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The effects of patterned breathing and continuous positive airway pressure on cardiovascular regulation in healthy volunteers

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1. Although the increased heart rate variability in healthy subjects in association with slow patterned breathing and continuous positive airway pressure is well documented, there is no general agreement regarding the underlying mechanism. The arterial baroreceptor stimulation due to greater blood pressure variability, the stimulation of pulmonary stretch and low pressure baroreceptors can play important role in this phenomenon.

2. In order to assess the interplay between blood pressure and heart rate changes we have studied nine healthy volunteers (mean age was 22 yrs. range 19–24), by applying 6/min patterned breathing, and continuous positive airway pressure of 10 cm of water. ECG and finger blood pressure (Finapres 2300) was continuously recorded. The oscillation amplitude of R–R intervals were analysed as well as the time and frequency domain indexes of heart rate variability. The oscillation amplitude and the corresponding frequency domain components of systolic blood pressure were also calculated.

3. The forced deep breathing caused significant increase in heart rate variability as indicated by time and frequency domain analysis of R-R intervals (LF HRV ms^2: spontaneous: 777.40±526.1, patterned breathing 6828.00±5468.0). The application of CPAP in the same rhythm during patterned breathing resulted in further enhancement in heart rate variability (LF HRV ms^2: 9052.00±4533.0). The analysis of the same frequency domain components of systolic blood pressure showed marked elevation of the total and low frequency power during patterned breathing. (LF BPV mm Hg^2: spontaneous: 8.24±6.2, patterned breathing: 16.22±9.7). Applying CPAP with the same breathing pattern elicited further significant increment in systolic blood pressure fluctuation (LF BPV mm Hg^2: deep breathing+CPAP: 27.11±9.8). The baroreflex sensitivity as calculated from spontaneous HR and BP sequences was 11.66±2.9 at baseline and increased to 17.66±6.1 and changed to 15.22±3.2 with the addition of patterned breathing and CPAP, respectively.

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4. Our findings indicate that the heart rate and blood pressure responses to slow patterned breathing may be interpreted as consequences of an altered baroreflex sensitivity. In contrast the active breathing with CPAP exerts mechanical effects which in turn present an augmented systemic baroreflex trigger, however, the baroreflex sensitivity remains unchanged.

Keywords: spectral power, heart rate variability, blood pressure variability, continuous positive airway pressure, autonomic nervous system

It has been long known that respiration is associated with heart rate and blood pressure changes [1, 2]. The respiratory sinus arrhythmia involves inspiratory heart rate acceleration, and expiratory (or postinspiratory) heart rate deceleration [3]. It has also been realized that the extent of the pulse rate fluctuations is related to the frequency and depth of breathing, the highest amplitude changes being reached around the breathing frequency of 6/min [4, 5]. The breathing rate also bears influence on the blood pressure responses. The amplitude of the blood pressure swings increases with decreasing respiratory rate, with a maximum again around the 6/min. respiratory frequency [3]. It has been noted by Dornhorst et al. that the relation between the blood pressure variation and the respiratory phase is a function of the respiratory rate [3]. More recently Laude et al. documented that systolic blood pressure decreases during inspiration with a time delay which increases as breathing frequency decreases [6].

The effects of the positive pressure ventilation and continuous positive airway pressure on the circulation and the cardiovascular autonomic regulation are well recognized, however the exact mechanisms remain subject of controversy [7, 8]. Continuous positive airway pressure (CPAP) exerts mechanical effects in healthy subjects by increasing the left ventricular filling pressure and the right atrial pressure, the downstream pressure for venous return [9]. The effect of active and passive breathing with CPAP may induce different responses [10], and the role of the breathing frequency while applying CPAP is not known.

Monitoring the oscillations of hemodynamic parameters is a new field in anesthesiology. Since the magnitude of blood pressure oscillation caused by the mechanical effect of positive pressure ventilation is dependent on the volume status of the patients, a number of recent studies focused on this relationship [11]. Another approach, the spectral assessment of heart rate variability, has already gained different applications [12, 13]. Although the hemodynamic effects of both components have been extensively studied their interactions remains unclear. In order to assess the additional effects of slow patterned breathing and continuous positive airway pressure a series of studies was performed on 9 healthy volunteers.

Methods

Subjects

Healthy subjects were recruited to participate in this study. The study group consisted of nine male volunteers who ranged from 19 to 24 years in age (mean: 22 years). Their assessment revealed no pulmonary, cardiovascular or neurologic disease. None were taking medications. The subjects were studied in supine rest position, in the afternoon, 3 to 4 hours after meal. For our recordings, the study was not begun until the most stable baseline of blood pressure and heart rate signals for each subject was obtained. The subjects were non smokers. All subject gave their written informed consent prior to the experiments.

Measurements and calculation

The electrocardiogram was continuously recorded with a Siemens Sirecust 730 monitor. Blood pressure was measured noninvasively with a Finapres 2300 (Ohmeda) device, which has been referred to reflect blood pressure spectral changes reliably. The R wave of the electrocardiogram and the plethysmographic signals were fed through an amplifier, filter and analog-digital converter into an IBM–AT compatible computer. Data were stored and analysed by a self-developed program written in Microsoft C/C++ 7.0. The computer measured the interval between successive R waves with a precision of 2 ms. The accuracy of ECG signals detection was 40 microvolts and 1 mm Hg. The cardiotachogram and trend-grams of the blood pressure values were continuously recorded on-line. The oscillation amplitude of R–R intervals and systolic blood pressure were analysed. The spectral analysis of R–R interval and systolic blood pressure variability were computed by using fast Fourier transformation, using the Hanning window over two frequency bands: low frequency power (0.04 to 0.14 Hz) and high frequency power (0.15 to 0.4 Hz). We considered a total frequency power range from 0.01 to 0.4 Hz. The short-term measures of time domain indexes as the average normal R–R intervals (mean R–R), the standard deviation of the consecutive normal R–R intervals (SD), % of consecutive normal R–R interval differences >50 ms (pNN5O), root mean square successive differences (rMSSD) were also calculated.

Baroreflex sensitivity (BRS) was characterised by the spontaneous sequences method as described earlier [14, 15]. It has been stated that 3 or more cardiac cycles of unidirectional BP increase or decrease with the corresponding lengthening or shortening of the interbeat intervals form spontaneous "up-" or "down-sequences" [15]. These sequences are analogous to those induced by pharmacological manoeuvres, and a spontaneous baroreflex sensitivity could be determined as an average of several

individual slopes [14]. In this study each step changes in systolic blood pressure (delta SBP) were paired with the changes in the subsequent RR intervals (delta RR). This method is referred as lag 1 technik [15].

Protocol

The subjects were studied in supine rest position. The subjects were asked to close their eyes, relax not to cough during the measurements. The R–R intervals and the finger blood pressure were monitored and recorded continuously over ten minutes baseline period followed by 30 minutes of study period. Three sets of measurements were taken: during spontaneous, then during patterned breathing (6/min) without CPAP, and finally during 6/min patterned breathing while applying CPAP. High flow system was used to keep CPAP level constant throughout the respiratory cycle, avoiding flow dependency of the airway pressure. Ten cm of water CPAP was applied through a mouthpiece using the Veolar (Hamilton) ventilator while the subject's nose was clamped. Subjects were instructed not to change the depth of breathing with the addition of CPAP. The magnitude of applied pressure was monitored by the Leonardo graphical computer program attached to the ventilator.

Statistical analysis

Sheffe's test was used to compare the three independent variables. For data showing non-normal distribution, and the skewness coefficient was >1 the statistical tests were done only after logarithmic transformation. We considered the differences statistically significant when p was less then 0.05. Data are given as means and standard errors.

Results

The effect of patterned breathing and CPAP on HRV (Fig. 1, Table I)

The mean of the R–R intervals did not show any changes between the three different situations. The standard deviation of the R–R intervals showed significant increment between the patterned (6/min) and spontaneous breathing. The other time domain parameters such as pNN5O and rMSSD did not change significantly between the spontaneous and patterned breathing. There were no significant, just tendentious differences between the time domain parameters measured at metronomic ventilation and during the application of CPAP with the same rhythm of respiration. Thus the time

domain indexes of the heart rate variability showed significant differences only between the spontaneous respiration and patterned breathing with CPAP. The frequency domain parameters of the HRV showed significant elevation on transition from spontaneous to patterned mode. Similar differences were seen between the spontaneous and CPAP mode in the total and low frequency range. In the high frequency range the HRV did not exhibit any significant modification throughout the study.

Table I

Heart rate and blood pressure variability and baroreflex sensitivity during the three different situations

	Spontaneous breathing	Scheffe F test	Metronom breathing	Scheffe F test	Metronom breathing+CPAP
R–R mean (ms)	847.5±34.7	0.85	798.6±26.1	0.51	786.7±14.0
SBP mean (mm Hg)	116.1±6.0	0.065	113.2±5.5	0.125	117.1±5.2
R–R oscill (ms)	80.7±10.1	**12.9***	238.1±26.72	1.63	294.1±24.9
SBP oscill (mm Hg)	6.83±1.16	**7.32***	14.5±1.36	**4.24***	20.3±1.66
SD RR (ms)	46.4±4.3	**6.61***	90.6±11.87	0.81	106.2±7.8
pNN50%	25.3±5.03	0.436	31.1±4.5	0.702	38.4±3.36
rMSSD (ms)	44.1±4.3	1.48	61.3±9.4	0.34	69.6±6.4
TFhrv (ms^2)	1703±256.7	3.87*	8059.8±2232.4	0.526	10402.1±1664.6
LFhrv (ms^2)	777.4±175.3	4.87*	6828±1822	0.65	9052.4±1511
HFhrv (ms^2)	595.8±173.8	0.52	1000±422.7	0.029	904.3±155.3
TFbpv (mm Hg^2)	15.46±3.2	0.85	23.3±4.79	**3.53***	39.3±4.6
LFbpv (mm Hg^2)	8.24±2.07	1.85	16.22±3.25	**3.46***	27.11±3.2
HFbpv (mm Hg^2)	2.37±0.91	0.28	1.63±0.38	1.324	3.23±0.61
BRS spont (ms/mm Hg)	11.6±1.2	**4.12***	17.6±2.06	0.649	15.2±1.09

CPAP=continuous positive airway pressure, SBP=systolic blood pressure, hrv (bpv)=heart rate (blood pressure) variability, BRS=baroreflex sensitivity, TF, LF, HF=total, low, high frequency power, *significant at 95%

The effects of the patterned breathing and CPAP on the blood pressure variability (Fig. 1)

The mean and the standard deviation of the systolic blood pressure remained unchanged during the study. The application of 6/min metronomic ventilation did not alter the BPV compared to spontaneous respiration. The application of CPAP caused marked increment in the power of the total and low frequency ranges of the blood pressure variability compared to both the baseline parameters and to those recorded during patterned breathing. The blood pressure power in the high frequency range did not change during the three different situations.

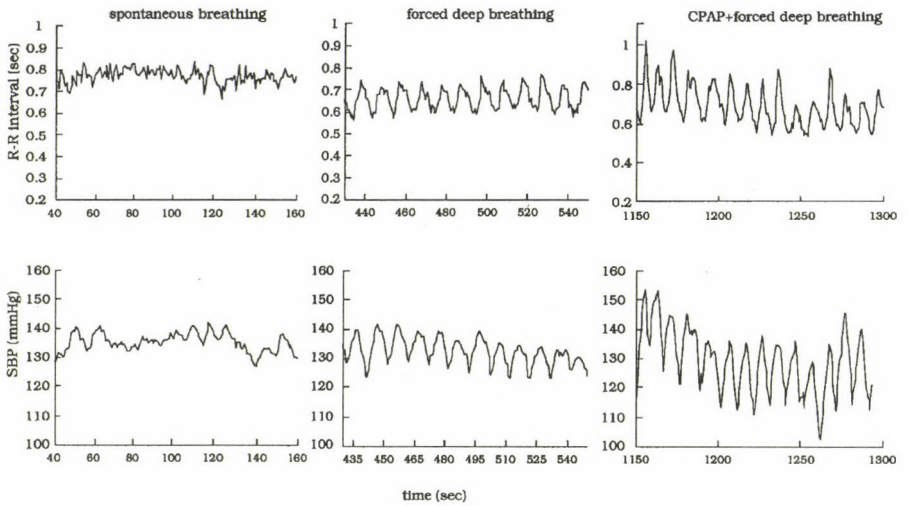

Fig. 1. The R–R intervals and systolic blood pressure (SBP) trendgrams are shown during the three breathing conditions in a single subject. CPAP: continuos positive airway pressure

The oscillation amplitude of the R–R intervals and the systolic blood pressure (Fig. 2)

The oscillation amplitude of the R–R intervals showed significant increase during 6/min breathing. The application of CPAP did not cause further increment in the oscillation of R–R intervals. In contrast, the oscillation amplitude of the systolic blood pressure increased during patterned breathing, and showed further significant elevation due to the application of CPAP.

Analysis of baroreflex sensitivity and baroreceptor gain

The baroreflex sensitivity determined by the method of spontaneous sequences showed statistically significant elevation on transition from spontaneous to patterned breathing. No further changes were detected while breathing with CPAP.

Discussion

The genesis of respiratory sinus arrhythmia is complex. The following mechanisms have been implicated: 1. Central cardiorespiratory coupling. 2. Reflexes from pulmonary stretch receptors. 3. Arterial baroreflexes. 4. Reflexes from the cardiac

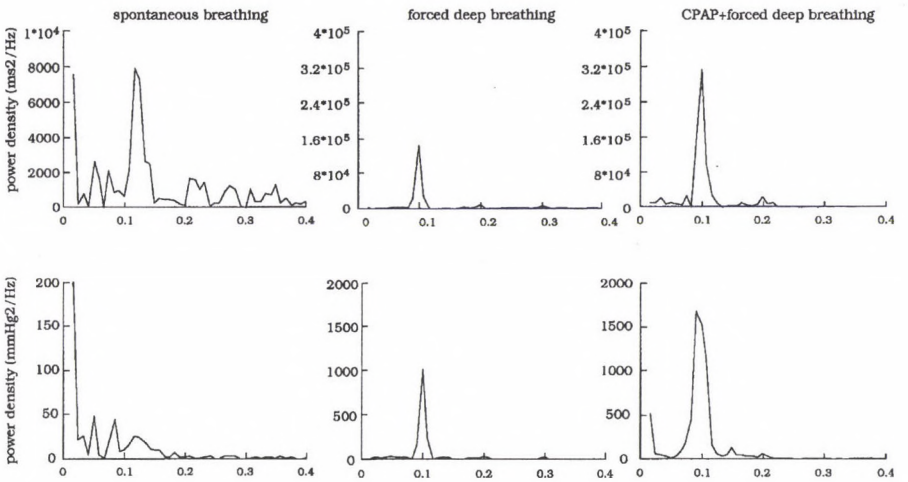

Fig. 2. The power spectral densities of R–R intervals and systolic blood pressure (SBP) are shown during the three breathing conditions in a single subject. CPAP: continuos positive airway pressure

(low pressure) baroreceptors [16]. The relative roles of these mechanisms remain unclear. The mechanism of augmented heart rate fluctuation at slow breathing rates – a proposed marker of cardiac vagal integrity – is also subject of debate. In our study, in concordance with previous publications, the patterned breathing with the frequency of 6 cycles/min. elicited a significant increase in the amplitude of both HR and BP fluctuations [3, 6]. The same effect has been reflected by the increment in the BPV and HRV total power. The marked respiratory effect appeared in the low frequency spectral band, corresponding to the slow breathing rate. There is a superposition of the breathing induced hemodynamic changes on the other low frequency oscillations governed mainly by baroreflex mechanisms [17]. This amplification, or resonating baroreflex control loop mechanism explains the accentuated 0.1 Hz BPV and HRV peaks. The efferent mechanisms of this regulations involve both parasympathetic influences on the heart rate, and sympathetic influences on the heart rate and peripheral vascular tone [17]. Thus the designation of this peak in the given setting as "sympathetic" or "parasympathetic" is meaningless. Although the amplitude of HR changes in response to the slow patterned breathing is widely used to characterize cardiac parasympathetic tone [18], there have also been controversies regarding this marker. Kollai and Mizsei demonstrated that respiratory sinus arrhythmia is a limited measure of cardiac parasympathetic control in man [19]. One of our most interesting results is that the baroreflex sensitivity was substantially higher during slow deep

breathing than during spontaneous breathing. The increased BRS during 6/min breathing could be expressed as a 50% greater HR change in response to a given rise or drop in blood pressure. The mechanism of the exaggerated baroreflex responsiveness might have been related to the altered activity of cardiopulmonary baroreceptors. Vatner et al. documented a progressive reduction of the arterial baroreflex sensitivity with acute volume loading [20], and Shi et al. found similar tendency using lower body positive pressure [21]. In contrast Bevegard et al. [22], Takeshita et al. [23], and Eiken et al. [24] reported that physiological variation of central venous pressure do not influence sinus node responses to arterial baroreceptor stimulation in man.

