bound thiol content (PB-SH) either decreased or remained at control level. The phosphorothioate WR-2721 was effective only after *in vitro* or *in vivo* dephosphorylation. Although the amount of NP-SH slightly increased, the PB-SH content decreased in the platelets isolated after intravenous administration of WR-2721. Moreover, platelet-poor plasma samples of WR-2721 treated animals also inhibited both the AC-activity and function of control platelets, too.

Based on these results it is suggested that aminothiol radioprotectors non-specifically inhibit platelet functions forming mixed disulfides with endogenous, mostly protein-bound thiols. The slight elevation of AC-activity caused by low doses of WR-1065 also suggest that the AC-complex consists of unevenly sensitive subunits (G-proteins, catalytic subunit).

Keywords: radioprotective compounds, blood platelet aggregation, adenylate cyclase, cAMP, SH-content

Jókay et al. reported first that the agglutination of thrombocytes (blood platelet aggregation) obtained *in vitro* by the addition of bacterial endotoxins could be inhibited by L-cysteine, β-mercaptoethanol and glutathione. At the same time a

[1] This paper is dedicated to the memory of Professor Ferenc Varga (1929–1994).

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reduction in the liberation of histamine and serotonine (5-HT) which accompanied agglutination was observed [19]. Sztanyik et al. found that S-2-aminoethyl-isothiuronium (AET) and some other radioprotective aminoalkylthiol compounds increased the clotting time of the recalcified rabbit plasma [29]. During further research evidence of hypocoagulability was proved in rats after intraperitoneal injection of cystamine [38] and AET [23], respectively. Mixed disulfide formation between the thiol (SH) groups of the radioprotector and pro-coagulant proteins were supposed as to be the reason of this effect at that time. After intraperitoneal injection of AET, we really observed local subcutaneous bleeding rather often in the neighbourhood of the site of the injection, indicating local hemostatic disturbances caused by the radioprotector. It was also found that AET, cysteamine (mercaptoethylamine or MEA) and S-2-(3-aminopropylamino)-ethylthiol (WR-1065) but not WR-2721, phosphorothioate derivative of the latter, decreased prostacyclin formation in rat aorta rings and malondialdehyde (MDA) formation from arachidonic acid by blood platelets, respectively [16, 17, 18]. Based on these data, aminoalkylthiol radioprotective compounds were expected to inhibit arachidonic acid-induced blood platelet aggregation, too. Indeed, recently we proved their anti-aggregating effectiveness against various platelet activators (pro-aggregators) including adenosine-diphosphate (ADP), arachidonic acid (AA), thrombin, collagen as well as the calcium ionophore A-23187. However, the mechanism of their anti-aggregating effect has not been revealed so far.

It is known that aggregability and adenylate cyclase (AC) activity is inversely proportional in platelets [1, 4, 11, 13, 14]. Elevation of the cAMP content in platelets will inhibit the phospho-inositol metabolism [10, 39], i.e. the other main signal transduction mechanism, as well as promote the sequestration of the intracellular ionized calcium into inner granules or storage pools [41].

The aim of our present work was to evaluate the potential involvement of this well-known platelet aggregation regulatory system in the *in vivo* anti-aggregating effect of this type of radioprotectors. As in other experiments AC enzyme complex proved to be rather sensitive to different thiol blocking agents [31, 32], along with cAMP level and the stimulability of AC by prostacyclin (PGI_2), platelet thiol content was also studied following intravenous WR-2721 treatment or after *in vitro* incubation with elevating concentrations of WR-1065, respectively.

Methods

RAxLE/F_1 and Wistar strain male rats and New Zealand male rabbits were used in these experiments. Animals were kept on standard diet and supplied by tap water. In order to prevent the disturbing effect of the large size plasma lipids, especially chylomicrons, animals were starved for 24 hours before the experiment.

Blood collection and platelet isolation [6]

Under diethyl ether anaesthesia blood was collected from rabbits by cardiac puncture or from the abdominal part of the aorta in the case of rats into plastic syringes containing ACD (2.5 g of trisodium citrate, 1.5 g of citric acid, 1.0 g glucose/100 ml H_2O) anticoagulant in the ratio of 1:10. In order to get platelet rich plasma (PRP), blood was centrifuged at 150 g for 20 minutes at room temperature. To remove the remaining red and white blood cell contamination PRP was centrifuged at 100 g for further 10 minutes. Platelet poor plasma (PPP) was obtained after the removal of PRP by centrifuging the remaining blood at 1500 g for 10 minutes.

Platelet suspension was prepared from the PRP by differential centrifugation and washing procedure described by Mustard [21] with some modifications. As rat platelets do not tend to activate spontaneously during gentle sedimentation, lowering the pH of the blood and the washing buffer to 6.5 by the use of ACD was generally enough to avoid unwanted platelet aggregation. Therefore, in contrast to the original method, inhibitors such as apyrase and heparin were not used during platelet isolation. In the case of rabbit platelets either the original centrifugal procedure, or gel-filtration method using Sepharose 2B [30, 35] was followed for platelet isolation. Washed or filtrated platelets were resuspended in calcium-free Tyrode-Hepes buffer (137 mmol/l NaCl; 2.7 mmol/l KCl; 11.9 mmol/l $NaHCO_3$; 0.5 mmol/l NaH_2PO_4; 1.0 mmol/l $MgCl_2$; 1 g/l dextrose; 10 mmol/l Na^+-Hepes; pH = 7.4) containing 2 mg/ml bovine serum albumin (BSA). Platelets were counted by Laborscale (Medicor, Hungary) cell counter, then the platelet suspension was diluted to the final platelet count 5×10^8/ml in the different incubation media.

Measurement of platelet aggregation

The *in vitro* effect of radioprotectors on platelet aggregation was tested on rat PRP using Chronolog (M 300) one-channel aggregometer. According to the original platelet aggregation method described by Born [2], stirred PRP samples were incubated with increasing concentrations of different radioprotectors at 37 °C for three minutes before inducing platelet aggregation by 25 μmol/l ADP. The nadirs of the aggregation curves were compared to the appropriate control and were expressed as percentage inhibition. On the basis of the anti-aggregating dose-effect curves, $IC_{50\%}$ concentration of the different aminoalkylthiol radioprotectors and their non-thiol derivatives was computed.

Measurement of platelet cAMP content

The *in vitro* and *ex vivo* effects of radioprotectors on the basal cAMP content and on the stimulability of AC by prostacyclin were studied. In the case of *in vitro* experiments aliquots of platelet suspension were incubated with WR-1065 at 37 °C. In order to stimulate adenylate cyclase 100 ng/ml prostacyclin (PGI_2) was added to one half of the samples one minute before terminating the incubation. Optimal prostacyclin concentration and incubation time were based on experiments (see Fig. 1). In some experiments a known phospho-diesterase enzyme inhibitor 1-methyl-3-isobutylxanthine (MIX) was used to get further elevation of platelet cAMP.

In the *ex vivo* experiments rats were treated with 200 mg/kg WR-2721 intravenously. Then a 10-min blood sample was collected, and isolated platelets were incubated with the same amount of platelet-poor plasma (PPP) prepared from either control or treated animals. In one half of the samples platelet AC was also stimulated by prostacyclin as described before.

Incubation was terminated by the addition of the same amount of ice-cold 10% (w/v) trichlor-acetic acid (TCA). After a few seconds of further stirring, samples were placed in ice-cold water bath until cooled down to 0 °C, then quickly sedimented by an Eppendorf-type centrifuge. In order to remove TCA, supernatants were extracted three times using water-saturated diethyl-ether. Finally, diethyl-ether contamination was removed by bubbling the samples with N_2 gas for ten minutes. The cAMP content was

determined by the RIA method of Brooker et al. [5, 33]. The amount of cAMP was expressed as pmol/10^8 platelets or as the percentage of the appropriate control.

Measurement of plasma and platelet thiols

Total thiol (T-SH) content of the different samples was determined as originally described by Ellman [12], using 5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB thiol reagent. Non-protein SH (NP-SH) subfraction was measured by the method of Sedlak et al. [25]. Protein-bound (PB-SH) content was computed as the difference of the former two. Either the SH-content or its change was expressed as nmol/mg protein.

Results

The *in vitro* 50 percent inhibitory concentrations ($IC_{50\%}$) of some aminoalkyl-thiol compounds and their non-thiol derivatives made on the adenosine-diphosphate-induced aggregation of rat platelets are listed on Table I.

It seems evident from the list that all compounds, which have at least one free SH-group was able to inhibit platelet aggregation at some extent. AET, which spontaneously transforms to the free SH-derivative, mercaptoethyl-guanidine (MEG), in waterous solutions around neutral pH proved to be the most effective with its $IC_{50\%}$ of 2.52 $\pm$ 0.39 mmol/l. The least effective thiol-compound was WR-151326 with its $IC_{50\%}$ value of 12.14 $\pm$ 0.61 mmol/l. For the datailed explanation of the reduced or lacking anti-aggregatory effectiveness of the non-thiol derivatives we refer to the discussion part of this article.