Toska and Eriksen first documented that the blood pressure changes during spontaneous breathing are related to the changes in stroke volume [25]. They found that the normal heart rate fluctuation exerts a buffering effect on mean blood pressure changes [25]. The anti-oscillatory role of HRV was further substantiated by Saul and Triedman [26], and Kardos et al. [27]. Beaussier et al. recently reported similar causative relationship between stroke volume and blood pressure transients during positive pressure ventilation [11]. There is a respiration related fluctuation of the preloads and afterloads of the ventricles, and pulmonary vascular capacitance. As Dornhorst pointed out that the changes in the filling of the left ventricle are determined but lags behind that of the right [3]. In addition there is a possible ventricular interdependence mediated by the displacement of the ventricular septum, and by the pericardial pressure. All of these factors may contribute to the stroke volume changes, and in turn to the blood pressure oscillations during spontaneous and positive pressure mechanical ventilation. Although application of PEEP causes marked changes in these parameters, Innes et al. [28], Yi-Hankala et al. [29], and Taha et al. [10] reported reduction of respiratory sinus arrhythmia at the time of positive pressure mechanical ventilation. Yi-Hankala et al. [29], and Taha et al. [10] also reported a phase shift of HR swings compared to spontaneous breathing. The explanation for this paradox was presented by Taha et al who demonstrated an altered profile of systolic blood pressure during passive positive pressure ventilation in comparison with spontaneous breathing. Thus, in turn the different responses were mediated by the systemic baroreflex. In our present experimental design we utilized spontaneous breathing with additional positive end expiratory pressure. Therefore the blood pressure fluctuation pattern remained normal during the test. The actual amplitude of these swings with CPAP were greater than during spontaneous and patterned breathing without positive end expiratory pressure. Thus the amplified HR oscillation with the application of CPAP could be explained by increased trigger of the systemic baroreflex. The role of low pressure baroreceptors again could not be excluded. Nevertheless the spontaneous BRS showed no further alterations compared to that of slow patterned breathing.

In conclusion our findings indicate that the well-known effects of slow patterned breathing may be interpreted as consequences of an altered baroreflex sensitivity. The exact mechanism of the phenomenon is not known and necessitates further studies. In contrast the active breathing with CPAP exerts mechanical effects which in turn present an augmented systemic baroreflex trigger. The involvement of low pressure baroreceptors and pulmonary stretch receptors remains unclear.

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Effect of neonatal triiodothyronine (T_3) treatment (hormonal imprinting) on the sexual behavior of adult rats

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Neonatal treatment with triiodothyronine increased the mounting and decreased the intromission of male rats. Number of inactive rats and number of ejaculations were not changed. In females a non-significant increase of Meyerson index was observed. Considering earlier results, the experiment demonstrates that neonatal T_3 treatment can influence different receptors for life. The results also supports earlier observations on the sexual behavioral effect of perinatal treatment with molecules being structurally different from steroids however able to bind to receptors of the steroid receptor superfamily.

Keywords: hormonal imprinting, delayed effects, sexual behavior, thyroid hormone, steroid receptors, perinatal treatment

Hormonal imprinting takes place neonatally at the first encounter of the appropriate hormone and receptor [3, 4] and this is needed for the normal maturation of the receptor. Nevertheless, in the perinatal critical period molecules similar to the adequate hormone can also bind to the receptor, provoking distorted imprinting [5–7]. These molecules could be synthetic hormones, hormone analogues and different molecules able to bind to the members of the same hormone family. The distorted imprinting resulted in lifelong consequences, as abnormal hormone binding and cellular response [3, 4, 7] and in deviation of some physiological or morphological parameters [1, 2, 14, 15, 19].

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Steroid receptors are highly sensitive to false imprinting. Single neonatal treatment with allylestrenol reduces the adequate hormone binding of uterine estrogen [8] and thymic glucocorticoid receptors [16] and strongly influences the sexual behavior of adult male and female animals for life. There are similar consequences after neonatal benzpyrene (which have a steroid-like structure) treatment [9].

Molecules not having steroid-like structure, however bound to receptors of the steroid receptor superfamily also can influence the binding capacity of receptors for life. Clofibrate, a peroxysome proliferator substance decreased the sexual activity of adult rats after neonatal treatment (10) and triiodothyronine (T_3) significantly increased the binding capacity of thymus glucocorticoid receptors [11]. However not all of the receptors were influenced by these two molecules. Clofibrate did not influence the thymic glucocorticoid and estrogen receptors and T_3 did not change the binding capacity of thymic and uterine estrogen receptors either. On the basis of these observations, the present experiment was undertaken in order to study the effect of neonatal T_3 treatment on the sexual behavior of adult rats.

Materials and Methods

Newborn Wistar rats were treated subcutaneously (strictly before 24 h of life) with 3 mg triiodothyronine (T_3, Fluka, Buchs, Switzerland) freshly dissolved in a solution containing 0.005 N NaOH in 0.9% NaCl, buffered and adjusted to the final concentration with physiological saline. The dose was chosen considering the results of previous pilot experiments (11). Control animals received 0.1 ml solvent only.

The rats had been weaned from the mother after 4 weeks and sexes were handled separately. Experiments were done with three months old rats.

Investigation of male animals

In the case of males five components of the sexual behavior were tested [12]:

1. without purpose of mounting, intromission and ejaculation, together with a receptive female rat during a 30 min period
2. mounting only
3. mounting and intromission without ejaculation
4. mounting, intromission and ejaculation
5. multiple ejaculation

In each group 10 males were tested, four times each.

Investigation of female animals

The test was carried out with indicator (experienced) male rats [17]. The Meyerson index and the lordosis quotient were measured. The observation of the two parameters was based on the responses of the females, that is when the female hollows the back (lordosis) as a response to the mounting of the male. The females' positive response to the first mounting makes the Meyerson index, while the number of these responses up to 10 mountings makes the lordosis quotient. Only females in estrus-checked by vaginal smears – were tested.

Fourteen females per group were in the experimental pool. Females in the estrus phase had been tested, so about 5–6 animals were tested per day. During the 2-week testing period one animal was tested four times as an average.

The statistical significance of the results was evaluated by χ^2 probe and Student's "t"-test, for both sexes.

Results and Discussion

There was no significant difference either in the number of inactive males or in the number of ejaculations (Fig. 1). However, the mounting was significantly ($p<0.05$) more frequent in the neonatally T_3 treated animals (almost doubled) than in the controls. At the same time the intromission rate dropped to less than half, which was also significant ($p<0.05$). This demonstrates an increase in libido, with a decrease in real actions.

In females there was a (non-significant) increase in the Meyerson index, without any difference in the case of lordosis quotient (Fig. 2).

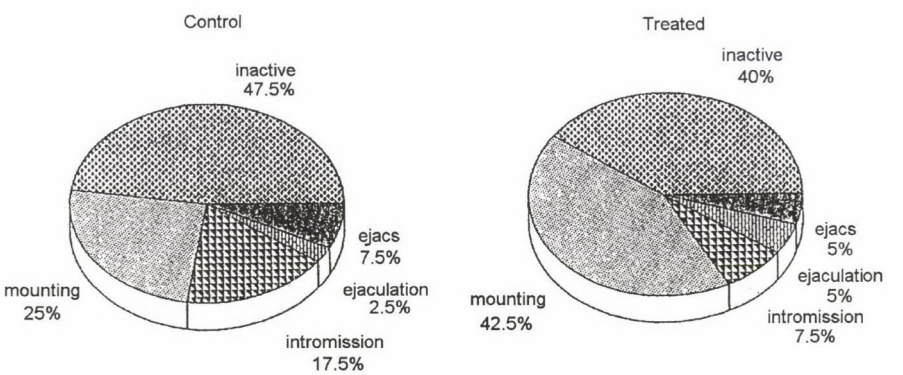

Fig. 1. Effect of neonatal treatment with T_3 on male sexual behavior (ejacs = multiple ejaculations). There is a significant difference ($p<0.05$) in the mounting and intromission of control and T_3 pretreated rats

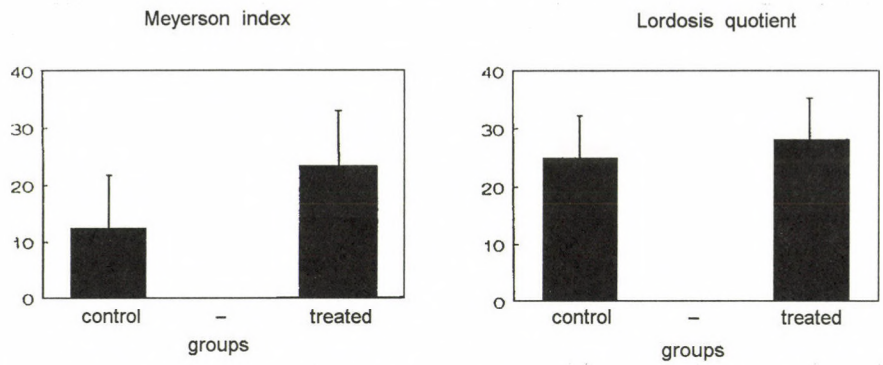

Fig. 2. Effect of neonatal treatment with T_3 on female sexual behavior. There is a difference in the Meyerson index of control and T_3 pretreated rats, however this is statistically not significant

In earlier experiments, the two sexes showed in general opposite directions in response to neonatal treatments [7]. This meant that the reaction was characteristic to the treatment, and its direction was sex dependent. In the present case, the reaction was not very strong (the weakest among the reactions given to all of the substances studied), and first of all only males were involved. On the basis of this observation we can conclude that neonatal T_3 treatment seems to have some effect on sexual behavior later in life. However this effect was weaker, than in the case of treatment with other – steroid-like – molecules.

T3 is a physiological molecule, present in the developing – fetal – organism in a very early time and its presence is needed for the development of the brain [13, 18]. It is possible that the hypothalamic receptors are not so sensitive to T_3 they are to foreign molecules such as allylestrenol, benzpyrene or clofibrate. Nevertheless this does not completely explain why the thymic glucocorticoid receptors were so sensitive to T_3 in a similar situation [11]. To explain this, it must be hypothesised, that the receptors of sexual steroids do not consider T_3 as "foreign" as much as those of glucocorticoids. This is supported by the ineffectiveness of neonatal T_3 treatment to influence the thymic and uterine estrogen receptors [11]. This might result from the fact that the T_3 receptor and estrogen receptor belong to the same subfamily of steroid receptor superfamily, while the glucocorticoid receptor belongs to a separate one [20].

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Glomerular prostanoid production is modified by plasma samples of hypertensive and diabetic patients

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The autocrin-paracrin prostanoid system plays a major role in the enhancement or inhibition of renal tissue damage. Our hypothesis was that there might be circulating factors in the plasma with a capability to modify renal (glomerular) prostanoid synthesis. We measured the synthesis of prostacyclin I–2 (PGI2) and thromboxan A–2 (TxA2) of isolated glomeruli, incubated in plasma samples obtained from hypertensive and diabetic (NIDDM) patients. It was found that these plasma samples decreased the renal PGI2/TxA2 ratio, mostly by decreasing glomerular PGI2 synthesis and, to a lesser extent, increasing the synthesis of TxA2. Our results demonstrate that circulating factors in hypertension and diabetes might play a role in renal damage seen in these conditions.

Keywords: hypertension, diabetes mellitus, NIDDM, kidney, plasma, isolated glomeruli, prostacyclin, thromboxane

The kidneys are not only the primary regulatory organs in blood pressure control, but are also among the targets of the end organ damage seen in hypertension [1–3]. Kidney damage also occurs in association with impaired glucose metabolism: although the incidence is different, impaired renal function may develop both in type I and type II diabetes mellitus [4, 5].

The risk for kidney damage increases when diabetes and hypertension are both present [5]. Moreover, alterations in glucose metabolism and blood pressure regulation

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might be associated with each other: a great percentage of the patients with essential hypertension also have impaired glucose tolerance or diabetes, and in diabetic patients the development of secondary hypertension is markedly increased [5–8]. Renal damage seen in hypertension, or in coexisting hypertension and diabetes, develops by three different pathways:

1. Secondary to the increased total peripheral resistance and blood pressure,
2. Genetically determined pathologic alterations in local cellular protein synthesis
3. By circulating factors inducing biochemical alterations in renal vessels and glomeruli.

Our goal was to test the third pathway: we intended to investigate whether plasma obtained from hypertensive or hypertensive and diabetic patients would modify the glomerular prostacyclin I–2 (PGI2) or thromboxane A–2 (TxA2) production. More specifically, we measured the PGI2 and TxB2 production of isolated glomeruli, in vitro. Productions were detected at baseline and in the presence of the vasoactive peptides bradykinin, angiotensin II and vasopressin. The autocrin-paracrin prostanoid system plays a major role in the enhancement or inhibition of renal tissue damage [9–14]. Prostanoid production of glomeruli is of basic clinical significance in most progressive renal lesion [15, 16]. Synthesis of PGI2 and TxA2 occurs mainly in the glomeruli along the nephron [17, 18].

The basis for our hypothesis was, that it has been published PGI2 production and PGI2/TxA2 index decrease in the kidney of patients with hypertension or long-standing diabetes mellitus [19–22]. As for background other additional findings could be related to the above-mentioned basic findings as follows. In our previous studies we were able to detect and characterize a circulatory factor that increases pressor activity in patients with essential hypertension [23]. Bachmann et al. [24] found that this factor modifies intracellular Ca++ transport. We hypothesize that since prostanoid production is a Ca++ dependent process, it might be modified by such circulating factors. Circulating factors with biochemical modifying effect on vessel wall processes are also likely to be present in non-insulin dependent diabetes (NIDDM).These processes may be linked to the regulation of prostanoid system too [28–32]. In diabetic animals alteration of the endothelial Ca transport has been demonstrated [26–27]. Clinical observations of Umeda et al. [22] and Tajiri et al. [23] can be connected to the above results: they found a decrease of PGI2 and partially an increase of TxA2 metabolites in the urine of patients with long-standing NIDDM.