Figure 1 shows the *in vitro* effect of prostacyclin (PGI_2), a well-known adenylate cyclase challenger, on the cAMP content of rat blood platelets. Being a rather unstable metabolite of the arachidonic acid, prostacyclin caused the rapid but transient activation of platelet AC, as it is indicated by the elevated level of cAMP. Therefore, it was very important to determine the concentration and incubation period of prostacyclin that provided optimal AC stimulation further on. Based on the curves depicted in Fig. 1, we generally added 100 ng/ml prostacyclin to the platelet samples at the beginning of the last minute of incubation.

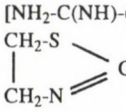
The upper part of Fig. 2 shows the *in vitro* effect of 25 μmol/l–20 mmol/l WR-1065 on the cAMP content of rat blood platelets.

Cyclic AMP content of 10^8 platelets was 3.783 $\pm$ 0.45 pmol in resting conditions and 92.6 $\pm$ 7.65 pmol after challenging platelet adenylate cyclase by 100 ng/ml prostacyclin for one minute, respectively. Basal platelet cAMP level did not differ from control until WR-1065 concentration exceeded 1 mmol/l. Beyond this drug level, a concentration-dependent decrease occurred resulting in the almost 60% loss of the starting platelet cAMP content at 20 mmol/l WR-1065. In contrast, stimulation of platelet adenylate cyclase by prostacyclin during the last minute of the incubation caused a relatively higher and statistically significant ($p < 0.01$) cAMP increase when WR-1065 was present at less than 0.5 mmol/l concentration. The highest cAMP ratio

of the treated versus control platelets was 1.35 ± 0.13 at 0.1 mmol/l WR-1065. In higher than 0.5 mmol/l the radioprotector caused a more pronounced decrease in the platelet cAMP ratio than it occurred in resting platelets. Although only the elevated dose-range of WR-1065 was used, the same inhibition was observed in rabbit platelets, as it is indicated on the bottom of Fig. 2.

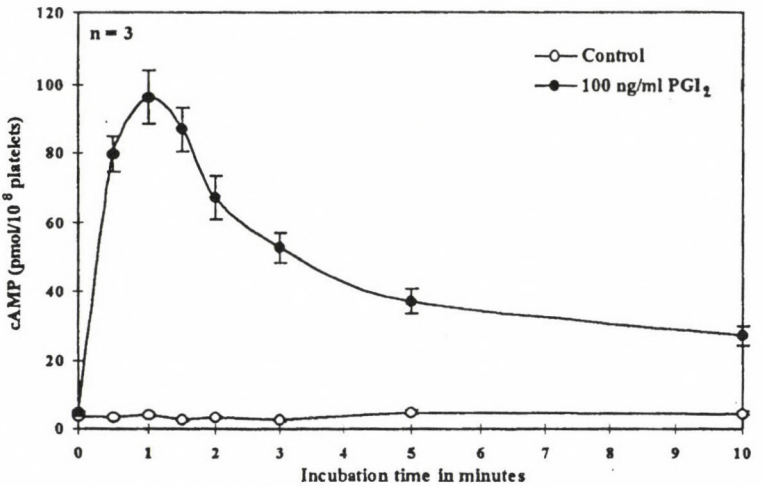
Table I

Effect of aminothiol radioprotective agents and their non-thiol derivatives on the ADP-induced platelet aggregation in rats

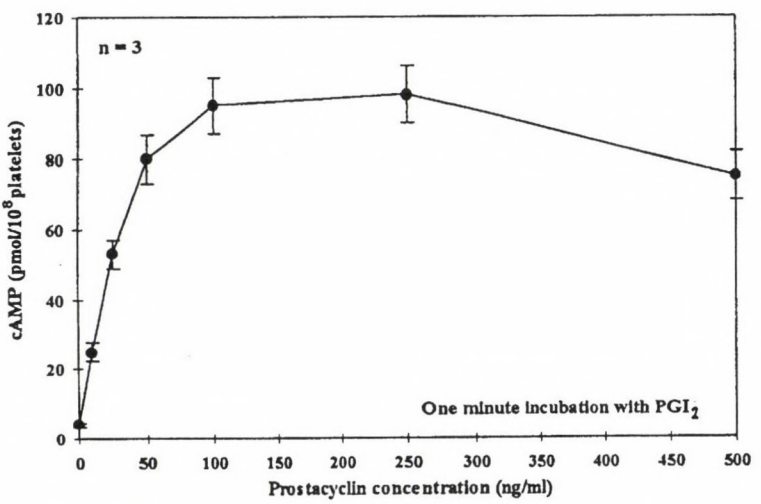
Aminothiol compounds and derivatives	Chemical structure	Free SH groups	Anti-aggregating $IC_{50\%}$ (mmol/l)
AET → MEG	NH_2-C(NH)-S-$(CH_2)_2$-NH_2 → NH_2-C(NH)-$(CH_2)_2$-SH	Yes	2.52 ± 0.39
GED (-S-S-)	[NH_2-C(NH)-$(CH_2)_2$-S-]$_2$	No	9.95 ± 0.45
2-aminothiazoline (2-AT)		No	Ineffective
Cysteine	COOH-CH(NH_2)-CH_2-SH	Yes	3.81 ± 0.41
Cysteamine (MEA)	NH_2-CH_2-CH_2-SH	Yes	5.53 ± 0.30
Cystamine (-S-S-)	NH_2-CH_2-CH_2-S-S-CH_2-CH_2-NH_2	No	ND*
Thiola (MPG)	CH_3-CH(SH)-CO-NH-CH_2-COOH	Yes	4.08 ± 0.53
Glutathione (GSH)	Propionyl-cysteinyl-glycine	Yes	3.42 ± 0.29
WR-2721 (Ethiofos)	NH_2-$(CH_2)_3$-NH-$(CH_2)_2$-SPO_3H_2	No	Ineffective
WR-1065	NH_2-$(CH_2)_3$-NH-$(CH_2)_2$-SH	Yes	8.18 ± 0.53
WR-3689	CH_3-NH-$(CH_2)_3$-NH-$(CH_2)_2$-SPO_3H_2	No	Ineffective
WR-151326	CH_3-NH-$(CH_2)_3$-NH-$(CH_2)_3$-SH	Yes	12.14 ± 0.61

*: No Data

Platelet-rich-plasma (PRP) was prepared from rat blood as described in Methods. Stirred PRP samples (0.5 ml) were incubated with the radioprotective drugs at 37 °C for three minutes before the addition of 25 μmol/l adenosine-diphosphate aggregating agent. Anti-aggregating dose-effect curves were completed using five different concentrations of each compounds and $IC_{50\%}$ concentrations were computed. Average values represent the means ± SD of at least three different experiments with multiple determinations.

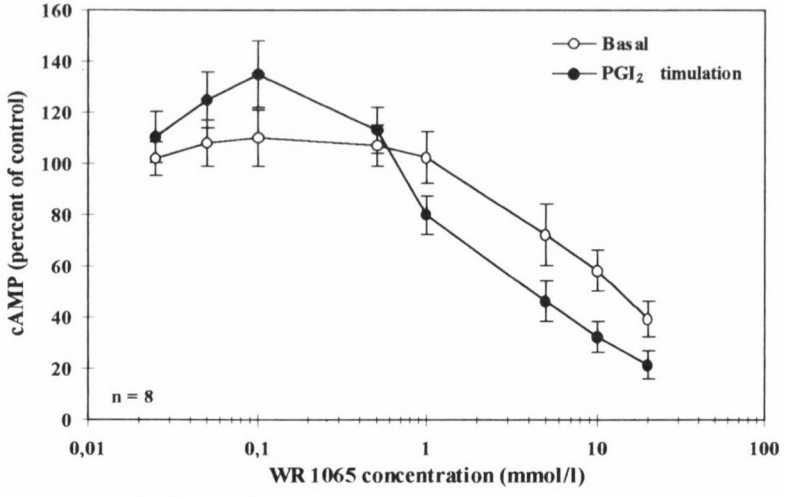


a: Time course of the PGI_2 stimulation

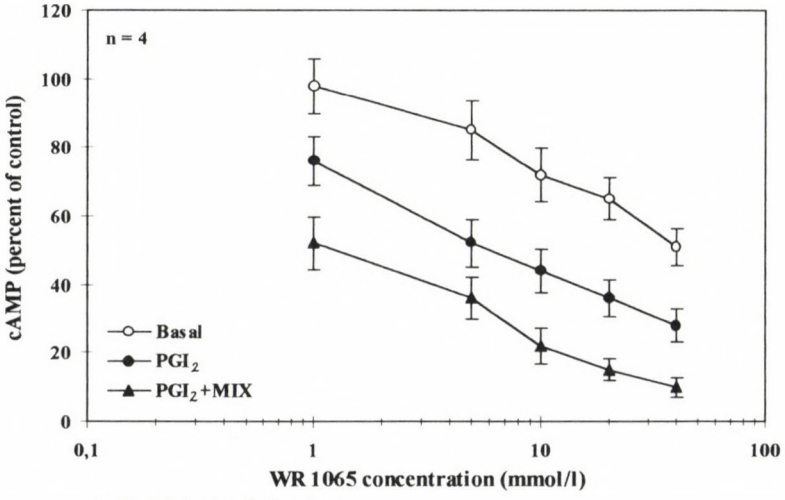


b: Concentration dependence of the PGI_2 stimulation

Fig. 1. Effect of adenylate cyclase stimulation by prostacyclin (PGI_2) on the cAMP content of rat blood platelets. Aliquots of rat blood platelets were incubated without or with elevating concentrations (a) of prostacyclin (PGI_2) for different time (b), and platelet cAMP content was determined. Based on these results, a one-minute incubation with 100 ng/ml PGI_2 was applied further on to reach optimal stimulation of platelet adenylate cyclase



a: Rat blood platelets



b: Rabbit blood platelets

Fig. 2. *In vitro* effect of WR 1065 on the cAMP content of blood platelets in rats and rabbits. Aliquots of rat and rabbit washed platelet suspensions were incubated with elevating concentrations of WR-1065 at 37 °C for 10 min. In order to stimulate platelet adenylate cyclase, 100 ng/ml prostacyclin was added to one of the series of the incubates at the end of the ninth minute. In case of rabbit platelets, a further series was also included, using 0.1 mmol/l 1-methyl-3-isobutylxanthine (MIX) as phosphodiestrase inhibitor to avoid the subsequent cAMP metabolism. The values are expressed as the percent of controls to facilitate the comparison of the effect of WR-1065 on the cAMP levels measured without and in the presence of PGI_2. Controls were incubated under the same conditions as treated samples but without WR-1065. For the absolute values of the appropriate controls, see the text

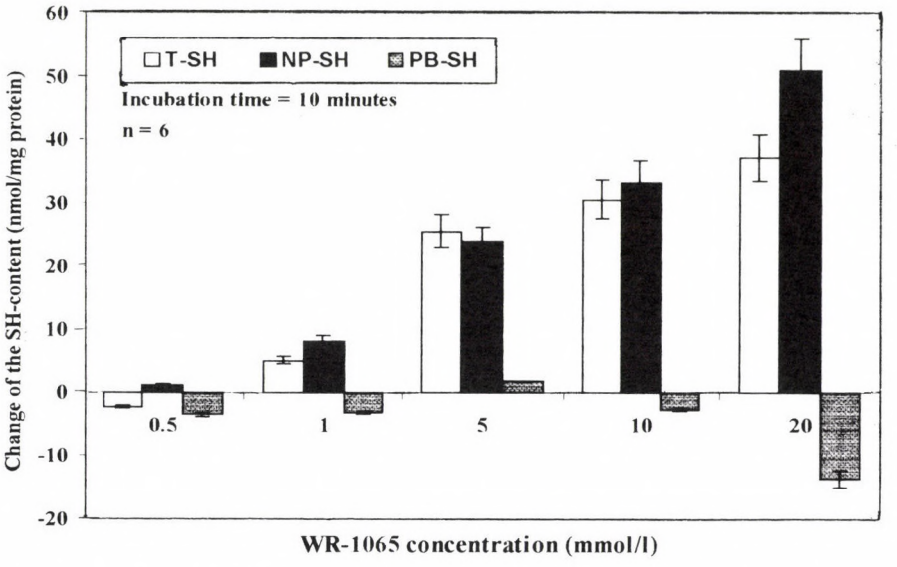


Fig. 3. *In vitro* effect of WR-1065 on the SH-content of rat blood platelets. Platelet suspension was incubated with elevating concentration of WR-1065 for 10 minutes at 37 °C under continuous stirring. Aliquots taken from the incubates were immediately cooled down to 0 °C and platelets were separated by an Eppendorf-type centrifuge. Total (T-SH) and non-protein (NP-SH) thiol content of the platelets were determined as described in Methods. Protein-bound thiol (PB-SH) content was computed from the above two values

Time and concentration dependent changes of the total (T-SH), non-protein (NP-SH) and protein-bound (PB-SH) thiol contents are depicted in Fig. 3 after incubating rat blood platelets with WR-1065 *in vitro*.

The drug uptake by the platelets seems evident even from the time and concentration dependent increase of the total and non-protein thiol content of the centrifuged and washed-out platelet pellet. Considering the mean volume and protein content of platelets, one can conclude that platelets are able to concentrate WR-1065 inside, i.e. the computed ratio of the intracellular versus outer concentration of the radioprotector was 2–5. On the other hand, the *in vitro* incubation of platelets with elevating concentrations of WR-1065 for different times did not affect the protein-bond thiol fraction as consequently as in the case of T-SH and NP-SH. Nevertheless, the main tendency has either remained unaffected or it has even decreased, suggesting the formation of mixed disulfide bounds.

When platelets were incubated with WR-2721 instead of WR-1065, none of the thiol fractions has changed significantly. However, after hydrolysing the washed-out platelet pellet with 0.1 mol/l HCL at 70 °C, the NP-SH content of the hydrolysate was significantly higher in comparison with the untreated control, i.e. the phosphorothioate derivative was taken up by platelets, as well. The reason for the *in vitro* ineffectiveness

of WR-2721 on platelet function might be their inability to dephosphorylate the drug to the effective thiol-compound (WR-1065) due to the lack of alkaline phosphatase activity.

In order to study whether the usual radioprotective dose of WR-2721 can alter cAMP formation *in vivo*, rats were treated with 200 mg/kg WR-2721 intravenously, then ten minutes later plasma and platelet thiol fractions as well as platelet cAMP contents were measured, respectively. Figure 4 summarizes the results of our cross-incubation experiments aimed at proving both the *in vitro* and *in vivo* effect of the radioprotector or its thiol derivative.

Platelets and platelet-poor plasma (PPP) samples were isolated from the treated and control animals. As the black columns indicate in Fig. 4 both the basal and PGI_2-challenged cyclic AMP content of platelets obtained from the WR-2721 treated animals, significantly decreased irrespective of being incubated in the PPP of either the control or the treated animals. On the other hand, cAMP formation also damaged in control platelets when they had been incubated in the PPP of the treated group. It

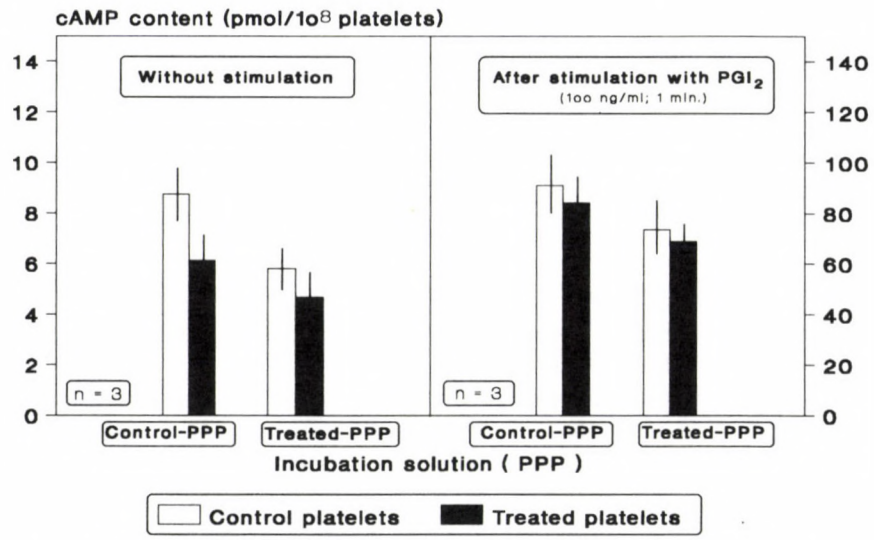


Fig. 4. Effect of WR-2721 treatment on the cAMP content of blood platelets in rats. 200 mg/kg WR-2721 was added intravenously to Wistar rats. Control animals were treated with physiological salt solution. After 10-min blood was collected, platelets were isolated by centrifugation and resuspended in calcium-free Tyrode-Hepes buffer (pH = 7.4) containing 2 mg/ml BSA with a platelet count of 10^9/ml. Platelet-poor plasma (PPP) was also preserved for later usage as incubation medium. The same amounts of platelet suspension and PPP were mixed and incubated at 37 °C for 10 minutes with continuous stirring in – as we called it – cross-incubation experiments. Platelets isolated from both the control and WR-2721 treated animals were incubated either in their own and the other group's PPP, respectively. In order to stimulate platelet adenylate cyclase, 100 ng/ml PGI_2 (prostacyclin) was added to one series of incubates at the end of the ninth min