Materials and Methods

Patients

All subjects signed a written, informed consent. A total of 63 patients were studied, aged 44–60 years. All patient groups were matched regarding age. Studies were performed on: 20 normotensive patients (12 males, 8 postmenopausal females); 20 patients with essential hypertension (12 males, 8 postmenopausal females), with diastolic blood pressure 100–115 mm Hg; 10 patients with accelerated essential hypertension (6 males, 4 postmenopausal females), with diastolic blood pressure >125 mm Hg; 12 patients (6 males, 6 females) with coexisting NIDDM and hypertension.

The patients were had not taken any medications 10 days prior to the study with the exception of hypoglycemic agents, diazepam, nepresol and short-acting Ca channel blockers. All medications were held 10 hours prior to the study. All patients were under continuous medical care during the preparation period.

Blood samples were drawn in the morning, fasting, prior to getting up. Twenty-four hours prior to blood collection, patients were give 1 g of aspirin p.o. to suppress platelet TxA2 production. Plasma was separated by centrifugation at 4 °C. Plasma specimens were stored at –20 °C until usage.

Animals, preparation of glomeruli

Glomeruli were obtained from male Wistar-Kyoto rats weighing 160–200 g. Under pentobarbital anesthesia (5,5 mg/100 mg body weight i.p.) kidneys were perfused with 50 ml of ice cold heparinized Hanks' balanced salt solution for 3 minutes. After nephrectomy, glomeruli were isolated by sieving technique [33], modified as described in the following manner.

Preparatory steps were done in ice cold Hanks balanced salt solution (HBSS) prebubbled with 95% O_2–5% CO_2 for 15 minutes. HBSS was supplemented with 5 mM glucose, 10 mM N-2-hydroxyethylpiperazine-N',2-ethanesulfonic acid (HEPES) and 0.2% bovine serum albumin (BSA) at pH 7.4. Cortex was separated, minced and transferred to a beaker containing solution of 0.3 mg/ml collagenase (Sigma Chemicals), type I, and incubated (bubbled with O_2+CO_2 and shaked cautiously at 120/min) for 45 min at 37 °C to remove the Bowman capsules. Differential sieving was than carried out by passing cortical tissue sequentially through 250-, 150-, 106- and 75 μm stainless steel sieves, and by interpolated washings and gentle centrifugations. The glomerular preparation that was obtained was >90% pure. Resuspended isolated glomeruli from rat kidneys were than pooled.

The above procedure assured good viability of glomerular cells.

Incubation

Glomeruli (triple samples) from the common homogen glomerulus-pool were incubated in a 1:1 mixture of modified HBSS + plasma samples from subjects N, EH, ACCH, and NIDDMH (1200 glomeruli/1 ml incub. suspension/tube) at 37 °C, for 60 min. The modified HBSS was prebubbled with 95% O_2 and 5% CO_2 for 30 min before the incubation. Incubation was terminated by sudden cooling and the addition of 60 µl of 2.8 mmol/l indomethacin. Suspensions were then centrifuged at 2700 g for 3 min at 4 °C. The supernatants were stored at –20 °C before RIA-s were performed. The protein content of pelleted (and 3x rewashed) glomeruli was assayed by colorimetric method.

Parallel incubations were carried out with angiotensin II, (arginin) vasopressin, and bradykinin in the incubation-medium (concentrations: 10^{-12}, 10^{-9}, 10^{-6} M).

PGI2 and TxA2 (assayed as the stable metabolites 6-keto-PGF 1 alfa and TxB2) production were determined in triplicate, using RIA kits (New England Nuclear, Boston, USA). The prostanoid content of the parallelly incubated HBSS+human plasma control tubes (without glomeruli) were distracted from the prostanoid content of the tubes containing also the glomeruli and the results were expressed as ng of prostanoid produced hour – 1 mg – 1 of glomerular protein.

All data are presented as mean ±SEM. Statistical analysis was performed with Student's *t*-test. Individual data represent mean values of three different measurements.

Results

The PGI2 and TxB2 production of isolated glomeruli, incubated in plasma samples of normotensive, hypertensive, accelerated hypertensive and hypertensive-diabetic patients is shown in Figure 1.

The incubation with plasma samples from the accelerated hypertension patient group resulted in significantly decreased glomerular PGI2 production, when compared to the incubation with plasma from the normotensive patient group: 142±24 ng/h/mg glomerular protein, vs. 122±25, $p<0.05$. Plasma specimens from the essential hypertension group, and from diabetic-hypertensive group did not result in significant alteration of glomerular PGI2 production: 156±32, vs. 126±34 ng/h/mg glomerular protein).

There was no statistically significant difference between the TxB2 production of glomeruli when incubated with plasma specimens of the four different groups. It was 318±70 ng/h/mg glomerular protein in the normotensive group, while 343±78 in the group with essential hypertension, 340±45 in the group with accelerated hypertension and 363±48 in hypertension and diabetes (p=n.s.).

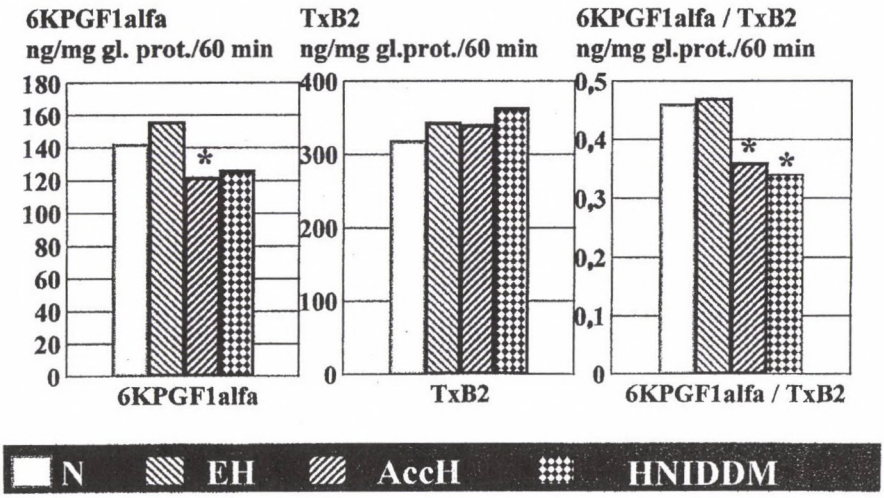

Fig. 1. PGI2 and TxA2 production of isolated glomeruli in the effect of plasma-medium of subjects N, EH, AccH, and H+NIDDM. [n(N)=20, n(EH)=20, nAccH:10, n(H+NIDDM)=13]. Significance of difference from group N: *p<0.05

When calculating the ratio of PGI2 and TxB2 production (PGI2/TxB2), we found that incubation with plasma samples from the group with essential hypertension and coexisting hypertension and diabetes resulted in a significant decrease when compared to the normotensive group: 0.36±0.08 ng/h/mg glomerular protein in the accelerated hypertension group, 0.34±0.07 ng/h/mg glomerular protein in the coexisting hypertension and diabetes group, versus 0.46±0.14 ng/h/mg glomerular protein in the normotensive group ($p<0.05$). The PGI2/TxB2 ratio was 0.47±0.12 ng/h/mg glomerular protein in the essential hypertension group, p=n.s.

In our prestudies we found that angiotensin II, bradykinin and vasopressin had to be present at an optimal concentration of 10^{-6} M to modify glomerular prostanoid production significantly. Therefore, in this study, we used the above peptides at that concentration. Angiotensin II-induced glomerular PGI2 and TxA2 productions are shown in Figure 2. In the presence of angiotensin II, the PGI2 production significantly decreased in all three groups: 183±29 ng/h/mg glomerular protein in the essential hypertension group, 116±28 in the accelerated hypertension group, 135±35 in the hypertensive-diabetic group, vs. 214±23 in the normotensive group ($p<0.01$). Angiotensin II resulted in significantly increased glomerular production of TxA2 only in the accelerated hypertension group when compared to the normotensive group: 78± 23 ng/h/mg glomerular protein in the normotensive group vs., 96±36 in the accelerated hypertension group ($p<0.05$), 73±32 in the essential hypertension group (p=n.s.), and

89±28 in the hypertensive-diabetic group (p=n.s.). The PGI2/TxB2 ratio in the presence of angiotensin II was significantly lower in the accelerated hypertension, and in the hypertensive-diabetic group, when compared to the normotensive group: 0.18±0.19 in the accelerated hypertension group, and 0.24±0.01 in the hypertensive-diabetic group, vs. 0.45±0.23, in the normotensive group ($p<0.001$). The ratio was 0.36±0.16 in the essential hypertension group, p=n.s. when compared to the normotensive group.

Vasopressin induced glomerular prostanoid production in the four different groups is shown in Figure 3. All three patient produced significantly less PGI2 than the normotensive group: 124±23 ng/h/mg glomerular protein in the essential hypertension group, 114±26 in the accelerated hypertension group, 90±23 in the hypertensive-diabetic group, vs. 186±21 in the normotensive group ($p<0.001$). The vasopressin induced TxB2 production was significantly higher in the accelerated hypertension group and hypertensive diabetic group, when compared to the normotensive group: 94± 21 ng/h/mg glomerular protein in the normotensive group, vs. 104±42 in the essential hypertension group, 140±39 in the accelerated hypertension group, 133±25 in the hypertensive diabetic group. The vasopressin induced PGI2/TxB2 ratio was significantly lower in all three groups, when compared to the normotensive group: 0.34±18 in the normotensive group, vs. 0.17±0.16 in the essential hypertension group, 0.13±0.07 in the accelerated hypertension group, and 0.09±0.09 in the hypertensive diabetic group ($p<0.01$ for all groups).

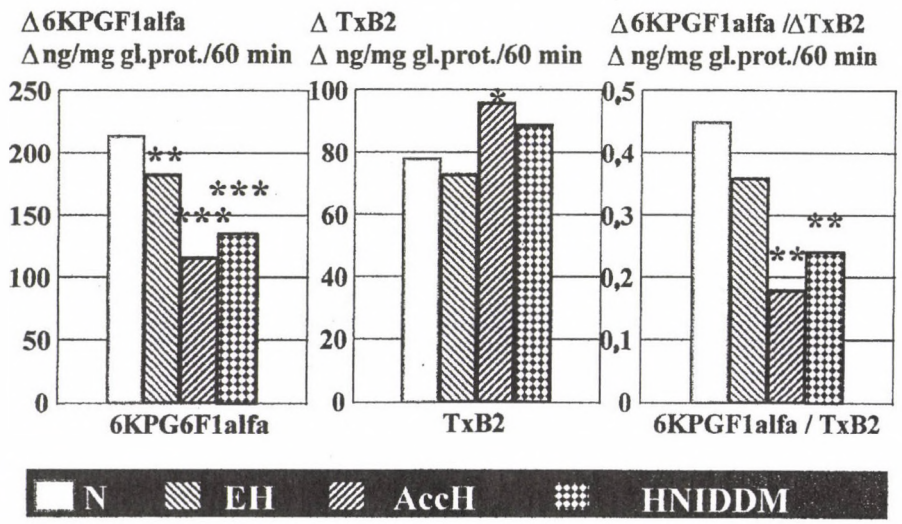

Fig. 2. Angiotensin II induced changes of PGI2 and TxA2 generation by isolated glomeruli in the plasma medium of subjects N, EH, AccH and H+NIDDM. Significance of difference from group N: *p<0.05

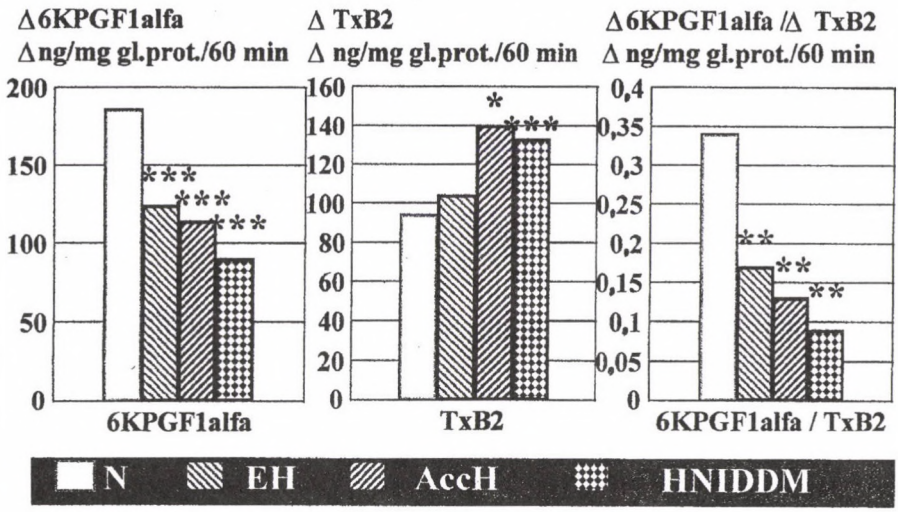

Fig. 3. Vasopressin induced alterations of prostanoid generation by isolated glomeruli in the plasma medium of subjects N, EH, AccH and H+NIDDM. Significance of difference from group N: *p<0.05, **p<0.01, ***p<0.001

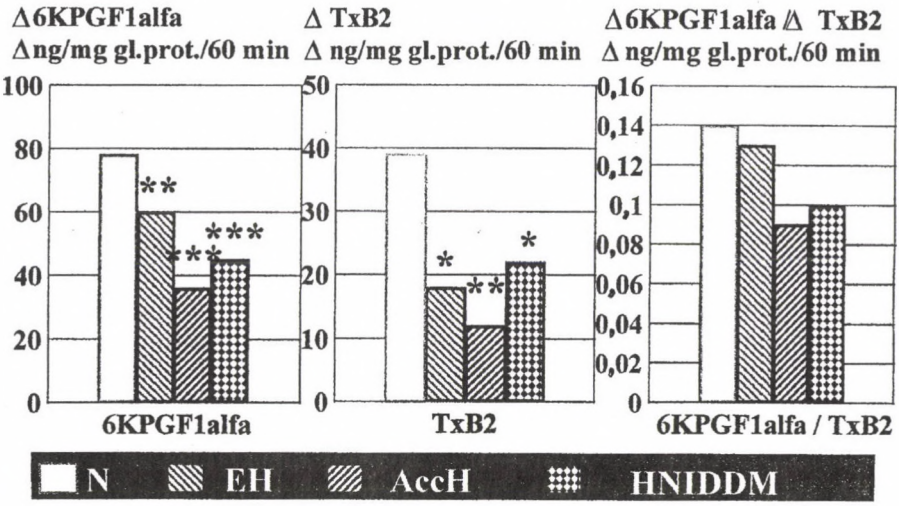

Fig. 4. Bradykinin induced alterations of prostanoid generation by isolated glomeruli in the plasma medium of subjects N, EH, AccH and H+NIDDM. Significance of difference from group N: *p<0.05, **p<0.01, ***p<0.001

Bradykinin induced changes in glomerular prostanoid production are shown in Figure 4. All three groups showed a significantly decreased bradykinin induced PGI2 and TxB2 synthesis, when compared to the normotensive group: PGI2 synthesis was 78±15 ng/h/mg glomerular protein in the normal group, vs. 60±21 in the essential hypertension group, 36±8 in the accelerated hypertension group, and 45±27 in the hypertensive diabetic group. TxB2 synthesis in the normal group was: 39±18 ng/h/mg glomerular protein, vs. 18±29 in the essential hypertension group, 12±21 in the accelerated hypertension group, and 22±19 in the hypertensive diabetic group. The PGI2/TxB2 ratio was: 0.14±0.13 in the normal group, vs. 0.13±0.12 in the essential hypertension group, 0.09±0.07 in the accelerated hypertension group, and 0.10±0.01 in the hypertensive diabetic group.