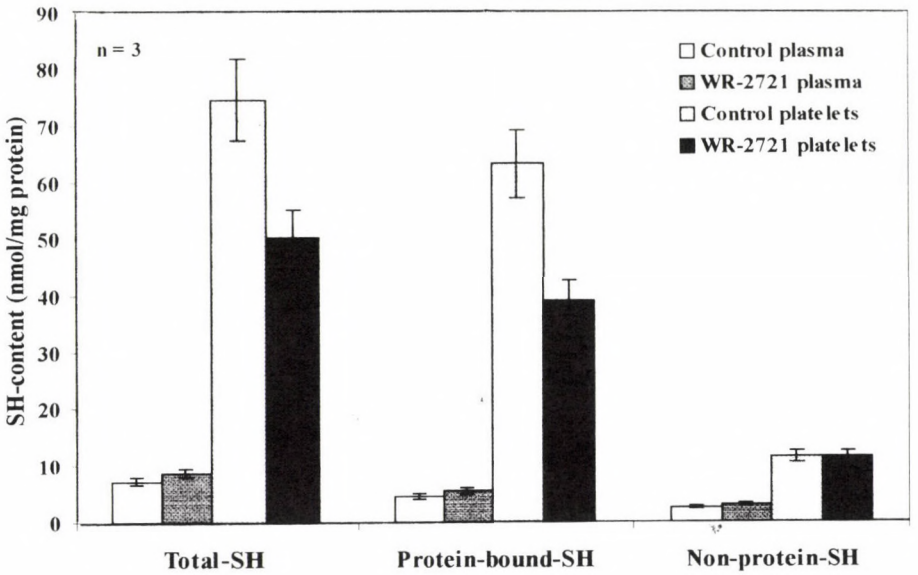


Fig. 5. *In vivo* effect of WR-2721 treatment (200 mg/kg i.v.) on the SH-content of the plasma and blood platelets in rats. Rats were treated with 200 mg/kg WR-2721 i.v. Following a 10-min blood collection was platelets were isolated from the plasma by centrifugation and resuspended in calcium-free Tyrode–Hepes buffer (pH = 7.4) with a platelet count of 10^9/ml. Total (T-SH) and non-protein (NP-SH) thiol content of the platelets as well as platelet-poor plasma samples (PPP) were determined and expressed in nanomol/mg protein

suggests that after ten minutes the amount of the effective, dephosphorylated thiol-metabolite in the PPP has been sufficient to inhibit adenylate cyclase, or to increase the cAMP metabolism by phosphodiesterase [34].

In Fig. 5 plasma and platelet thiol fractions are compared in control and WR-2721 treated rats. In the plasma the level of all thiol fractions slightly increased in the treated animals, however, it was not statistically significant. In contrast, under the effect of WR-2721 treatment, the amount of protein-bound thiols and consequently the total-SH content of the platelets markedly decreased, meanwhile their non-protein-SH content remained unchanged.

Discussion

Our first results that confirmed the cyclo-oxigenase inhibitory and anti-aggregating effectiveness of the best-known and formerly broadly investigated two aminoalkylthiol radioprotective compounds, AET and MEA date back to the mid eighties. Similar but weaker effects were observed with WR-1065 somewhat later. Since that time, further aminoalkylthiol compounds and a few of their non-thiol

derivatives have been studied in order to evaluate the possible involvement of the SH-groups in this process. Indeed, their anti-aggregating effect highly related to the presence or intactness of the free thiol groups, as it was emphasized in Table I. In the case of the phosphorothioate compounds, such as S-2-(3-aminopropylamino)-ethyl-phosphorothioic acid (WR-2721), or its methylamino relative, WR-3689, the appropriate as well as effective free thiol derivatives are only formed when the thiophosphate compound is subjected to enzymatic dephosphorylation. Nevertheless, our previous experiments proved that although blood platelets can take up significant amounts of WR-2721, they cannot convert it to WR-1065 due to the lack of platelet alkaline phosphatase activity (not reported data). On the other hand, symmetric disulfides of certain aminothiol compounds – especially cystamine – can be reduced even by platelets, hence inhibiting their response to pro-aggregators. During these experiments cystamine was not investigated, but previously we had found on rabbit PRP, that its anti-aggregating effectiveness was stronger than the appropriate free-SH derivative, cysteamine. A moderate reduction of guanido-ethyl-disulfide (GED) may also occur in platelets resulting in some amounts of the free SH-form of AET.

The role of cyclic nucleotides in chemical radioprotection, especially their involvement in the protective effect of aminothiol compounds is still debated, based on the diverse and rather contradictory experimental data reported so far. Treating animals with cAMP or dibutyryl cAMP prior to irradiation a significant protection was provided to various tissues, including bone marrow stem cells [24] and parotid gland [9, 27]. The increase of the survival of irradiated mice also was noted [20]. There is a body of clear evidence supporting the existence of a direct relationship between radioprotection provided by beta-adrenerg compounds (first of all isoproterenol), and their stimulating action on the AC–cAMP system [8].

As for the aminothiol radioprotective compounds, the picture is not so uniform. Langendorff et al. reported that the beta-adrenerg blocking Visken decreased the radioprotective effectiveness of AET, cysteamine and even of the *E. coli* endotoxin. As they achieved increased survival with cAMP and ATP treatment, they also suggested that "direct relation exists between the radioprotectors and the cyclic AMP mechanism" [20]. Some further results seemed to support this idea, as either the cyclic nucleotide content or the cAMP-dependent protein phosphorylation was found to be elevated in mouse tissues after aminothiol treatment [7]. The other half of the experimental results rather contradict the concept of cyclic nucleotide involvement in aminothiol radioprotection. Sodicoff et al. found that the radioprotection conferred by WR-2721 was not reduced by propranolol, showing that the beta-adrenerg receptors and consequently the AC activation played no part in WR-2721's action [28]. Trocha et al. observed a fluctuation with dominating decrease in cyclic nucleotide levels of rat liver and spleen after ip. administration of WR-2721 [36, 37]. It was also reported that iv. infusion of WR-2721 decreased the urinary excretion of cAMP in a patient with parathyroid carcinoma [15]. *In vitro*, 0.02–2.0 mmol/l WR-2721 caused a progressive decrease in intracellular cAMP in dispersed bovine parathyroid cells. In plasma membranes of the same cells a dose-dependent decrease in AC-activity was noted. WR-

2721 inhibited both basal and NaF, Gpp(NH)p, forskolin and pertussis toxin-stimulated adenylate cyclase [40]. Finally, in contrast to the *in vivo* cAMP elevation, neither AC nor cAMP phosphodiesterase activities of membrane preparations from various tissues had been affected by the *in vitro* administration of aminothiol radioprotectants [26]. These results suggest rather an indirect (due to occasional beta-adrenerg stimulation as pharmacological side-effect of some aminothiols) influence on the AC-cAMP system than a direct stimulation of the enzyme complex.

In our experiments we found that both the basal cAMP content of blood platelets and the effectiveness of the *in vitro* prostacyclin stimulation decreased following the intravenous administration of 200 mg/kg WR-2721. In contrast to the moderate increase of plasma thiols, platelet T-SH and PB-SH content also decreased at the same time.

In vitro WR-1065 – the thiol-derivative of WR-2721 – within the dose-range of 0.025–0.5 mmol/l did not affect the basal cAMP content of platelets, while the prostacyclin-induced AC stimulation increased. In the case of higher WR-1065 concentration both the basal and especially the stimulated cAMP levels dropped in a dose-dependent manner. In addition to the AC inhibition, the amount of protein bound thiols also decreased.

It is known that the different subunits of AC complex (the stimulatory and inhibitory G proteins, catalytic subunit) contain a significant amount of SH amino acids, especially cysteine. Reduction of the disulfide bounds of the inactive enzyme by, for example, prostaglandins or other reducing agents seem to be essential for activity and stimulability [22]. In contrast, thiol blocking agents such as N-ethyl-maleimide cause the non-specific inhibition of AC-complex [31, 32]. Our results suggest that aminothiol radioprotective compounds may also exert their AC-blocking effect by the so-called 'mixed-disulfide' forming mechanism [3]. The increased stimulability of AC caused by low WR-1065 concentrations also suggests the existence of unevenly sensitive compartments within the enzyme complex. On the basis of our results, obtained on chicken embryo brain membrane preparate [32, 33], inhibitory G proteins of the AC complex are supposed to be the most sensitive ones against thiol blocking. At higher radioprotector concentration the inhibition tends to become more general resulting in the progressive decrease of platelet cAMP content.

Since WR-1065 concentrations necessary to inhibit platelet aggregation also decrease the AC activity and consequently the cAMP content, it is more likely that other mechanisms than AC-system are responsible for the anti-aggregating effect of aminothiol compounds.