Discussion

The autocrine-paracrine prostanoid system plays an important role in determining the long-term outcome of renal-vascular diseases [9–16, 23]. Glomerular synthesis of prostacyclin, thromboxan A2 and their ratio are of special importance in that respect. The basis of their importance is their different role in modifying vascular tone, the adhesion of blood cells to the endothelium, endothelial damage or protection [34–40]. The major source of prostanoid metabolites 6-keto-PGF 1 alfa and TxB2 in urine is the local synthesis of PGI2 and TxA2 in the kidney [41–43]. Renal prostanoid production is highly changeable and it reacts sensitively to changes in circulation and circulatory regulatory factors. In our earlier clinical study we observed significant changes in the production of several prostanoids of the kidney when controlled acute reduction of blood pressure was carried out by various drugs [44–45]. We could also demonstrated in rat and cat in vitro experiments, that the release of PGI2, TxA2 and other prostanoids from the vascular tissue reacts in a highly differentiated pattern to vasoactive agents [46].

The main goal of our study was to determine whether there would be factors circulating in the plasma of hypertensive and diabetic patients to modify glomerular prostanoid synthesis. We examined glomerular prostanoid production not only at baseline, but also in the presence of important renal-circulatory mediators – angiotensin II, bradykinin and vasopressin.

The results have proven our hypothesis:

- In patients with essential hypertension, especially if accelerated or coexisting with NIDDM, the circulating plasma decreases the glomerular PGI2/TxA2 ratio.
- The dominating effects of the plasma media are:

- decreased baseline production of PGI2 (mostly seen in our patients with accelerated hypertension or coexisting hypertension and diabetes);
- suppression of peptid induced glomerular PGI2 synthesis (seen in all of our three patient groups);

• The other component of the decreased PGI2/TxA2 ratio seen in our study is a mild stimulation of vasopressin induced glomerular TxA2 release (seen mostly in accelerated hypertension and coexisting hypertension and diabetes.

The highest rate of PGI2 synthesis is in the glomerulus along the nephron [18]. Thus the above detailed main trends of our experimental findings on PGI2 release by isolated glomeruli incubated in plasma media originated from humans with hypertension and diabetes were in good coincidence with previous clinical observations on decreased urinary excretion of the stable prostacyclin metabolite in long-standing hypertension [19] and in chronic NIDDM [21, 22]. In our present experiment we found that the more marked decrease in the basic and peptid induced PGI2 synthesis occurred rather in patients with severe or metabolically complicated hypertension. This tendency is compatible with our previous clinical findings which proved negative correlation between the diastolic blood pressure and the excretion rate of 6-keto-PGF 1 alfa in the urine of patients with essential hypertension [44], or to recently published clinical data that proved a reduced 6-keto-PGF 1 alfa excretion in the urine in human NIDDM nephropathy [21–22]. The clinical importance of the diminished capability of glomeruli to produce PGI2 was also justified by clinical studies where the effects of the metabolically stable PGI2 analog was tested on endothelial function, platelet aggregation and microalbuminuria in patients with NIDDM [45]. Nishimura and Nozaki found that the short-term administration of Beraprost sodium caused a decrease in the presumed promoting factors of angiopathy, a reduction in the microalbuminuria, and an increase in the protecting endothelial factor of angiopathy, tissue type plasminogen activator [45].

Changes detected in glomerular synthesis of TxA2 in the plasma samples from humans with accelerated hypertension can be brought into connection to the results of Hornych et al. [20], who observed a slightly enhanced TxB2 excretion in the urine of patients with hypertension. At the same time the facilitated baseline and peptid induced release of TxA2 from isolated glomeruli incubated in the plasma medium of NIDDM (and hypertensive) patients can explain one possible partial mechanism of the increased TxB2 excretion found in long standing NIDDM by Umeda et al. and Tajiri et al. [22, 23]. On the basis of our experimental conditions we can not decide on wether alterations in glomerular TxA2 production mean a direct plasma stimulus on TxA2 synthesis or they are explained by a substrate shift [46] for thromboxane synthase from the suppressed process of PGI2 synthesis. The significance of the altered TxA2

generation by renal tissues in diabetes mellitus have been illuminated also by some therapeutic results. Recently Umeda et al. [22] published that pharmacological inhibition of TxA2 in NIDDM patients with nephropathy and decreased 6-keto-PGF l alfa/TxB2 index in their urine significantly diminished the albuminuria and improved the creatinine clearance. Similar data from animal experiments are also available: the appearance of the proteinuria in streptozotocin-diabetic rats could be prevented by TxA2 inhibition [13].

Our results indicate that the alterations in prostanoid production caused by factors circulating in the plasma may play role in the development of renal damage seen in hypertension and diabetes. However, more work needs to be done to clarify the nature and relative significance of these factors because in addition to some plasma factors which have remained occasionally unrevealed, several circulating plasma constituens could be considered as potential candidates for those actions on glomerular prostanoid production [8, 26–30, 50–52].

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Oxidative stress in experimental diabetes induced by streptozotocin

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It is known that streptozotocin (STZ) penetrating into the organism generates nitrogen monoxide (NO). Therefore, it is justified to presume, that in beta-cell destruction thereby induced, peroxinitrit resulting from NO and superoxide (O_2^-) reaction has an important role. It has also been studied how pro- and antioxidant systems change in STZ induced experimental diabetes in rat organs. Beside pro- and antioxidant systems of plasma and red blood cell hemolysates, changes in homogenates of the following organs were studied: liver, kidney, heart, lungs, spleen, brain, muscles and pancreas. We tested and compared antioxidant enzymes (superoxide dismutase-, glutathione peroxidase- and catalase activities) glutathione reductase activity regenerate reduced glutathione (GSH). The oxidized, reduced glutathione values and lipid peroxidation changes were measured. From our studies it has appeared that STZ treatment generally induces an oxidative predominance in tissues. Changes in this model thereby, can be compared to changes occurring in type 1 human diabetic patients.

Keywords: oxidative stress, diabetes, streptozotocin, antioxidant system

Animal models of diabetes mellitus can be produced with use of chemicals for example by Streptozotocin (STZ) [2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glyco-pyranoside] as a diabetogenic agent to rats [11]. The mechanism of STZ-induced diabetes is considered as follows:

STZ causes: DNA strand breaks and methylation in pancreatic islets; Stimulates nuclear poly(ADP-ribose)synthase (EC 2.7.7.19), and thus depletes the intracellular

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NAD(P) levels. (It inhibits the proinsulin synthesis and thus induces diabetes.) It is a similar model in pathological and biochemical aspects to type 1 diabetes in humans [22].

(iii) Simultaneously, activated oxygen species, superoxide (O_2^-) anion, hydrogen peroxide (H_2O_2) and hydroxyl radical (HO·) have important roles in diabetogenic neurovascular dysfunction heart failures and in some respect surfactant protein gene expression [4, 8, 20] and so on. Some authors proved [22] that in STZ-induced diabetes in rats HO· radicals are the main factors in oxidative stress and cell death. HO· radicals induce the inhibition of ATP synthesis and mitochondrial respiratory enzymes.

(iv) Lipid peroxidation increase, on the other hand, damage plasma and mitochondrial membranes (in general the membranes) [14].

(v) Recent results prove that during intracellular STZ decomposition nitric oxide (NO) formation occurs [9].

In this report we addressed the questions: How is the pro- and antioxidant balance changing in rats early after the STZ treatment? Which pro- and antioxidant changes are typical in different organ homogenates after the treatment?

It was studied and compared the changes of the reduced and oxidized glutathione (GSH and GSSG), lipid peroxidation values and antioxidant enzymes (superoxide dismutases, catalase, glutathione peroxidase and -reductase activities).

Materials and Methods

All chemicals, enzymes and other reagents were of analytical grade or purest quality purchased from Sigma, Merck, Aldrich and Reanal (Budapest, Hungary).

Experiments were carried out with male Wistar rats [200–250 g body weight (b.w.)] housed individually at room temperature (23 °C), and maintained on a 12/12-hour light-dark cycle. Food and water was given *ad libitum* The rats after 24 hours fasting were made diabetic with a single injection of STZ (85 mg/kg b.w.) intraperitoneally (i.p.). The streptozotocin was freshly dissolved in 50 mM citric acid solution (85 mg STZ/mL 50 mM citric acid). Control rats were treated with equal volume and concentration of citric acid solution. Forty-eight hours after STZ injection, when hyperglycemia was confirmed, the diabetic and control rats were anesthetized with i.p. injection of sodium hexobarbital (50 mg/kg b.w.) and sacrificed.

Abbreviations: C-ase: catalase; DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid); GPx-ase: glutathione peroxidase; GR-ase: glutathione reductase; GSH: reduced glutathione; GSSG: oxidized glutathione; LP: lipid peroxidation; RBC: red blood cell; SOD: superoxide dismutase; Cu, Zn-SOD, Mn-SOD: copper, zinc- or manganese superoxide dismutase; STZ: streptozotocine; TTBARS: total thiobarbituric acid reactive substance.

Before the STZ treatment the animals were divided into two groups in every case because the experiments were repeated three times: Nondiabetic control animals (n=5; blood glucose level were 6.32±0.65 mM/L), STZ diabetic animals (n=6; their blood glucose levels were: 20.90±1.26 mM/L).

Blood glucose was determined using o-toluidine method [7]. Blood samples were taken from the left ventricle of the heart and collected in heparin-containing tubes. Following centrifugation at 3000 g for 10 min, plasma was prepared for measurements. The red blood cells (RBCs) were collected and washed three times with physiological saline (ph.s.). The packed RBCs were hemolysed by cold distilled water (ratio 1:10).

Heart muscle, skeletal muscle, liver, kidney, lung, spleen and brain rapidly dissected washed with ph.s., frozen until analysis. Samples [about 1 g wet weight (w.w.)] of different tissues were weighed and homogenized with 10 vol./w.w. cold ph.s. solution. The homogenates became suitable for lipid peroxidation (LP) analyses [19]. The homogenates were centrifuged at 15.000 g for 10 min. The aliquots of the supernatants were used for the determination of other parameters.

Enzyme activity measurements

Superoxide dismutase (SOD; EC 1.15.1.1) activities were determined by the epinephrine–adrenochrome method [15, 13].

In the case of hemolysate 0.5 ml of centrifuged and packed RBCs were hemolysed with 4.5 ml cold distilled water. After centrifugation the supernatant aliquots were used for the enzyme measurements. Before the determination of SOD activity, the hemolysates were treated with chloroform:ethanol (1:2). The supernatants were used for the measurements of Cu, Zn-SOD activity [16].

The meaning of the method is the superoxide (O_2^-)-dependent epinephrine-adrenochrome transformation in the presence of air at pH 10.2. The transformation of epinephrine to adrenochrome is inhibited to an extent depending on the amount of SOD. Unit SOD can be regarded as the amount of enzyme which causes a 50% inhibition in the extinction change/min compared to control. Measurements were made at 25 °C at 480 nm.

In the presence of CN^- (5×10^{-3}M KCN) Mn-SOD could be determined with the same method [1].

In the case of tissue samples the supernatant aliquots were used for enzyme activity measurements without any pretreatment.

Glutathione peroxidase (GPx-ase; EC 1.11.1.9) activity was by the so-called "chemical" method [18] in the presence of cumene hydroperoxide and reduced glutathione (GSH). In this case we estimated the residual GSH remaining after the action of enzyme in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid) [DTNB or

Ellman's reagent] at 412 nm [21]. In details: For incubation blank tube contained 700 µl TRIS buffer (0.05 M, pH 7.6), 100 µl GSH solution (2 mM) and 100 µl sample. After 5 min incubation we added 1 ml of 15% TCA solution and 100 µl cumene hydroperoxide (3.28 mM). Test tube contained 700 µl TRIS buffer (0.05 M, pH 7.6), 100 µl GSH solution (2 mM), 100 µl cumene hydroperoxide (3.28 mM) and 100 µl sample. After 5 min incubation we added 1 ml of 15% TCA solution to stop enzyme reaction. The reaction mixtures were centrifuged for 10 min at 15000 g. Supernatants were used for determination of GSH content. One unit of GPx-ase converts 1 micromole of GSH into GSSG/min at 30 °C at pH 7.4.

In the case of **catalase** (C-ase; EC 1.11.1.6) activity determination the extinction decrease was measured at 240 nm every min for 10 min at 30 °C [2]. The C-ase activities were given in Bergmeyer Unit (BU). One BU is the amount of C-ase which can decompose 1000 mg H_2O_2 in 1 min.

Glutathione reductase (GR-ase; EC 1.6.4.2) was measured as a decrease of NADPH at 340 nm at 30 °C and pH 7.4 [18]. The enzyme needs NADPH for reduction of GSSG. One unit of GR-ase was defined as 1 micromole of GSSG reduced/min.

GSH was determined spectrophotometrically with Ellman's reagent [15] and GSSG by the method of Tietze [23].

Protein content was measured with the Folin reagent using plasma albumin as standard [10].

Statistical evaluation: the reported data are means and SEM of the measurements. For statistical evaluation Student's *t*-test was used. Differences were considered significant if $p<0.05$.

Results

The fasting blood sugar levels of control rats were: 6.32±0.65 mM. 48 hours after the single injection of 85 mg/kg b.w. STZ, blood glucose elevated significantly 20.90±1.26 mM as mentioned earlier.

Table I presents in columns the amounts of plasma and hemolysate total thiobarbituric acid reactive substances (TTBARS or LP) in nM MDA/mg protein, oxidized and reduced glutathione values of diabetic and control rats. Forty-eight hours after STZ treatment in our case the LP, GSH and GSSG did not change in the plasma, but the last two decreased significantly in the hemolysate of STZ-treated animals. Columns of Table show the antioxidant enzyme activities in the hemolysate of control and diabetic rats. After STZ treatment all of the measured antioxidant enzyme activities decreased significantly.