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REFERENCES

1. Aktories, K., Jakobs, K. H.: Regulation of platelet cyclic AMP formation. In: The Platelets: Physiology and Pharmacology. Academic Press, New York, 1985, pp. 243–260.
2. Born, G. V. R.: Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature, **194,** 927 (1962).
3. Brandwein, H. J., Lewicki, J. A., Murad, F.: Reversible inactivation of guanylate cyclase by mixed disulfide formation. J. Biol. Chem., **256**(6), 2958–2962 (1981).
4. Brass, L. F.: Regulation of biological responses in platelets. In: Blood Cells and Arteries in Hypertension and Atherosclerosis. eds P. Meyer and P. Marche, Raven Press, New York, 1989, pp. 59–92.
5. Brooker, G., Harper, J. F., Terasaki, W. L., Moylen, R. D.: Radioimmunoassay of cyclic AMP and cyclic GMP. In: Advances in Cyclic Nucleotide Research. Vol. 10. ed. G. Brooker, P. Greengrand and C. A. Robinson, Raven Press, New York, 1979, pp. 1–33.
6. Catalfamo, J. L., Dodds, W. J.: Isolation of platelets from laboratory animals. Methods in Enzymology, **169**, 27–34 (1989).
7. Chirkov, Yu. Yu., Chesnokova, L. P., Sobolev, A. S.: Effect of radioprotectors on cAMP-dependent protein phosphorylation. Biull. Eksp. Biol. Med., **87**(3), 230–232 (1979) (in Russian).
8. Chirkov, Yu. Yu., Malatsidze, M. A.: Modification of radiosensitivity and the cyclic AMP system. Biol. Nauki, **1985**(9), 14–27 (1985) (in Russian).
9. Conger, A. D., Sodicoff, M., Samel, A.: Comparison of cAMP with other radioprotectors against chronic damage to the rat parotid gland. Radiat. Res., **102**(1), 99–105 (1985).
10. de Chaffoy de Courcelles, D., Rovens, P., van Belle, H.: Agents that elevate platelet cAMP stimulate the formation of phosphatidylinositol 4-phosphate in intact human platelets. FEBS Lett., **195**, 115–118 (1986).
11. DiMinno, G., Silver, M., DeGaetano, G.: Prostaglandins as inhibitors of human platelet aggregation. Br. J. Haematol., **43**, 637–647 (1979).
12. Ellman, G. L.: Tissue sulfhydryl groups. Archs. Biochem. Biophys., **82**, 70–77 (1959).
13. Fitzpatrick, F. A., Gorman, R. R.: Regulatory role of cyclic adenosine 3',5'-monophosphate on the platelet cyclooxygenase and platelet function. Biochim. Biophys. Acta, **582**, 44–58 (1979).
14. Haslam, M., Davidson, M.: Guanine nucleotides decrease the free $[Ca^{2+}]$ required for secretion of serotonin from permeabilized platelets: evidence of a role for GTP-binding protein in platelet activation. FEBS. Lett., **174**, 90–95 (1984).
15. Hirschel-Scholz, S., Jung, A., Fischer, J. A., Trechsel, U., Bonjour, J. P.: Suppression of parathyroid secretion after administration of WR-2721 in a patient with parathyroid carcinoma. Clin. Endocrinol. Oxf., **23**(3), 313–318 (1985).
16. Horváth, Gy., Benkő, Gy.: Prostacyclin formation after the treatment of rat aorta with indomethacin and radioprotective compounds. Acta Physiol. Hung., **64**(3–4), 371–372 (1984).
17. Horváth, Gy., Bodó, K., Rónai, É.: Calcium dependency of antiaggregatory effect of aminothiol radioprotective compounds. 22nd Ann. Meet. of ESRB, Brussels (1989).
18. Horváth, Gy., Rónai, É., Bodó, K.: Effect of radioprotectors on the metabolism of arachidonic acid in arteries and blood platelets. Role of free radicals in biological systems, Balatonaliga (1991).
19. Jókay, I., Kassay, L., Kiss, A.: Inhibition of endotoxin induced platelet agglutination, histamine, and serotonine release in vitro by thiol-compounds. Experientia, **20**, 315–316 (1964).
20. Langendorff, H., Langendorff, M.: Chemical radiation protection and the cAMP mechanism. Int. J. Radiat. Biol., **19**(5), 493–495 (1971).
21. Mustard, J. F., Perry, D. W., Ardlie, N. G., Packman, M. A.: Preparation of suspensions of washed platelets from humans. Br. J. Haematol., **22**, 193–204 (1972).

22. Peterson, D. A., Kelly, B., Mehta, N., Gerrard, J. M.: Prostaglandins as reducing agents: a model of adenylate cyclase activation? Prostaglandins, **36**(5), 667–671 (1988).
23. Praga, C., Jean, G., Ferrone, S.: Effet du bromure de 2-aminoethylisothiuronium (AET) sur la coagulation sanguine. Thromb. Diath. Haemorrh., **15**, 131–142 (1966).
24. Pushkareva, N. B., Nikolskii, A. V., Koterov, A. N., Filippovich, I. V.: Effect of agents for the chemical prevention of radiation sickness on the cyclic nucleotide content in the bone marrow of mice. Biull. Eksp. Biol. Med., **107**(2), 191–193 (1989) (in Russian).
25. Sedlak, J., Lindsay, R. H.: Estimation of total, protein-bound and nonprotein sulfhydryl groups in tissue with Ellman's. Anal. Biochem., **25**, 192–205 (1968).
26. Sobolev, A. S., Chirkov, Yu. Yu., Tertov, V. V., Kazarov, A. R.: Effects of radioprotectors on the cAMP and cGMP systems. Radiat. Environ. Biophys., **23**(2), 79–93 (1984).
27. Sodicoff, M., Conger, A. D.: Radioprotection of the rat parotid gland by cAMP. Radiat. Res., **96**(1), 90–94 (1983).
28. Sodicoff, M., Conger, A. D.: Radioprotection of the rat parotid gland by WR-2721 and isoproterenol and its modification by propranolol. Radiat. Res., **94**(1), 97–104 (1983).
29. Sztanyik, B. L., Geszti, O.: Potenciális sugárvédő vegyületek hatása a véralvadásra (Effect of potencially radioprotective compounds on the blood clotting). Honvédorvos, **8**(3), 205–212 (1965) (in Hungarian).
30. Tangen, O., Berman, H. R., Marfey, P.: A new technique for separation of blood platelets from plasma. Thromb. Diathesis Haemorrhagica, **25**, 268–278 (1971).
31. Takáts, A., Binh, V. H., Bertók, L.: Potential role of SH groups in the radiosensitivity of adenylate cyclase. Acta Physiol. Hung., **76**(4), 265–272 (1990).
32. Takáts, A., Binh, V. H., Offenmüller, K., Bodó, K., Bertók, L., Köteles, Gy.: Adenylate cyclase – the more membrane associated, the less radiosensitive. J. Radiat. Res., **34**, 141–147 (1993).
33. Takáts, A., Offenmüller, K., Horváth, Gy., Fülöp, N., Bertók, L.: Effect of irradiation on the GTP binding kinetics of chicken embryo brain plasma membranes. Acta Physiol. Hung. (in press).
34. Thompson, W. J., Tan, B. H., Strada, S. J.: Activation of rabbit liver high affinity cAMP (type IV) phosphodiesterase by a vanadyl-glutathione complex. Characterization of the role of the sulfhydryl. J. Biol. Chem., **266**(26), 17011–17019 (1991).
35. Timmons, S., Hawiger, J.: Separation of human platelets from plasma proteins including actor VIII by a combined albumin gradient-gel filtration method using Hepes buffer. Thromb. Res., **12**, 297–301 (1978).
36. Trocha, P. J., Catravas, G. N.: Effect of WR-2721 on cyclic nucleotide levels and lysosomal enzyme activities. Int. J. Radiat. Oncol. Biol. Phys., **8**(3–4), 543–546 (1982).
37. Trocha, P. J., Catravas, G. N.: Effect of radioprotectant WR-2721 on cyclic nucleotides, prostaglandins, and lysosomes. Radiat. Res., **94**(2), 239–251 (1983).
38. Van Caneghem: Shock and Blood Composition. In: Sulfur-containing Radioprotective Agents, Pergamon Press, 1975, pp. 77–90.
39. Watson, S., McConnel, R., Lapetina, E.: The rapid formation of inositol phosphates in human platelets by thrombin is inhibited by prostacyclin. J. Biol. Chem., **259**, 13199–13203 (1985).
40. Weaver, M. E., Morrissey, J., McConkey, C. Jr., Goldfarb, S., Slatopolsky, E., Martin, K. J.: WR-2721 inhibits parathyroid adenylate cyclase. Am. J. Physiol., **252** (2 Pt 1), E197–201 (1987).
41. Zavoico, G., Feinstein, M.: Cytoplasmic Ca^{2+} in platelets is controlled by cyclic AMP: antagonism between stimulators and inhibitors of adenylate cyclase. Biochem. Biophys. Res. Commun., **120**, 579–585 (1984).

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Intrarenal distribution of renal blood flow after acute and chronic administration of nitric oxide-synthase inhibitor[1]

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The effects of acute and chronic inhibition of endothelium derived relaxing factor (EDRF/NO) synthesis were investigated on the intrarenal blood flow in anaesthetized rats. N_{ω}-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg) was used for inhibition of NO-synthase.

In acute experiments L-NAME was infused, while in chronic experiments L-NAME (4-day pretreatment) was dissolved in the drinking water. Blood flow was measured by 86-Rubidium accumulation method.