Table I

The effect of STZ treatment on the pro- and antioxidant systems measured in the plasma, hemolysate, spleen, heart and skeletal muscles. (All values are expressed as mean ± SE. A value of $p<0.05$ or $p<0.01$** were considered to be significant)*

Organs	Plasma		Hemolysate		Spleen		Heart muscle		Skeletal muscle	
Parameters	Control	STZ	Control	STZ	Control	STZ	Control	STZ	Control	STZ
GSH nM/mg	1.689 ±0.061 (n=4)	1.739 ±0.086 (n=5)	62.45 ±1.02 (n=4)	56.75 ±1.02 (n=4)**	10.48 ±0.36 (n=4)	9.28 ±0.20 (n=4)**	11.53 ±0.30 (n=4)	10.24 ±0.31 (n=4)**	13.77 ±0.25 (n=4)	12.74 ±0.34 (n=3)**
GSSG nM/mg	0.086 ±0.025 (n=4)	0.080 ±0.051 (n=5)	1.17 ±0.39 (n=4)	0.43 ±0.16 (n=4)**	0.15 ±0.08 (n=4)	0.17 ±0.08 (n=4)	0.11 ±0.09 (n=4)	0.22 ±0.15 (n=6)	0.13 ±0.06 (n=4)	0.13 ±0.06 (n=4)
LP nM MDA/mg	0.272 ±0.008 (n=4)	0.268 ±0.026 (n=5)	0.92 ±0.08 (n=4)	0.89 ±0.03 (n=4)	1.02 ±0.21 (n=7)	0.996 ±0.03 (n=5)	0.45 ±0.01 (n=3)	0.57 ±0.05 (n=6)**	1.06 ±0.06 (n=3)	0.86 ±0.07 (n=6)**
t-SOD U/mg	–	–	21.34 ±2.19 (n=3)	10.23 ±1.59 (n=4)**	6.47 ±0.51 (n=4)	4.06 ±0.43 (n=4)**	16.83 ±1.41 (n=5)	11.10 ±1.62 (n=7)**	11.34 ±1.06 (n=5)	8.73 ±0.77 (n=4)**
Mn-SOD U/mg	–	–	–	–	0.15 ±0.07 (n=4)	0.13 ±0.04 (n=4)	2.65 ±0.26 (n=5)	2.23 ±0.56 (n=7)	1.73 ±0.50 (n=5)	1.93 ±0.10 (n=4)
Cu, Zn-SOD U/mg	–	–	–	–	6.33 ±0.46 (n=4)	3.94 ±0.39 (n=4)**	14.18 ±1.24 (n=5)	8.87 ±1.09 (n=7)**	9.60 ±0.66 (n=5)	6.80 ±0.67 (n=4)**
GPx-ase $U/mg \times 10^{-2}$	–	–	64.5 ±0.5 (n=4)	49.3 ±3.8 (n=4)**	2.63 ±0.01 (n=4)	2.11 ±0.02 (n=4)**	5.55 ±0.12 (n=3)	6.16 ±0.32 (n=3)	1.39 ±0.24 (n=3)	1.29 ±0.19 (n=6)
C-ase $BU/mg \times 10^{-4}$	–	–	4.10 ±0.21 (n=3)	1.97 ±0.01 (n=4)**	1.20 ±0.15 (n=4)	1.11 ±0.09 (n=4)	1.17 ±0.05 (n=5)	3.30 ±0.35 (n=7)**	0.28 ±0.02 (n=5)	0.41 ±0.01 (n=5)**
GR-ase $U/mg \times 10^{-2}$	–	–	–	–	7.2 ±0.8 (n=6)	7.16 ±0.52 (n=3)	2.23 ±0.13 (n=3)	2.25 ±0.34 (n=6)	1.80 ±0.08 (n=3)	1.44 ±0.07 (n=6)**

Further in the Table I the control and diabetic rat tissue antioxidants were compared in the following organs: spleen, heart and skeletal muscle homogenates.

The following comparisons were made between the control and STZ-treated spleen homogenates:

LP mildly decreased in spleen.

GSH amount decreased significantly in the organ homogenates.

GPx-ase activity decreased significantly and GR-ase activity mildly in the spleen.

Decrease of SOD activities were significant in t- and Cu, Zn-SODs.

In the heart and skeletal muscle homogenates (samples taken from the right posterior tight muscle) if we are comparing the diabetic parameters with control the following conclusions can be drawn:

The LP increased in the heart muscle and decreased significantly in the skeletal muscle after the STZ treatment.

GSH amount decreased significantly in both muscles.

The activities of GPx-ase and GR-ase did not change markedly in the muscles, but the GR-ase activity decreased significantly in skeletal muscle. The increase of C-ase activity was significant in both muscles.

Decrease in activities of SODs was also general and significant in both muscles.

Table II shows the measured and compared pro- and antioxidant changes in rats after STZ treatment. The following results are summarized in the Table: the pro- and antioxidant changes are observed in liver, brain, kidney, lung and pancreas homogenates. In details, in the liver:

The LP, GSH and GSSG didn't change in the liver homogenate.

GPx-ase, GR-ase and C-ase activities were significantly decreased.

The t- and Cu, Zn-SOD decreases were significant too.

In general the CN^- resistant (mainly Mn-SOD) activities increased in the homogenates after the toxic effects of STZ [5].

In the brain:

The LP mildly increases as well as the amount of GSH and GSSG.

GPx-ase activity increased and GR-ase decreased after the STZ treatment.

The same activity enlarger effect is the STZ on the t-, and Cu, Zn-SOD activities.

In kidney homogenates the following activity changes are shown after STZ treatment:

The LP decrease was significant as well as the decrease of GSH and GSSG quantities.

GPx-ase activity' increase and the GR-ase and C-ase activities' decrease were significant in the kidney homogenates.

Table II

Pro- and antioxidant changes measured in the liver, brain, kidney, lung and pancreas after STZ treatments

Organs	Plasma		Hemolysate		Spleen		Heart muscle		Skeletal muscle	
Parameters	Control	STZ	Control	STZ	Control	STZ	Control	STZ	Control	STZ
GSH	5.78	5.66	24.18	27.28	10.04	9.56	11.17	10.46	9.76	76.92
nM/mg	±0.10	±0.51	±0.82	±7.22	±0.09	±0.19	±0.17	±0.30	±1.70	±18.17
	(n=4)	(n=6)	(n=4)	(n=4)	(n=4)	(n=6)**	(n=3)	(n=4)*	(n=4)	(n=8)**
GSSG	0.43	0.37	3.34	4.69	0.13	0.25	0.09	0.11	0.21	0
nM/mg	±0.30	±0.25	±0.56	±1.05	±0.05	±0.09	±0.05	±0.16	±0.11	0
	(n=4)	(n=6)	(n=4)	(n=4)	(n=4)	(n=6)	(n=3)	(n=4)	(n=2)	(n=3)
LP	0.42	0.45	2.16	2.32	1.28	1.08	0.83	0.79	0.39	0.27
nM MDA/mg	±0.01	±0.02	±0.06	±0.54	±0.01	±0.05	±0.01	±0.05	±0.03	±0.01
	(n=4)	(n=6)	(n=4)	(n=4)	(n=4)	(n=6)**	(n=3)	(n=6)	(n=4)	(n=3)**
t-SOD	40.53	30.11	13.29	16.34	36.11	26.78	4.16	2.93	9.37	5.82
U/mg	±1.45	±2.05	±1.19	±1.89	±3.84	±2.01	±0.58	±0.47	±0.57	±0.79
	(n=3)	(n=6)**	(n=3)	(n=5)	(n=3)	(n=6)**	(n=5)	(n=7)**	(n=4)	(n=4)**
Mn-SOD	1.76	1.90	2.64	2.41	1.69	2.22	1.06	0.81	0.95	0.97
U/mg	±0.11	±0.49	±0.34	±0.45	±0.27	±0.24	±0.18	±0.17	±0.06	±0.11
	(n=3)	(n=6)	(n=3)	(n=5)	(n=3)	(n=6)*	(n=5)	(n=7)*	(n=4)	(n=4)
Cu,Zn-SOD	38.77	28.21	10.65	13.94	34.47	24.57	3.10	2.13	8.43	4.85
U/mg	±1.36	±1.71	±0.82	±1.51	±3.66	±1.80	±0.41	±0.32	±0.51	±0.69
	(n=3)	(n=6)**	(n=3)	(n=5)**	(n=3)	(n=6)**	(n=5)	(n=7)**	(n=4)	(n=4)**
GPx-ase	5.08	4.14	2.57	2.94	1.62	2.16	5.85	5.02	1.59	0.87
$U/mg \times 10^{-2}$	±0.08	±0.40	±0.07	±0.35	±0.06	±0.17	±0.18	±0.53	±0.05	±0.05
	(n=4)	(n=6)**	(n=3)	(n=4)	(n=4)	(n=6)**	(n=3)	(n=6)	(n=4)	(n=3)**
C-ase	34.7	21.4	0.46	0.34	22.7	9.7	1.98	2.57	3.85	0
$BU/mg \times 10^{-4}$	±2.8	±1.3	±0.08	±0.08	±0.08	±1.1	±0.32	±0.25	±0.21	
	(n=5)	(n=6)**	(n=4)	(n=4)	(n=4)	(n=4)**	(n=5)	(n=9)**	(n=5)	
GR-ase	10.47	8.02	5.19	4.98	22.20	12.83	6.68	6.03	5.70	5.00
$U/mg \times 10^{-2}$	±0.57	±0.74	±0.16	±0.32	±2.54	±3.27	±0.53	±0.21	±0.35	±0.06
	(n=3)	(n=6)**	(n=3)	(n=4)	(n=3)	(n=6)**	(n=3)	(n=6)	(n=3)	(n=4)

(*p<0.05; **p<0.01)

The SODs activities in general decreased significantly. The significant increase of Mn-SOD activity shows the toxic oxidative stress effect of STZ [5].

In the last part of Table II the pro- and antioxidant changes in the lung and pancreas after STZ treatment are compared.

The LP activities decreased in both organs. In the pancreas LP decrease was significant. It looks so, that in the pancreas the significant GSH increase tries to prevent the LP increase.

Significant GSH increase was observed in the pancreas, but decrease in lung. GSSG values decreased in pancreas, but increased in lung.

GPx-ase and GR-ase activities decreased significantly in pancreas.

The SODs also decreased significantly in both organ homogenates.

Discussion

During the studies of various human diabetic cases, stressed that in red blood cell (RBC) hemolysates of these patients superoxide dismutase activity is significantly decreased. Thereby, enzymatic antioxidant defence is deteriorated [12].

Later our result were complemented and confirmed by more extended studies on antioxidant enzyme activities (superoxide dismutase, glutathione peroxidase and catalase) and on lipid peroxidation.

Parallel, pro- and antioxidant studies and comparisons were performed in two experimental types of diabetes (i) in experiments with rats with diabetes induced by alloxan; and (ii) in rat organ homogenates in STZ diabetes. Approximately similar conclusions could be drawn from these comparative studies [14].

In our present communication data obtained in studies of STZ induced rat diabetes are summarized.

Recently publications have been released assuming the decisive role of hydroxyl radical (HO·) in development of STZ induced diabetes [22, 17].

As to its action mechanism, a paragraph of our earlier paper published in 1982, is worth citing "It emerged that SOD activity is significantly reduced in the 'large' organs and in the organs participating in the hematopoiezis in chemically-induced experimental diabetes; This may be direct consequence of the superoxide (O_2^-) or H_2O_2 accumulation [3], but a direct nucleic acid influence on protein synthesis by SOD is also conceivable" [12].

In the other hand, the presumption was expressed, that STZ is a "nitric oxide" (NO) scattering and exerts its effect on beta cells in the form of peroxinitrite ($ONOO^-$) either with the help of NO, or through its reaction with superoxide radical produced by alloxan, inducing diabetes [9].

The two presumptions can be reconciliated, since through the following decomposition peroxinitrite can produce HO· radical:

$$ONOO^- + H^+ \rightarrow NO_2 + HO\cdot$$

The same has been confirmed by recent clinical data in two human diabetes and in experimental diabetes [6].

From the present results it turnes out that in STZ diabetes of rats a prooxidant dominance (oxidative stress) develops, which is responsible in experiments for secondary injuries, e.g. increased injuries of DNA, protein and lipids. The main reason of oxidative stress in experimental diabetes is induced by high blood glucose level.

Further experiments are scheduled to prove the role of peroxinitrite in the development of experimental and human diabetes.

Acknowledgements

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Prostaglandin E receptors in myometrial cells

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Prostaglandins (PGs) exert their effects via binding to specific cell surface receptors and influencing second messenger systems through G-proteins. PGE_2 may interact with at least four receptor subtypes (EP1, EP2, EP3, EP4), each showing different pharmacological profiles. The second messengers calcium, inositol phosphates (InsPs) and cyclic nucleotides play decisive roles in uterine contractility. The question in this investigation was, which EP receptors, G-proteins and second messenger systems transmit PGE_2 induced signals in human myometrium. We have measured changes in InsPs and cAMP formation and also in intracellular calcium concentration ($[Ca^{2+}]_i$) induced by PGE_2 and receptor subtype selective analogues in cultured human myometrial cells. PGE_2 increased cAMP level and this effect was shared by the EP2 receptor subtype selective agonist Butaprost and by Misoprostol (EP3>EP2>EP1). Sulprostone (EP3>EP1) did not stimulate adenylyl cyclase activity *per se*, but inhibited forskolin-stimulated adenylyl cyclase in a pertussis toxin (PT) sensitive way. PGE_2, GR63799X (EP3 selective), Sulprostone and Misoprostol activated phospholipase-C (PLC), this effect was resistant to PT treatment. PGE_2 also elevated $[Ca^{2+}]_i$ from the resting level of 60–90 nM up to 350 nM. Low concentrations (1–300 nM) of PGE_2 increased $[Ca^{2+}]_i$ without PLC activation. The selective EP1 inhibitor AH6809, Nifedipine, Verapamil and PT treatment inhibited this effect of PGE_2. In cultured human myometrial cells PGE_2 interacts with EP1 receptors, which elevate $[Ca^{2+}]_i$ independently from PLC, but involving a Gi protein and plasmamembrane calcium channels; EP2 receptors which stimulate adenylyl cyclase; EP3A receptors, which inhibit adenylyl cyclase activity through Gi activation and EP3D receptors which activate PLC through a PT-insensitive pathway and also elevate $[Ca^{2+}]_i$.

Keywords: prostaglandin, prostaglandin receptors, G-proteins, PGE_2, calcium, inositol phosphates, cyclic nucleotides

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Although prostaglandins are of nonpolar, lipophilic nature, these paracrine substances do not exert their effects the way the typical lipoid hormones, the steroids or the thyroide hormones do, i.e. freely diffusing through the cell membrane, binding to cytosol receptors and influencing gene activation, but act more like the peptide hormones and the catecholamines: they bind to cell surface receptors and influence intracellular second messenger systems [33].

Specific binding sites for prostaglandins have been described in a wide variety of tissues [29]. Nevertheless the existence *of bona fide* prostaglandin receptors was always questioned, and their effects were partly explained by biophysical interactions: direct ionophore effects or intercalation between the fatty acid chains of the membrane lipids. The final decision in this question came in 1991 when the cDNA sequence of the first prostanoid receptor (the thromboxane receptor) was published [17]. The deduced amino acid sequence has shown a typical rhodopsin-type protein with seven nonpolar membrane-spanning segments. Since, at least one receptor for each of the major prostanoids has been sequenced. All these receptors belong to the G-protein binding hormone receptor family [8].

The scheme for classification of prostanoid receptors suggested by Coleman and coworkers has gained general acceptance [7]. This is a pharmacological system based on the one hand on clear cut effects, observed by prostaglandins in several animal organs/tissues, and on the other hand on the analysis of more or less receptor-selective agonists and antagonists. Accordingly, there are separate receptors for each of the five naturally occurring prostanoids, PGs D_2, E_2, $F_{2\alpha}$, I_2 and TxA_2. These receptors are called P receptor (P for prostaglandin) and are designated by the letter of the prostaglandin series (DP, EP, FP, IP and TP, respectively, Fig. 1). The system is very selective, each of these receptors is at least one orders of degree more sensitive to its own ligand than to the others. The amazingly variable effects of PGE_2 are explained by the existence of receptor subtypes. Four such subtypes: EP1, EP2, EP3 and EP4 can be found in different tissues, for instance EP1 receptors form homogenous population in guinea pig ileum, EP2 in cat trachea, EP3 in chicken ileum and EP4 in piglet saphenous vein smooth muscles. Each receptor subtype shows a characteristic pharmacological profile, selective agonists and antagonists are available for some but not every receptor subtype. The effects and the second messenger systems activated are different: EP2 and EP4 receptors in smooth muscle are inhibitory through the activation of adenylyl cyclase, EP1 and EP3 receptors increase smooth muscle contractility by phospholipase C (PLC) activation and calcium mobilization from intracellular stores, and also the stimulation of calcium influx through the cell membrane. EP3 receptors may have an additional myometrial stimulatory action by inhibiting cAMP production (Fig. 1) [7].