Renal blood flow decreased markedly after both acute and chronic L-NAME treatment. (Acute: RBF-control: 679±122, RBF-L-NAME: 333±65 ml/min/100 g, $p<0.01$; Chronic: RBF-control: 527±133, RBF-L-NAME: 315±75 ml/min/100 g, $p<0.01$). The consequences of the NO-synthase blockade are different in the cortical and medullary vessels. The increase in the vascular resistance in the medulla (in acute experiments 228%, R-control: 2.76±0.76, R-L-NAME: 9.08±4.36 R, $p<0.01$; in chronic experiments 113%, R-control: 3.89±1.20, R-NAME: 8.31±3.75 R, $p<0.01$) is appreciably greater than in the cortex (acute blockade: 112%, R-control: 1.12±0.29, R-L-NAME: 2.38±0.47 R, $p<0.01$; chronic blockade: 62%, R-control: 1.61±1.03, R-L-NAME: 2.61±0.61 R, $p<0.01$).

The percentile distribution of intrarenal blood flow shifts from the medulla toward the cortex following acute NO-synthase blockade (MBF-control: 16.8±1.11%; MBF-NAME: 12.3±2.66%, $p<0.01$). The proportion of cortical and medullary blood flow remains unaltered after chronic NO-synthase inhibition.

Conclusion: The nitric oxide plays an important role in determining the renal, cortical and medullary blood flow. The effect of EDRF in influencing the vascular resistance is more pronounced in the medulla than in the cortex.

Keywords: nitric oxide, renal cortical blood flow, renal medullary blood flow, L-NAME

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It has been proved by a great number of investigations the after inhibition of endothelium derived relaxing factor (EDRF/NO) synthesis the renal blood flow decreases as a consequence of an elevated renal vascular resistance. The decrease of blood flow was established after acute inhibition of nitric oxide synthesis *in vivo* [5–7, 9, 10] and *in vitro* [8], and following chronic inhibition of synthesis, too [3, 12]. The renal vasoconstriction following the diminution of nitric oxide synthesis has been described to be independent of the experimental conditions (either in anaesthetized [5, 4, 10] or in conscious [2, 3, 12] animals).

The studies mentioned above contain no data concerning the regional renal blood flow (intrarenal circulation). Factors influencing the intrarenal haemodinamics are of importance, since sodium and water output of the kidney can be influenced per se by change of distribution of the cortical and medullary blood flow.

Therefore, in the present study the renal, cortical and medullary blood flow were simultaneously investigated after treatment with nitric oxide synthesis blocker L-NAME (N_ω-nitro-L-arginine-methyl ester) either as a bolus injection (acute experiment), or in the drinking water for four days (chronic experiment).

The following questions arose

- Are the consequences of nitric oxide synthase inhibition in the cortical and medullary vessels during acute and chronic synthesis-blockade the same?
- Is there any alteration in the distribution of intrarenal blood flow following acute and long term blockade of nitric oxide synthesis?

Materials and Methods

The experiments were carried out in female Wistar rats weighing 200–250 g. Before the experiments rats were fed a normal diet and tap water ad libitum. The animals were divided in four groups.

1. Acute experiment

These experiments were carried out in sodium penthobarbital anaesthesia

a. L-NAME-treated rats (n=10)
   L-NAME was infused (10 mg/kg/7.5 ml 0.9% NaCl/30 min).
b. Control group (n=11)
   The animals were infused with saline (7.5 ml 0.9% NaCl/kg/30 min)

2. Chronic experiment

The animals were kept in individual cages for four days.

a. L-NAME-treated animals (n=16)
   The drinking water contained L-NAME (0.1 mg L-NAME/ml H_2O). As the mean daily water intake of the rats amounted 9.47±3.41 ml/100 g, the calculated mean L-NAME uptake of the animals was about 10 mg/day.
b. Control group (n=13)
   Animals were kept on a normal diet and tap water *ad libitum*.

The circulatory parameters in the chronic experiments were estimated on the fifth day in the same manner as they were in the acute experiments described later.

Blood flow investigations

Surgery

In anaesthesia (sodium penthobarbital 42 mg/kg) from cervical midline incision the right external jugular vein and the left common carotid artery were exposed, and a polyethylene cannula was introduced into the right atrium through the right external jugular vein. The cannula inserted into the common carotid artery served for measuring the mean arterial blood pressure and for collecting blood samples. The mean arterial blood pressure was continuously measured by an electric manometer (Medicor EM-61) and recorded (Radelkis OH-827). Blood clotting was prevented by heparin (250 U/kg, i.v.). The infusion was given into a tail vein after the stabilization of mean arterial blood pressure.

Cardiac output, renal and intrarenal blood flow was determined 30 min after starting the infusion. 86-RbCl (0.5 MBq) dissolved in 0.15 ml physiological saline was injected via the jugular cannula into the right atrium, and arterial blood samples were collected at a rate of 0.75 s for 10 s. In the 90th sec following 86-Rb administration animals were decapitated and the kidneys were removed. Renal cortical, external and internal medullary zones were separated under macroscopic control. The radioactivity of blood samples and kidney specimens were determined by Beckman gamma scintillation counter. Among the experiments those were evaluated, in which the decrease of the blood pressure following the blood sample collection was less than 10 mmHg.

Calculations

Cardiac output was estimated on the basis of Stewart–Hamilton's principle [15], renal and intrarenal (cortical and medullary) blood flow was calculated according to Sapirstein [14] using the following equation:

$$\text{CO, or BF} = \frac{60 \times Q}{\int_0^t C dt}$$

where

CO: cardiac output

BF: blood flow (renal, cortical or medullary)

Q: – radioactivity injected (at the calculation of cardiac output, cpm)
– the radioactivity of the kidney, or the cortex, or the medulla (at the calculation of renal, cortical or medullary blood flow, cpm/100 g)

The denominator represents the area of the first arterial dilution curve during the first circulation.

The renal fraction of cardiac output (%) was calculated as a quotient of radioactivity of the kidney and the radioactivity injected.

The total peripheral resistance (TPR) was calculated as a quotient of the mean arterial blood pressure and the cardiac output. The renal, and regional vascular resistances were calculated as a quotient of mean arterial blood pressure and renal, or cortical, or medullary blood flow.

The percentile distribution of renal blood flow was calculated assuming that the renal cortex is 69%, the outer medulla is 28%, and the inter medulla is 32% of the kidney mass.

Statistics

Values are given as means ± SD. Data were analysed by analysis of variance. Student's unpaired *t*-test was used where appropriate. Statistical significance was considered to be $p < 0.05$.

Results

1. Effect of a single dose of L-NAME (acute experiment) on the renal and intrarenal blood flow (Table I)

The mean arterial blood pressure and the TPR increased, the cardiac output decreased significantly following L-NAME infusion.

The renal blood flow diminished to 49% of the control value under the effect of L-NAME treatment (RBF-control: 697±122; RBF-L-NAME: 333±65 ml/min/100 g, $p<0.001$). The renal and cortical blood flow decreased in equal proportion (51%), but the decrease in the medullary blood flow (to 37% of the control level) was greater than the decrease in the cortical blood flow (MBF-control: 349±86, MBF-L-NAME: 130±50 ml/min/100 g, $p<0.001$).

After L-NAME infusion the renal vascular resistance was enhanced by 114% while the cortical vascular resistance by 112%, respectively, but the increase of the medullary vascular resistance (228%) was remarkably greater than the cortical one. (R-medulla-control: 2.76±0.76, R-medulla-L-NAME: 9.08±4.36 R, $p<0.01$).

A larger ratio of RBF flows through the cortex in acute inhibition during L-NAME infusion, it means, that in this case the blood flow shifts from the medulla toward the cortex. (Intrarenal distribution of RBF. Cortex control: 83.2±1.13%, cortex L-NAME: 87±2.66%, $p<0.001$; medulla control: 16.8±1.11%, medulla L-NAME: 12.9±2.66%, $p<0.001$, respectively.)

2. Effect of long term L-NAME treatment (chronic experiment) on the renal and intrarenal blood flow (Table I)

The degree of blood pressure elevation was the same on the occasion of chronic NO-synthase blockade than it was observed during acute synthesis blockade. The diminution of cardiac output (to 59% and 64% of the control level), and the increase in the TPR (by 82% and 85% as compared to the control level) are practically the same in acute and chronic nitric oxide blockade in spite of the fact that the controls in the two types of experiments differ from each other. (CO acute-control: 33.1±7.95, CO chronic-control: 22.8±4.13 ml/min/100 g, $p<0.001$; TPR acute-control: 29.5±10.6 R, TPR-chronic-control: 39.9±7.67 R, $p<0.02$.)

The renal, cortical and medullary blood flows decrease under the effect of chronic L-NAME treatment, but this decrease was slighter than that observed in acute experiments. The decrease in the renal and regional blood flow was proportional in chronic experiments (to 59% of the control level in the kidney and the cortex, and to 55% in the medulla) in contradiction with the acute experiments where the inhibition of acute nitric oxide synthesis failes to induce a proportional diminution in renal and regional blood flows.

Table I

Effect of acute and chronic L-NAME treatment on the intrarenal haemodynamics

	Acute		Chronic	
	Control, n = 11	Name, n = 10	Control, n = 13	Name, n = 16
	$\bar{x}\pm SD$	$\bar{x}\pm SD$	$\bar{x}\pm SD$	$\bar{x}\pm SD$
Blood pressure	150±13.7	166±6.21*	150±19.8	167±13.6+
Cardiac output	33.1±7.95	19.6±4.72**	22.8±4.13	14.6±4.69++
TPR	29.5±10.6	53.8±12.9**	39.9±7.67	74.2±21.3++
RBF	679±122	333±65.7**	527±133	315±75.2++
CBF	850±181	435±65.7**	674±173	403±91.5++
MBF	349±86	130±50**	254±79	140±51++
R-kidney	1.38±0.32	3.10±0.62**	1.81±0.49	3.34±0.81++
R-cortex	1.12±0.29	2.38±0.47**	1.61±1.03	2.61±0.61+
R-medulla	2.76±0.76	9.08±4.36*	3.89±1.20	8.31±3.75++
Renal% of CO	13.4±1.64	12.3±1.89	13.6±2.02	12.9±2.62
Intrarenal distribution				
Cortex %	83.2±1.13	87.1±2.66**	84.2±2.34	85.5±3.42
Medulla %	16.8±1.11	12.9±2.66**	15.7±2.34	14.5±3.40

* Acute experiment, NAME vs. control *p<0.01 **p<0.001

\+ Chronic experiment, NAME vs. control +p<0.01 ++p<0.001

Cardiac output, blood flow: ml/min/100 g

TPR, R: mm Hg×ml^{-1}×s×kg^{-1} Blood pressure: mm Hg

As a result of the chronic L-NAME administration a 113% increase in the vascular resistance of the medulla could be recorded. (R medulla-chronic-control: 3.89±1.20 R; R medulla-chronic-L-NAME: 8.31±3.75 R, $p<0.01$). This increase is significantly smaller than it has been established in the acute experiment (228%).

From the results described above follows that the chronic inhibition of NO-synthase fails to influence the distribution of cortical and medullary blood flow (no blood flow redistribution), while this influence after an acute NO-synthase blockade can be observed (intrarenale blood flow redistribution).

Discussion

In the present study the acute inhibition of nitric oxide synthase decreased the renal blood flow to 49% of the control value, while the blood pressure increased. These findings are in good agreement with the results of Tolins et al. [16], Beierwalters et al. [4] and Baumann et al. [1]. In their experiments the renal blood

flow decreased, the mean arterial blood pressure increased and they have concluded, that a basal secretion of nitric oxide has a tonic effect on the resistance vessels of the kidney [1].

According to our *simultaneous* investigation in the cortex and the medulla the vasoconstriction induced by a single dose of L-NAME proved to be more pronounced in the medulla than in the cortex: the vascular resistance increased by 112% of the control value in the cortex and by 228% of that in the medulla, respectively. According to the altered vasoconstriction in the cortex and medulla the decrease of the regional blood flow was different, too: the decrease of the medullary blood flow exceeded the decrease of the cortical blood flow. Following a single dose of L-NAME, the percentile distribution of the intrarenal blood flow indicates a shift from the medulla towards the cortex.

Only a few experiments investigating the blood flow in the renal cortex and medulla after nitric oxide synthase blockade have been known so far. A 39.5% decrease of the blood flow in the outer renal cortex has been reported by Chen at al. [2] in anaesthetized rats after NLA infusion, while a 29% decrease of renal cortical blood flow has been found by Walder et al. [17] using an other blocker of nitric oxide synthase. In our experiments a 51% decrease of the total renal cortical blood flow could be recorded. According to Majid et al. [10] the intrarenal infusion of NLA caused a 25% decrease of the renal outer cortical blood flow in dogs. The renal medullary blood flow was not determined in the experiments above.

The renal medullary blood flow has been investigated by Mattson et al. [11] with an excellent special technique. It was found that the medullary vasodilation following bradykinin is nitric oxide dependent. This study, however, does not include data concerning the cortical blood flow.

To our knowledge *we determined at first in the same kidney simultaneously the cortical and the medullary blood flow investigating the role of nitric oxide.* It was found that the acute blockage of the nitric oxide synthase shifted the blood flow to the cortex from the medulla (intrarenal redistribution of renal blood flow).

Following a semi-chronic (4 days) L-NAME administration the decrease of renal blood flow was smaller than in acute experiments, although the decrease of renal blood flow was 59% vs. control after a 4-day pretreatment. The enhancement of the vascular resistance in the medulla was smaller than after acute nitric oxide synthase inhibition. The difference is especially large in the medulla: in chronic experiments the increase in the medullary vascular resistance proved to be only 113%, vs. to the 228% measured in the acute investigations.

In chronic experiments a lower plasma level of L-NAME might cause a smaller increase of renal and regional vascular resistance. (In acute experiments 10 mg L-NAME/kg b.m. was infused in 30 min, while in chronic ones a daily dose of 10 mg L-NAME/kg b.m. was taken in the drinking water.) The enhancement of TPR, however, was the same in the two series (82% or 85%). It seems that the increase in the vascular tone after acute or chronic inhibition of nitric oxide synthase is similar in most of the organs, but not in the kidney. In the kidney, especially in the medulla, the

vessels are less sensitive to the decreased secretion of nitric oxide if the nitric oxide synthesis is chronically blocked; or other vasoconstrictor effects may be involved in the case of acute nitric oxide synthase inhibition, too.

The mechanism of the various vasoactive effects of acute and chronic L-NAME treatment is not known yet. Our results presented fail to clarify it; the aim of this study has been only to describe this phenomenon.

The vasoconstrictor effect of L-NAME lasts long. Long-lasting high blood pressure (P_m = 170 mm Hg) developed in the experiments of Ribeiro et al. [13], in which rats were infused 50 mg L-NAME/day for 4–6 weeks. A five-day long L-NAME infusion into the renal medullary interstitium of conscious dogs caused a selective decrease (30%) in medullary blood flow, a rise in blood pressure and sodium retention in the investigation of Mattson et al. [12]. They supposed that the medullary NO plays an important role in the regulation of renal blood flow, sodium excretion and blood pressure. In our experiments a higher decrease of medullary blood flow (45%) was obtained after a 4-day L-NAME pretreatment. The difference could be explained by species differences (dog–rat), dissimilar experimental conditions (conscious–anaesthetized) and various application of L-NAME (infusion–per os).

In conclusion, the acute and chronic inhibition of nitric oxide synthase is followed by a pronounced increase of the renal and renal regional vasoconstrictor tone. The decrease of renal, cortical and medullary blood flow is higher in the case of acute nitric oxide synthase blockade. The redistribution of intrarenal blood flow to the cortex develops only in acute but not in chronic inhibition of nitric oxide synthase.

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REFERENCES

1. Baumann, J. E., Person, P. B., Ehmke, H., Nafz, B., Kirchheim, J. R.: Role of endothelium – derived relaxing factor in renal autoregulation in conscious dogs. Am. J. Physiol., **263**, F208–213 (1992).
2. Baylis, C., Engels, K., Samsell, L., Harton, P.: Renal effects of acute endothelium relaxing factor blockade are not mediated by angiotensin II. Am. J. Physiol., **264**, F74–78 (1993).
3. Baylis, C., Harton, P., Engels, K.: Endothelium-derived relaxing factor controls renal haemodynamics in the normal rat kidney. J. Am. Soc. Nephrol., **1**, 875–881 (1990).
4. Beierwaltes, W. H., Sigmon, D. H., Carretero, O. A.: Endothelium modulates renal blood flow but not autoregulation. Am. J. Physiol., **262**, F943–949 (1992).
5. Chen, C., Mitchell, K. D., Navar, L. G.: Role of endothelium-derived nitric oxide in the renal hemodynamic response to amino acid infusion. Am. J. Physiol., R510–516 (1992).
6. Chintala, M. S., Chin, P. J., Vemulapalli, S., Watkins, R. W., Sybertz, E. J.: Inhibition of endothelial derived relaxing factor (EDRF) aggravates ischemic acute renal failure in anaesthetized rat. Naunyn Schmiedebergers Arch. Pharmacol., **348**, 305–310 (1993).