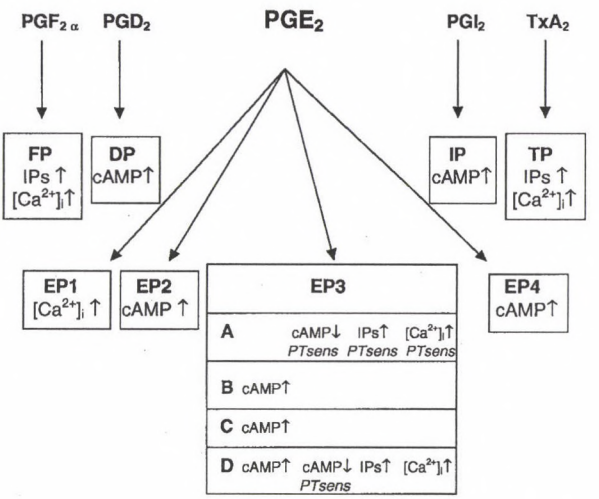

Fig. 1. Prostanoids and their receptors. See the Introduction for explanation. ↑: stimulation, ↓: inhibition, *PT sens*: sensitive to pertussis toxin treatment

An unexpected result emerged from the cDNA sequencing studies: EP3 receptors have been identified in isoforms, products of alternative splicing of the mRNA coding for the receptors. The deduced amino acid sequences of these isoforms are identical from the amino terminus to a glutamine in position 10 after the 7th transmembrane sequence, but have different carboxyl terminal (cytosolic) chains. As many as ten isoforms are differentially expressed in various species/tissues. In human cDNA libraries three [1], five [2, 28] and seven [30] EP3 isoforms have been identified. The most comprehensive study to date describes the EP3 receptors of bovine adrenal chromaffin cells, where the single gene encoding the receptor produces four receptor isoforms: EP3A, B, C and D [26]. These isoforms show no difference in drug sensitivity, what may not be surprising since they share the same transmembrane and extracellular domains, but couple through different G-proteins to different second messenger systems. EP3B, C and D receptors stimulate adenylyl cyclase, EP3A and D variants inhibit cAMP formation. This latter effect is pertussis toxin sensitive, the interaction is supposed to be transmitted through Gi protein. Besides, isoform A and D stimulate PLC and elevate $[Ca^{2+}]_i$ in a pertussis toxin sensitive and insensitive way, respectively (Fig. 1).

Existence of receptor subtypes among FP, IP and TP receptors has been also raised, probably different TP subtypes exist on platelets and on vascular smooth muscle cells, but this question is still unresolved [8].

Several lines of evidence support the view that prostaglandins (PGs) play a pivotal role in the mechanism of parturition: endogenous levels of PGs derived locally from gestational tissues rise sharply in amniotic fluid and maternal plasma during labour [18], exogenously administered PGs can terminate pregnancy [22], inhibitors of PG synthesis extend the period of gestation and delay preterm parturition [19]. PGE_2 is probably a key factor in the onset of parturition and a powerful stimulant of uterine contractility and cervical ripening *in vivo*. However, *in vitro* PGE_2 provokes complex biphasic responses in human myometrium: stimulation at low concentrations, and initial stimulation followed by relaxation at high concentrations [6, 31, 32, 37]. Uterine contractility is stimulated by increasing intracellular calcium level ($[Ca^{2+}]_i$), resulting in myosin light chain kinase (MLCK) activation [23, 34, 39]. On the other hand cyclic nucleotides (cAMP, cGMP) promote relaxation by decreasing the affinity of MLCK for the calcium-calmodulin complex and stimulating mechanisms to decrease $[Ca^{2+}]_i$ [9, 38].

The question in this investigation was, which EP receptors, G-proteins and second messenger systems transmit PGE_2-induced signals in human-myometrium. To answer this question we have measured changes in inositol phosphates (InsPs) and cAMP formation and also in $[Ca^{2+}]_i$ induced by PGE_2 and receptor subtype selective analogues in cultured human myometrial cells.

Materials and Methods

Materials

Sulprostone was kindly provided by Dr Sprzagala, Schering AG, Berlin, Germany; GR63799X and AH6809 were generous gifts from Dr R. A. Coleman and Dr B. M. Bain, Glaxo, UK; Butaprost was a gift from Dr Ph. Gardiner, Bayer, UK. Misoprostol was the product of Searle, UK. Myo-[^{3}H]-inositol and [2,8-^{3}H]-adenosine-3'5'-cyclic phosphate were obtained from Amersham International plc (Amersham, Bucks, U.K.). Fura-2 acetoxymethyl ester and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR, USA). Pertussis toxin, 1,1,2-trichlorotrifluoroethane (freon), tri-n-octylamine, elastase (type 1), DNAse I (type IV) and PGE_2 were bought from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). Dispase (type XIV) was from Boehringer Mannheim (Lewes, East Sussex, U.K.). Collagenase CLS 2 (200–250 U/mg) was from Cambridge BioScience (Cambridge, U.K.). Tissue culture reagents were from Gibco BRL (Inchinan, Renfrewshire, U.K.). All other reagents were commercial preparations of the highest available purity.

Myocyte isolation and culture

Samples of myometrium were obtained from non-pregnant pre-menopausal women at hysterectomies performed for benign gynaecological disorders. This study had the approval of the Central Oxfordshire Research Ethics Committee and all patients gave informed consent. Myocyte isolation was carried out as described earlier [27]. Myometrial cells were cultured in Waymouth MB 752/1 medium containing 10% (v/v) fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Myometrial cells in culture demonstrate characteristic features of uterine smooth muscle cells: fusiform shape, expression of smooth muscle specific actin and hormone responsiveness over several subcultures as described by Phaneuf et al. [27]. Experiments were performed on subcultures number 2–5.

Intracellular Ca^{2+} estimation

Myometrial cells were cultured to confluency on 13 mm diameter glass microscope coverslips, washed twice in 2 ml Hanks' balanced salt solution (HBSS) for 15 minutes and loaded with 5 μM Fura-2 AM in HBSS, in the presence of 0.1% w/v Pluronic F-127 detergent, at 37 °C in the dark for 30 minutes. Fluorimetric measurements were made by the method of Grynkiewicz et al. [16], in a Shimadzu RF 5001 spectrofluorimeter (Howe, Bicester, U.K.) controlled by a Viglen 386 SX PC, equipped with a dual wavelength determination software (Shimadzu). Determinations were made in 2.5 ml HBSS at 37 °C, excitation wavelengths were 340 nm and 380 nm; emission was detected at 510 nm. Intracellular calcium concentration ($[Ca^{2+}]_i$) was calculated from the fluorescence ratio data [16].

Inositol phosphates determination

Labelling of phosphoinositides was performed as described earlier [27]. Briefly, confluent myocytes grown in 24- or 48-well plastic plates were incubated with 2–10 μCi [^{3}H]-myo-inositol per well in medium/penicillin/streptomicin/2% v/v fetal calf serum for 48–60 hours. Stimuli were added for 30 minutes in a final volume of 250 μl HBSS, in the presence of 12 mM LiCl. The reaction was stopped by adding 1 ml chloroform/methanol/HCl (50:100:1, v/v). Further addition of 0.3 ml chloroform and 0.3 ml water separated the mixture into a water phase and an organic phase. Aliquots from the water phase were applied to Dowex AG 1-X8 resin columns and inositol phosphates (InsPs) were separated and quantified according to the method of Bone et al. [3].

Measurement of cAMP

Cells were incubated at 37 °C in HBSS containing 5 mM theophylline. Stimuli were added for 7 minutes in a final volume of 500 µl. The reaction was stopped by the addition of ice cold 5% perchloric acid, followed by centrifugation at 1000×g. The supernatants were extracted with 1.3 volume of freon:tri-n-octylamine (1:1) and cyclic AMP was measured by the competitive protein-binding assay of Brown et al. [4] as described earlier [21] using ^{3}H-cAMP as tracer.

Results and Discussion

Effects of PGE_2 on cyclic AMP formation in cultured human myometrial cells

PGE_2 stimulated the formation of cAMP in a concentration dependent manner up to a maximum of 50 fold above control. This effect of PGE_2 is well known in several systems [5, 21, 26, 33]. PGE_2 stimulates cAMP production by EP2, EP3B, C, D and EP4 receptors (Fig. 1) [7, 8, 26]. An increase in cAMP level and consequently the activation of protein kinase-A (PKA) decreases the affinity of MLCK for the calcium-calmodulin complex and also stimulates plasma membrane and endoplasmic reticulum calcium pumps, therefore decreases $[Ca^{2+}]_i$ [9, 38]. These effects are likely to result in smooth muscle relaxation. Misoprostol (EP2>EP3) and the selective EP2 agonist Butaprost also activated adenylyl cyclase up to 30–70 fold (Fig. 2), the role of EP2 receptors in this pathway is firmly established. The EP1–EP3 agonist Sulprostone did not stimulate cAMP production, but effectively inhibited Forskolin induced cAMP synthesis and this effect was pertussis toxin sensitive, indicating a role for Gi protein (Fig. 2). The Gi-coupled inhibition of adenylyl cyclase is a typical EP3 mediated effect and could be ascribed to EP3A or D type receptors (Fig. 1) [26, 28].

Effects of PGE_2 and receptor-subtype-selective analogues on inositol phosphates formation in cultured human myometrial cells

PGE_2 stimulated the formation of InsPs up to 500% above basal. There was no detectable stimulation of InsPs formation below 1 µM PGE_2. Similar effects have been shown in human myometrial explants [10] and in rat myometrium [15]. The EP3 selective agonist GR63799X and Sulprostone (EP3>EP1>EP2) had similar stimulatory effects on InsPs formation whereas Misoprostol (EP3>EP2>EP1) was only weakly stimulatory (150%). The effect of PGE_2 could not be inhibited by 20 µM AH6809.

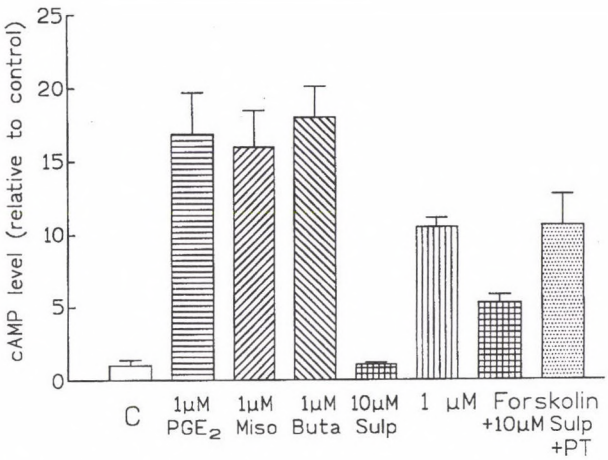

Fig. 2. Effects of PGE_2 and analogues on cAMP formation in cultured human myometrial cells. Cultured human myometrial cells were incubated in Hanks' balanced salt solution in the presence of 5 mM theophylline. C: control, 1 μM PGE_2, Miso: 1 μM Misoprostol, Buta: 1 μM Butaprost, Sulp: 10 μM Sulprostone. When present, 1.25 μg/ml PT was added to cultured myometrial cells for 16 hours before stimulating the cells with 1 μM Forskolin ±10 μM Sulprostone. Results are shown as stimulation relative to control. Values are means ±SEM from three independent experiments

These results indicate that PGE_2 stimulation of PLC activity is mediated primarily by EP3 type receptors in cultured human myometrial cells. Human myometrium has EP3 receptor type pharmacological responses [31, 32]. To test for the involvement of G-proteins in PLC activation by PGE_2, myometrial cells were treated with 1.25 μg/ml PT for 16 hours. This treatment did not modify the effects of PGE_2 on InsPs formation. The PT insensitive activation of PLC is in agreement with activation of the EP3D isoform [26, 28], which has also been named EP3II [2] or EP3c [30] and is analogous to the bovine EP3D [26] and the mouse EP3γ [8]. A likely candidate for the G-protein involved may be G_q which is expressed at high levels in human myometrium [11, 27].

Effect of PGE_2 on intracellular calcium concentration

Addition of PGE_2 resulted in a transient increase in $[Ca^{2+}]_i$ from the resting level (60–90 nM) up to 350 nM. This effect is likely to result in increased myometrial contractility [23, 34, 39]. PGE_2 provokes contractions in pregnant and nonpregnant human and rat myometrium [15, 31, 32, 37]. The peak value of $[Ca^{2+}]_i$ was concentration dependent at about the same concentration range, at which PGE_2 stimulated PLC activity. Lower concentrations of PGE_2 increased cytosolic calcium to

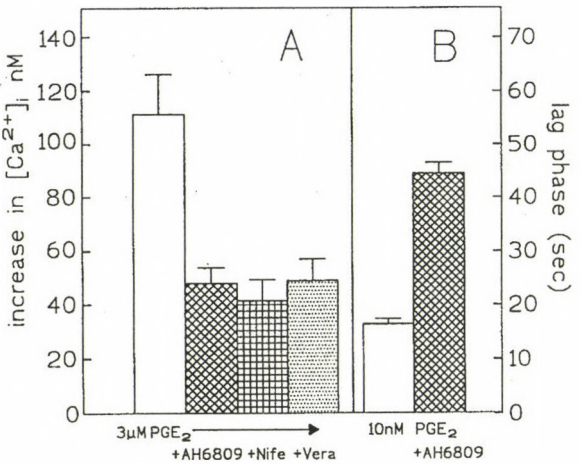

Fig. 3. PGE_2-induced intracellular $[Ca^{2+}]_i$ in cultured human myometrial cells. Inhibition by the EP1 antagonist AH6809 and calcium channel blockers. Panel A: Inhibition of 3 μM PGE_2-induced increase in $[Ca^{2+}]_i$ by 10 μM AH6809, 10 μM Nifedipine (Nife) or 100 μM Verapamil (Vera). Y-axis shows the PGE_2-induced increase of $[Ca^{2+}]_i$ in nM, means ±SD from quadruplicate determinations are shown. Panel B: Inhibition of the effect of 10 nM PGE_2 on $[Ca^{2+}]_i$ by 10 μM AH6809. The time elapsed from the addition of the agonist until half maximal stimulation of $[Ca^{2+}]_i$ is shown, values should read form the Y-axis on the right side, in sec. Results are means ±SD from quadruplicate determinations

about 200 nM, a value well within the range required for maximal contractions in human myometrium [34]. The height of the calcium peak was almost concentration independent between 3–300 nM. The contractile effects of PGE_2 in pregnant human myometrium appear to be dose-independent over a similar concentration range [32]. GR63799X, Sulprostone and Misoprostol increased $[Ca^{2+}]_i$ in the micromolar range as effectively as PGE_2. This shows the role of EP3 receptors in the elevation of $[Ca^{2+}]_i$ transients.