7. Deng, A., Baylis, C.: Locally produced EDRF controls preglomerular resistance and ultrafiltration coefficient. Am. J. Physiol., **264**, F212–215 (1993).
8. Gardes, J., Gonzales, M. F., Alhene-Gelas, F., Ménard, J.: Influence of sodium diet on L-NAME effects on renin release and renal vasoconstriction. Am. J. Physiol., **267**, F798–804 (1994).
9. Lahera, V., Solom, M. G., Miranda-Guardiola, F., Moncada, F., Romero, J. C.: Effects of N^G-nitro-L-arginine methyl ester on renal function and blood pressure. Am. J. Physiol., **261**, F1033–1037 (1991).
10. Majid, D. S., Williams, A., Navar, L. G.: Inhibition of nitric oxide synthesis attenuates pressure-induced natriuretic responses in anaesthetized dogs. Am. J. Physiol., **264**, F79–87 (1993).
11. Mattson, D. L., Cowley, A. W. Jr.: Kinin actions on renal papillary blood flow and sodium excretion. Hypertension, **21**, 961–965 (1993).
12. Mattson, D. L., Lu, S., Nakanishi, K., Papanek, P. E., Cowley, A. W. Jr.: Effect of chronic renal medullary nitric oxide inhibition on blood pressure. Am. J. Physiol., **266**, H1918–1926 (1994).
13. Ribeiro, M. O., de Nucci, G., Zatz, R.: Persistent arterial hypertension by chronic blockade of nitric oxide synthesis. Am. J. Soc. Nephrol., **2**, 512A (1991).
14. Sapirstein, L.: Regional blood flow by fractional distribution of indicator. Am. J. Physiol., **193**, 161–168 (1958).
15. Stewart, N. G.: Researches on the circulation time and on the influences which effect it. IV. The output of the heart. J. Physiol. (London), **22**, 159 (1898).
16. Tolins, J. P., Palmer, R. M. J., Moncada, S., Raij, L.: Role of endothel derived relaxing factor in regulation of renal haemodynamic responses. Am. J. Physiol., **258**, H655–662 (1990).
17. Walder, C. E., Thiemerman, C., Vane, J. R.: The involvement of endothelium-derived relaxin factor in the regulation of renal cortical blood flow in the rat. Br. J. Pharmacol., **102**, 967–973 (1991).

Acta Physiologica Hungarica, Volume 83 (4), 411–412 (1995)

In memoriam Tibor Kovács (1929–1994)

We are saddened and terribly shocked by the news of the sudden death of Professor Tibor Kovács who was active for over 40 years at the Department of Physiology, University Medical School, Debrecen, Hungary.

He was born in Debrecen and after finishing the primary and secondary schools, he studied medicine at the University Medical School, Debrecen. He got his M.D. degree in 1954. Professor Kovács obtained two further scientific degrees from the Hungarian Academy of Sciences, Ph.D. in 1968 and D.Sc. in 1980.

As a student he became coworker of Professor Went and was researching the possible role of humoral counterregulation in the cardiovascular system. Later on Tibor Kovács was involved in comparative physiological, biochemical and immunological studies on contractile proteins of striated muscle under different conditions. Professor Kovács's main research topic was the study of both passive and active ion transport processes in the skeletal muscle and in the heart. In the last period of his life he turned toward investigating the changes in ion transport processes, excitability and contractility of different types of muscle in streptozotocin induced diabetes. Over 200 publications, posters, oral presentations and 3 books reflected his activity.

In 1972–73 Tibor Kovács was a research fellow of Richard D. Keynes at the ARC Institute of Animal Physiology, Babraham, Cambridge. From this time on he built fruitful relations with many leading representatives of physiological and molecular biological research.

He was also a very good and highly popular organizer, lecturer, chairman and speaker at many conferences and symposia in different professional meetings in Hungary or anywhere abroad.

He, as one of the staff-members of the Physiological Department, had interest not only in broadening the scientific theoretical background of students, but also in preparing them for the next century, making their knowledge versatile and capable of development. He extended his activities to the postgraduate, and later to postdoctoral education, attracting many young scientists from abroad.

He was the first head of the Educational and Admission Office of his University helping foreign students not only in their studies but in all aspects of life. Several years ago he was elected president of the Membrane Transport Division of the Hungarian Physiological Society and that of the curatory of the foundation in the memory of Professor Gy. Romhányi to support a honourable membrane researcher every year for delivering a plenary lecture at the Membrane Transport Conference in Sümeg. His had distinctive merits in the maintenance of our interdisciplinary meetings.

He could visualize the future direction of medicine and education. His personal dedication highly contributed to disseminating knowledge, promoting the welfare of

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the society and a better understanding between people all over the world. He fought for these goals in all walks of life: as a simple member or as president of various scientific societies or even as initiator to organize conferences. He was also a good and devoted husband, father, grandfather.

During his life he was many times honoured with several awards, medals, acknowledgements, scientific grants evaluating his professional activity and his personalments.

For him the fight ended in the December 1994, and we must say that he emerged victoriously. He had accomplished many things in life – more than one can hope for – even though he probably wished to do more. Losing him has been a painful blow that one can never recover from. We only hope that we will be capable of following in his footsteps and fulfilling the dreams he could not.

The following papers are dedicated to the memory of our friend and colleague, Tibor Kovács.

András Kövér János Somogyi

Acta Physiologica Hungarica, Volume 83 (4), 413 (1995)

In memoriam Ferenc Varga (1929–1994)

Ferenc Varga was born on April 20, 1929 in Péterréve. After the primary and secondary schools he studied at Pécs University and got his M.D. degree in 1954. He began working in the Department of Pharmacology already as a medical student and was working there really up to the last minute of his life. He was appointed chairman of the Department of Pharmacology at University Medical School of Pécs in 1975. During the years of his chairmanship (1975–1994) he created a friendly but stimulating environment. He was very patient and helpful with all members of the staff and faculty, especially with young colleagues and coworkers. He was a member of the leadership of University Medical School of Pécs for six years (1985–1991). He was extremely successful in the organization and management of research work in different fields of medical sciences.

His research area was the study of the role of pharmacokinetic parameters in drug actions, especially the investigation of intestinal absorption, biotransformation and biliary excretion of drugs. The study of these questions are closely related to problems of toxicology, therefore he was an active participant of scientific meetings of toxicology, as well. For several years he was the president of the Hungarian Toxicological Society.

Ferenc Varga was one of the 13 researchers who founded the Hungarian Membrane Transport Conferences. His contribution to the organization and continuation of this conference series proved to be very significant.

The teaching activity of Ferenc Varga was really excellent and unforgettable for the medical students and his colleagues, as well. He was a rigorous and consistent teacher, he tried to reach and keep the highest level of teaching activity of the Department of Pharmacology with undiminished energy. The students realized and respected his effort and personality. He received the posthumous appreciation "The best lecturer of the University Medical School of Pécs".

Ferenc Varga died suddenly on August 29, 1994. He was 65 years old and full of energy and plans. After the termination of his chairmanship he hoped to devote more time to summarizing some aspects of his research and teaching activity and to enjoying more family life with his wife, children and lovely grandchildren. Unfortunately, he was already unable to realize these ideas. His loss is most acutely felt by those of us who knew him personally and respected his honest and modest personality, but his loss is felt by several generations of medical students, as well.

Emil Fischer

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In memoriam Lysle Henry Peterson (1920–1994)

Honorary Member of the Hungarian Physiological Society

Lysle Henry Peterson, M.D., died November 11, 1994, while visiting in Baltimore. Born Minneapolis, Minnesota, January 21, 1920, the son of Oscar Emil and Lucy Ireland Peterson. He graduated from the University of Minnesota in 1943, served in the U.S. Navy in the Pacific Theater during World War II and graduated from the University of Pennsylvania School of Medicine in 1950 where he served as a member of the faculty for more than 25 years. During this period he was director of the Bockus Research Institute and the Philadelphia City Science Center. He then served as Vice President of the University of Texas Health Science Center in Houston and later as Chairman of the Houston Cardiovascular Rehabilitation Center. During his long professional career he was the recipient of many honours, including honorary membership in scientific organizations in Hungary, Czechoslovakia, Argentina, France, Mexico and the Philippines. He was recently made an honorary citizen of Hungary. He was a Fellow of the American College of Cardiology and was President of the International Society of Cardiology, 1975–76. He was a Board member of the Downtown Kiwanis Club and a member of the Houston Committee on Foreign Relations, which he chaired in 1993–94. He was a member of the Texas Corinthain Yacht Club, the Houston Yacht Club and the Cosmos Club, Washington, D.C. Dr. Peterson was the author of more than 150 scientific articles and of a major textbook on cardiovascular rehabilitation. He is survived by his wife, Sara Meredith Peterson; his children, Christopher, New Orleans; Nancy Hallett and her husband, Robert, Baltimore; Douglas, London, England; and Hope, Houston; his grandchildren, and nephews. Dr. Peterson's contribution to the field of cardiovascular physiology both as scientist and organizer is recognized worldwide. His attention toward his Hungarian colleagues and friends has always been highly appreciated. The members of the Hungarian Physiological Society shall keep his memory.

Emil Monos M.D.
President of the
Hungarian Physiological Society

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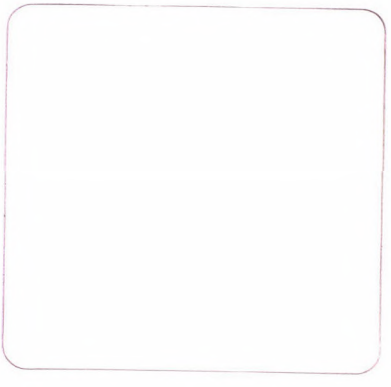

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