The EP1 selective antagonist AH6809 decreased the height of the calcium peak induced by 3 μM PGE_2 by 60% and also elongated the lag phase induced by 10 nM PGE_2 from 14 sec to 44 sec, an effect equivalent to 88% inhibition, suggesting the involvement of EP1 receptors in PGE_2-induced increase of $[Ca^{2+}]_i$ (Fig. 3). Human EP1 receptors produced an increase in $[Ca^{2+}]_i$ [12], with very little increase in InsPs formation [36]. Under our experimental conditions PGE_2 did not stimulate PLC activity below 1 μM, yet elicited an increase in $[Ca^{2+}]_i$. Nifedipine (10 μM) and Verapamil-HCl (100 μM) inhibited partially the calcium peak induced by 3 μM PGE_2 (Fig. 3), and blocked completely the calcium transients evoked by 10 nM PGE_2. Many reports show a complete dependency of PG induced $[Ca^{2+}]_i$ increases on the presence of extracellular Ca^{2+} ions [25, 35, 36]; indeed in the original classification EP1 receptors

were proposed to act by increasing calcium influx through plasma membrane ion channels [7]. Pertussis toxin treatment had dual effects on PGE_2-induced changes in $[Ca^{2+}]_i$. Calcium transients induced by μM concentrations of PGE_2 were not affected by PT pretreatment, similarly to the PLC activating effects of PGE_2, in contrast $[Ca^{2+}]_i$ transients induced by lower concentrations (nM) of PGE_2 were inhibited by PT. PGE_2 induces contractions in rat myometrium at submicromolar concentrations via a PT sensitive pathway, while micromolar effects are PT resistant [15]. The presence of G proteins which are substrates for PT (G_{i1}, G_{i2} and G_{i3}, but not G_0) has been demonstrated by immunoblotting with specific antibodies and ADP ribosylation in fresh human myometrium [11] and cultured myometrial cells [27].

The increase in $[Ca^{2+}]_i$ occurred after a delay of up to 30 sec, and the length of this lag phase was inversely proportional to the doses of PGE_2 used. By contrast even the lowest effective concentration of oxytocin or $PGF_{2\alpha}$ induce instantaneous increases in $[Ca^{2+}]_i$ [27], therefore the lag time observed with PGE_2 is unlikely to be the consequence of nonspecific (e.g. diffusion-related) delays at low concentration of the agonist. An inversely concentration-dependent lag phase was also apparent in cells expressing human EP1 receptors [12]. It is tempting to speculate that the rise in $[Ca^{2+}]_i$ depends on the formation of a mediator which activates $InsP_3$ insensitive calcium pools, such as cyclic ADP ribose [13], sphingosin phosphate [14] or nicotinic acid adenine dinucleotide phosphate [20]. The lack of PLC stimulation by EP1 receptors in RCCP cells has been also explained by the activation of $InsP_3$-independent intracellular calcium stores [8]. Alternatively these observations may represent delayed activation of plasma membrane calcium channels [24]. Our results with the calcium channel blockers favour the latter hypothesis.

Conclusion

In human myometrial cells PGE_2 exerts its effects via several signal transducing mechanisms. PGE_2 in a broad concentration range activates adenylyl cyclase through EP2 receptors, this pathway is likely to have inhibitory effects on smooth muscle contractility. There is also a high affinity mechanism present, with EP1 receptors, which couple through a PT-sensitive G-protein (probably G_i) to elevation of $[Ca^{2+}]_i$ and involves plasma membrane calcium channel opening, but does not activate PLC. PGE_2 also interacts with a lower affinity mechanism, through EP3D receptors, active in the micromolar concentration range, which stimulate PLC via a PT-insensitive pathway and also elevate $[Ca^{2+}]_i$. These pathways are likely to increase uterine contractility *in vivo*. Furthermore, there is a third stimulatory mechanism present, via EP3A receptors, which inhibit adenylyl cyclase activity through a Gi protein.

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The effect of adrenoreceptor antagonists and agonists on LHRH release from the stalk-median eminence in the pig

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A release of radio-immunoassayable LHRH from the stalk-median eminence of neonatal piglets and prepubertal gilts was measured using an *in vitro* incubation system. The stalk-median eminence was collected from one-week-old male (n=19) and female (n=21) piglets and from 6-month-old prepubertal ovariectomized gilts given oestradiol benzoate (20 μg/kg b.w.; n=52) or left untreated (control; n=25) 30 or 68 h before slaughter. Each vial, containing the stalk-median eminence in 2 ml of Krebs-Ringer bicarbonate buffer, was incubated for 30 min, followed by 30 min incubations during which either basal release or the effect of adrenoreceptor antagonists and agonists on LHRH output was evaluated.

There were no differences between the basal release of LHRH (x±SEM; pg/ml) from the stalk-median eminence of male (65.5±9.8) and female (66.3±9.6) newborn piglets. The addition of propranolol (10^{-6} M) caused a 250% increase in LHRH release from the stalk-median eminence explants of neonatal males ($p=0.08$) and females ($p<0.05$). Neither norepinephrine nor phentolamine affected LHRH release from the stalk-median eminence of newborn males and females.

The basal release of LHRH (pg/ml) from the stalk-median eminence explants collected from ovariectomized gilts given oestradiol benzoate 30 and 68 h before slaughter or left untreated was similar (147.5±36.1, 236.4±77.7 and 202.0±41.6, respectively). Propranolol evoked a significant increase in LHRH secretion from the stalk-median eminence in the control group, but not in the groups given oestradiol benzoate. Norepinephrine (10^{-6} M) increased LHRH release from the stalk-median eminence collected from the control animals, 30 h and 68 h after oestradiol benzoate treatment by 48, 78 and 73 percent, respectively. Phentolamine (10^{-6} M) did not affect LHRH release from the stalk-median eminence in control animals and ovariectomized gilts primed with oestradiol benzoate.

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Urapidil (10^{-6} M, α_1-adrenoreceptor antagonist) did not affect the basal LHRH release from the stalk-median eminence of gilts from the control group and group slaughtered 30 h after oestradiol benzoate treatment, but caused a rapid increase of LHRH release from the stalk-median eminence 68 h after oestradiol benzoate treatment. Phenylephrine (10^{-6} M) did not affect LHRH output from the stalk-median eminence collected at various time periods after oestradiol benzoate administration *in vivo*.

These results suggest that in pigs, nerve terminals releasing LHRH at the stalk-median eminence level are sensitive to adrenergic stimulation or inhibition and that the adrenergic system can be modulated by estrogens in the prepubertal gilts.

Keywords: LHRH, adrenoreceptor antagonists and agonists, pig

LHRH is a key factor in the control of reproductive functions in mammals. Many papers [1–3, 10, 14] indicate that the central noradrenergic system plays an important role in LH secretion. Recently, it has become obvious that neurotransmitters can act at multiple brain sites influencing LHRH release. Interestingly, activation of the central noradrenergic system may inhibit, as well as facilitate, LH release. Norepinephrine is excitatory in the presence of estrogen and inhibitory in its absence [18]. In the rat and rabbit [2, 13] α-adrenergic agonist stimulated LH and LHRH release in ovarian-intact or ovariectomized, estrogen-primed animals, but did not stimulate LHRH release in unprimed ovariectomized animals [15].

The presence of a steroid is not obligatory for the stimulatory influence of norepinephrine on LHRH release in rhesus monkey [8]. The apparent diversity of catecholaminergic actions (negative vs positive) might be explained on the basis of different receptor subtypes.

This report utilizes an *in vitro* system to study the LHRH release by the stalk-median eminence complex in pigs under the influence of adrenoreceptor antagonists and agonists during two different periods of development. A preliminary report has been presented [19].

Materials and Methods

Animals and experimental procedures

Investigations were performed on two groups of animals: one-week-old piglets and 6-month-old prepubertal gilts. Animals were exsanguinated and the brain tissues were collected within 2 min after death. Stalk-median eminences were collected from one-week-old males (n=19) and females (n=21) and from ovariectomized gilts given oestradiol benzoate (20 μg/kg b.w.; n=52) 30 or 68 h before slaughter or left untreated (control; n=25). The stalk-median eminence was easily detached from the medial basal hypothalamus without cutting and was then cut away at its junction with the pituitary.

In vitro incubation of the stalk median eminence

Immediately after the collection of the stalk-median eminence, tissues were transferred to glass incubation vials (20 mm × 40 mm) containing 2 ml of Krebs-Ringer bicarbonate buffer (KRB, pH 7.4) supplemented with 1 mg glucose ml^{-1}. Stalk-median eminences were transported to the laboratory and upon arrival (approximately 10–15 min after the collection) the medium was replaced with fresh KRB and the pre-incubation period began. Incubation vials were maintained in a water-bath at 37 °C with constant agitation (45 cycles min^{-1}) in the atmosphere of 95% O_2 and 5% CO_2. After a 30 min preliminary equilibration of the tissue, KRB was replaced and a 30 min incubation was performed to establish the basal release of LHRH. Subsequently, the 30 min incubation was carried out in the presence or absence of various adrenoreceptor antagonist and agonist as specified in the description of experiments. The tested drugs were dissolved in KRB and added to the total volume of 20–50 µl to the incubation vial at the beginning of the incubation after removing the same volume of medium. Thereafter the stalk-median eminence tissue was incubated (30 min) in the fresh KRB without drugs and finally exposed for two 30 min periods to an increased concentration of KCl (56 mM) in KRB. The buffer removed from the vial after each incubation was transferred to plastic tubes and centrifuged at 2000 g for 20 min at 4 °C. The supernatant was decanted and the stalk-median eminence tissue was weighed and frozen at –70 °C.

Experimental designs

Experiment No. 1

Stalk-median eminence tissues from one-week-old piglets were incubated with 10^{-6}M phentolamine (α-antagonist; males n=5 and females n=4), 10^{-6}M propranolol (β-antagonist; males n=5 and females n=7), 10^{-6}M norepinephrine (α- and β-agonist; males n=5, females n=6) or without any drug (control; males n=4 and females n=4).

Experiment No. 2

Stalk-median eminence tissues from prepubertal ovariectomized gilts given vehicle (control; n=15), oestradiol benzoate 30 h (30EB; n=15) or 68 h (68EB; n=15) before slaughter were incubated with phentolamine (control, n=5; 30EB, n=5; 68EB, n=5; respectively), propranolol (control, n=5; 30EB, n=5; 68EB, n=5; respectively) or norepinephrine (control, n=5; 30EB, n=5; 68EB, n=5; respectively) at a concentration of 10^{-6}M.

Experiment No. 3

Stalk-median eminence tissues from prepubertal ovariectomized gilts given vehicle (control, n=10) and oestradiol benzoate 30 h (30EB; n=10) or 68 h (68EB; n=12) before slaughter were incubated with urapidil (α_1-antagonist, control, n=5; 30EB, n=6; 68EB, n=6; respectively) or phenylephrine (α_1-agonist; control, n=5; 30EB, n=4; 68EB, n= 6; respectively) at a concentration of 10^{-6}M.

LHRH radioimmunoassay

LHRH concentration in the incubation medium was determined by a single antibody radioimmunoassay as described earlier by Ziecik et al. [20] and modified by Sesti and Britt [16]. Synthetic LHRH (acetate salt, Sigma, St. Louis, USA) was radioiodinated by the iodogen method of Fraker and Speck [7]. The iodinated LHRH was separated from free iodine by ion-exchange chromatography using a QAE-Sephadex (Sigma, St. Louis, Ho, USA) column. The anti-LHRH antiserum AZJR-2 used in this assay was raised in our laboratory by immunizing rabbits against the native LHRH conjugated to bovine serum albumin (BSA). AZJR-2 is a polyclonal antibody that does not crossreact (<0.1%) with LH, FSH, CRF and oxytocin. Antiserum (50 µl) diluted at 1:2000 in 10 mM PBS-EDTA, pH=7.4 was added to the 100 µl of unknown and standard samples followed 30 min later by 50 µl of iodinated LHRH (about 20 000 cpm). After incubation for 36 h at 4 °C, free labelled LHRH was separated from the bound by adding 2 ml of ice-cold 96% ethanol, incubating for 15 min, centrifuging at 3000 g for 20 min and decanting the supernatant. Bound ^{125}I labelled LHRH was estimated by counting the pellet. The range of LHRH standards used was 0.15–100 pg per tube and the average assay sensitivity was 9.8 pg/ml^{-1} at 95% binding. The intra- and interassay CVs (coefficients of variation) were 10% and 13%, respectively.

Drugs

The following adrenoreceptor agonists and antagonists were used: norepinephrine, propranolol, phentolamine – all purchased from Sigma Chemical Company, St. Louis; urapidil (Ebrantil, Byk-Gulden, Germany) and phenylephrine (Neo-synephrine, Polfa, Poland).

Statistics

The significance of differences was evaluated by a one-way analysis of variance and Student's *t*-test or Bonferoni's test.

Results

Experiment No. 1

The *in vitro* release of LHRH from the stalk-median eminence of newborn piglets (average wet weight of the stalk-median eminence = 7.5±0.6 mg) varied from 17.5–94.0 pg/ml with no differences between males (65.5±9.8 pg/ml) and females (66.3±9.6 pg/ml).

The changes of *in vitro* LHRH release from the stalk-median eminence of one-week-old males are depicted in Fig. 1. Propranolol caused a significant (over 250%) increase ($p=0.08$) of LHRH efflux from the stalk-median eminence tissue to the incubation medium. Norepinephrine slightly increased LHRH release, but due to variations between incubations the difference was not significant ($p>0.05$). Phentolamine did not show any influence on LHRH release from the stalk-median eminence of newborn males. KRB alone did not change the pattern of LHRH release during the next incubation, though LHRH efflux from tissue was statistically significant ($p<0.05$). K^+ elicited a higher LHRH release from the stalk-median eminencies than the initiating concentrations.

The changes of *in vitro* LHRH release from the stalk-median eminence of one-week-old females are depicted in Fig. 2. Propranolol also caused 250% ($p<0.05$)

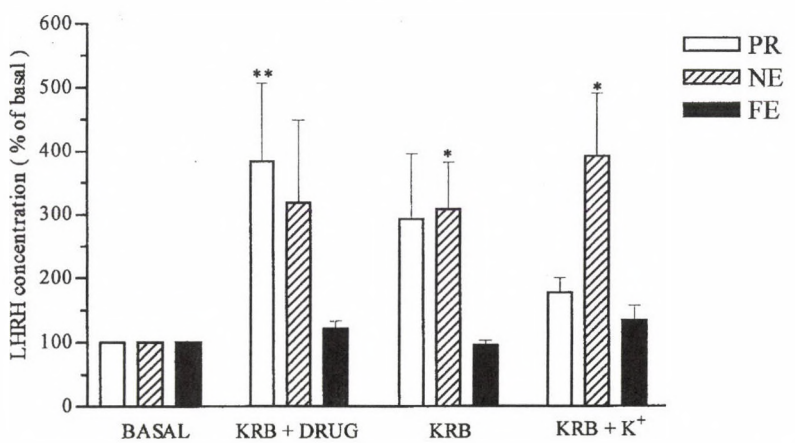

Fig. 1. Basal and in response to 10^{-6} M propranolol (PR), norepinephrine (NE), phentolamine (FE) and potassium (56 mM) release of LHRH (±SEM) *in vitro* from the stalk-median eminence explants of neonatal males (Experiment No. 1). BASAL – basal release of LHRH; KRB+DRUG – tested drugs dissolved in KRB; KRB – KRB without drug and KRB+K^+– 56 mM KCl in KRB. Asteriks indicate significant differences when compared with the appropriate basal values *$p<0.05$; **$p<0.01$

increase in LHRH release from the stalk-median eminence tissue. Neither norepinephrine nor phentolamine affected LHRH release from the stalk-median eminence of neonate gilts. Interestingly, K^+ evoked the highest LHRH release from the stalk-median eminence after incubation with norepinephrine both in males and females (Fig. 1 and Fig. 2, respectively).

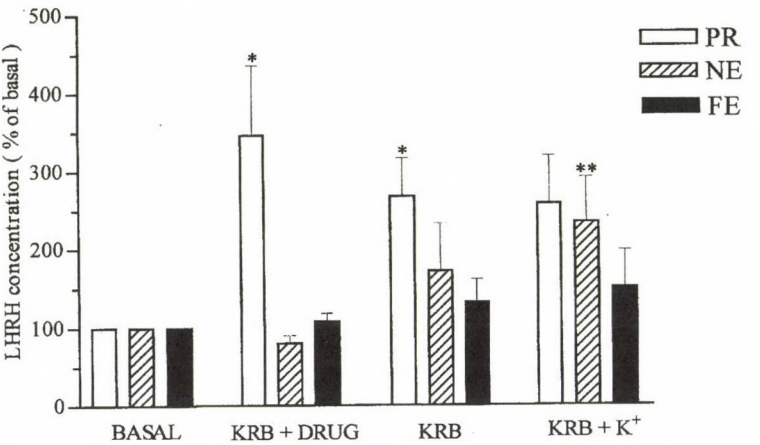

Fig. 2. Basal and in response to 10^{-6} M propranolol (PR), norepinephrine (NE), phentolamine (FE) and potassium (56 mM) release of LHRH (±SEM) *in vitro* from the stalk-median eminence explants of neonatal females (Experiment No. 1). Asteriks indicate significant differences when compared with the appropriate basal values *$p<0.05$; **$p<0.01$

Experiment No. 2

In all groups (control group, 30 h and 68 h) of ovariectomized gilts (average wet weight of the stalk-median eminence = 22.0±1.9 mg) the basal release of LHRH from the stalk-median eminence was similar (202.0±41.6, 147.5±36.1 and 236.4±77.7 pg/ml, respectively). Norepinephrine increased LHRH release from the stalk-median eminence collected at 0 h (control), 30 h (30EB) and 68 h (68EB) after oestradiol benzoate treatment by 48, 78 and 73 percent, respectively (Fig. 3). However, the increase observed in the group primed with oestradiol benzoate 30 h before the stalk-median eminence collection was not significant. The most evident stimulation of LHRH release was noted in the group of gilts primed with oestradiol benzoate 68 h before the stalk-median eminence collection ($p<0.01$).

Propranolol administration into the incubation media also invokes an increase in LHRH release from the stalk-median eminence (Fig. 4), but the most clear elevation of

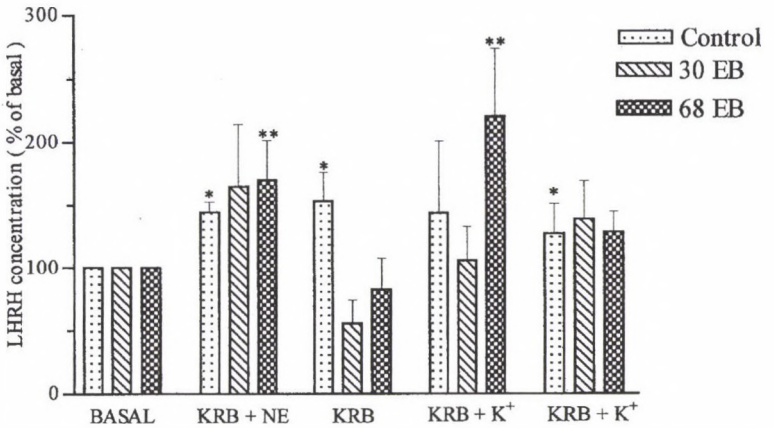

Fig. 3. Basal and in response to 10^{-6} M norepinephrine (NE) and potassium (56 mM) release of LHRH (±SEM) *in vitro* from the stalk-median eminence explants of ovariectomized gilts treated with vehicle (control) or primed with oestradiol benzoate 30 h (30EB) and 68 h (68EB) before tissue collection. Asteriks indicate significant differences when compared with the appropriate basal values *p<0.05; **p<0.01 (Experiment No. 2)

LHRH in the incubation media was found in the control group, i.e. not primed with oestradiol benzoate. The increases observed in groups 30EB and 68EB were not significant (Fig. 4).

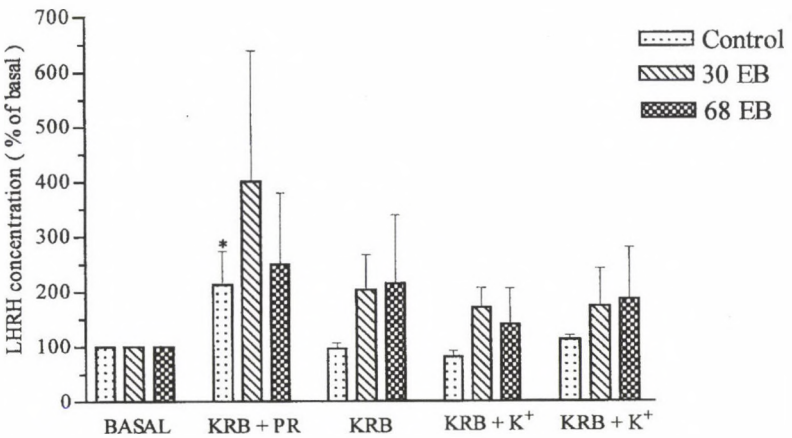

Fig. 4. Basal and in response to 10^{-6} M propranolol (PR) and potassium (56 mM) release of LHRH (±SEM) *in vitro* from the stalk-median eminence explants of ovariectomized gilts treated with vehicle (control) or primed with oestradiol benzoate 30 h (30EB) and 68 h (68EB) before tissue collection. The asterisk indicate significant differences when compared with the appropriate basal values *p<0.05 (Experiment No. 2)

Phentolamine did not affect LHRH release from the stalk-median eminence in the control group, the unprimed oestradiol benzoate group of ovariectomized gilts (Fig. 5) and those primed with oestradiol benzoate 30 h before tissue collection (Fig. 5). A significant, 50% decrease of LHRH release ($p<0.05$) to the stalk-median eminence incubation tissue medium collected 68 h after oestradiol benzoate injection was observed during the post phentolamine treatment period (Fig. 5).

Experiment No. 3

Alpha-1-adrenoreceptor antagonist urapidil did not affect significantly the basal LHRH release from the stalk-median eminence of control gilts and gilts given oestradiol benzoate 30 h later (Fig. 6). However, in group 30 EB a 56% elevation of LHRH concentration in incubation media was observed ($p>0.05$; Fig. 6). At 68 h after oestradiol benzoate injection (68 EB), urapidil caused a rapid increase of LHRH release (108.6±21.2 vs 41.4±4.5 pg/ml, $p<0.01$; Fig. 6).

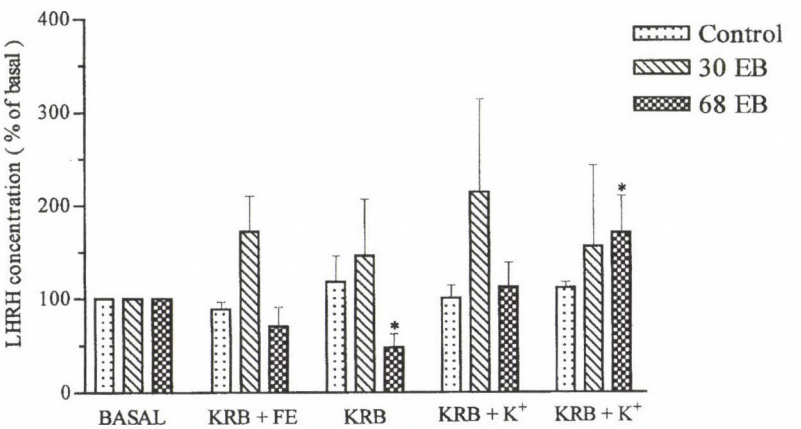

Fig. 5. Basal and in response to 10^{-6} M phentolamine (FE) and potassium (56 mM) release of LHRH (±SEM) *in vitro* from the stalk-median eminence explants of ovariectomized gilts treated with vehicle (control) or primed with oestradiol benzoate 30 h (30EB) and 68 h (68EB) before tissue collection. Asteriks indicate significant differences when compared with the appropriate basal values *$p<0.05$ (Experiment No. 2)

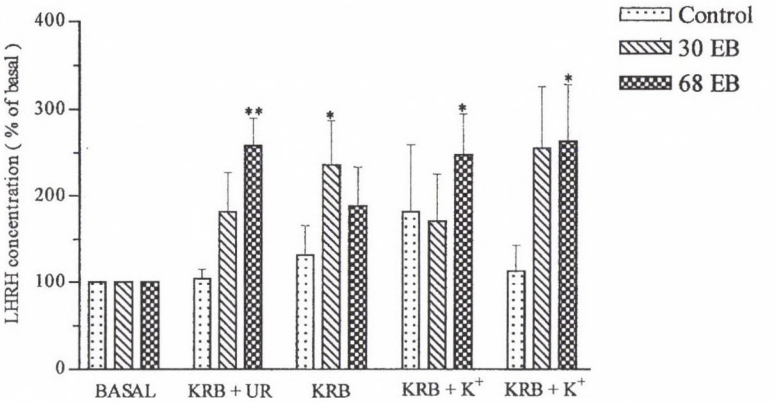

Fig. 6. Basal and in response to 10^{-6} M urapidil (UR) and potassium (56 mM) release of LHRH (±SEM) *in vitro* from the stalk-median eminence explants of ovariectomized gilts treated with vehicle (control) or primed with oestradiol benzoate 30 h (30EB) and 68 h (68EB) before tissue collection. Asteriks indicate significant differences when compared with the appropriate basal values *p<0.05; **p<0.01 (Experiment No. 3)

Phenylephrine-α_1 receptor agonist (Fig. 7) did not significantly affect LHRH release from the stalk-median eminence collected at various time periods after oestradiol benzoate administration *in vivo*.

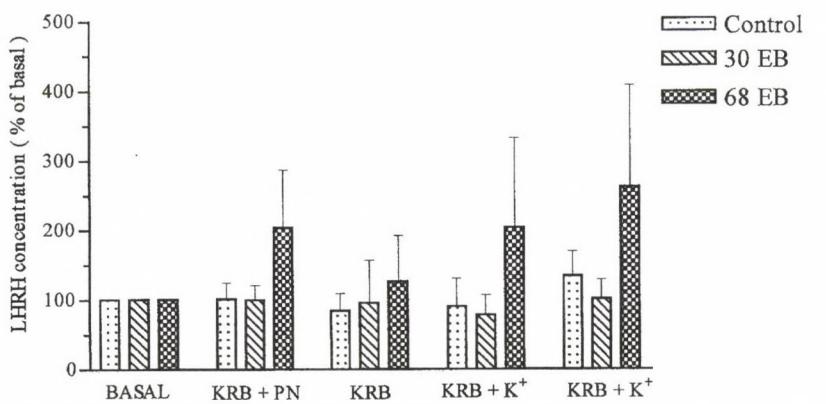

Fig. 7. Basal and in response to 10^{-6} M phenylephrine (PN) and potassium (56 mM) release of LHRH (± SEM) *in vitro* from the stalk-median eminence explants of ovariectomized gilts treated with vehicle (control) or primed with oestradiol benzoate 30 h (30EB) and 68 h (68EB) before tissue collection (Experiment No. 3)

Discussion

In our studies, two different animals models: neonatal piglets and prepubertal gilts were utilized to study the LHRH secretion from the stalk-median eminence in pigs. In the first model, one-week-old male and female piglets were used, because there is a considerable difference between the sexes in steroid production at this period of postnatal development. The concentrations of oestradiol and testosterone in males is high whereas the steroids are not produced in females, contributing to the quiescent state of ovarian development during late gestation and early neonatal life [9, 11, 20]. The basal LHRH release from the stalk-median eminence of neonatal pigs found in our study was similar to that reported earlier [20]. The previous study [20] indicated that the stalk-median eminence, a structure in which LHRH is predominantly contained in nerve terminals, can release much greater amounts of LHRH than similarly incubated medial basal hypothalamus fragments of porcine brain. Setalo et al. [17] showed that LHRH containing elements in the median eminence are particularly sensitive to the stimulatory actions of two catecholamines, dopamine and norepinephrine.

In our study on neonatal piglets norepinephrine, the applied doses, did not significantly affect LHRH release in females and males, although the release of this neuropeptide in males was considerably higher in females. Phentolamine (α_1- and α_2-antagonist) also did not influence LHRH release from the stalk-median eminence collected from the piglets of both sexes. On the other hand, propranolol – a standard β-receptor blocker – clearly increased LHRH release from the stalk-median eminence tissues of boars as well as of gilts. The stimulatory effect of β-receptors blockade in the stalk-median eminence on LHRH release was also maintained in ovariectomized, prepubertal gilts. The activation of α- and β-receptors by norepinephrine affected LHRH release both with and without the presence of oestradiol benzoate in the gilts blood circulation before tissue collection i.e. during the positive feedback action of estrogens on LH secretion in pigs. Similarly, as in neonates, phentolamine was not effective in prepubertal gilts. The results of our experiments on postnatal and prepubertal pigs indicated that stimulation of α-rather than β-adrenoreceptor is responsible for LHRH release from the stalk-median eminence. In the last experiment on prepubertal gilts urapidil (α_1-antagonist) and phenylephrine (α_1-agonist) were used to elucidate the role of α_1-adrenergic receptors in LHRH release in pigs. The stimulatory effect of α_1-antagonist was noticed in the stalk-median eminence collected from gilts given oestradiol benzoate 30 h before tissue collection (LHRH release during post-incubation period with urapidil) and 68 h (positive feedback period) after oestradiol benzoate administration. At the same time a_1-agonist phenylephrine at the dosage applied did not show any effect.

While the stimulatory action of propranolol and norepinephrine on LHRH release both in neonatal and prepubertal gilts was steroid independent, the positive effect of urapidil seems to depend on earlier estrogen stimulation of gilts *in vivo*. The question that immediately arises is: Why does the blocking of β-receptors by propranolol or α_1-receptors by urapidil without any additional stimulation causes a higher LHRH release from the stalk-median eminence? Several possibilities can be considered to explain this fact. One is that the release of LHRH by the stalk-median eminence terminals is tonically affected by endogenous compound(s). When the stalk-median eminence is separated from the rest of hypothalamus the stimulation of the above factors is removed resulting in a lower (basal) release of LHRH. The addition of β- and α_1-blockers *in vivo* again restores the high LHRH release.

LHRH release from the stalk-median eminence terminals by norepinephrine, especially in the prepubertal gilts, corresponds with the results reported by Negro-Vilar et al. [10] in rats. However, microinjections of norepinephrine to the area of the arcuate nucleus in adult male miniature pigs [12] resulted in a lowering of serum LH. The presence of catecholamine-sensitive facilitatory or inhibitory neurones within the hypothalamus may explain the differences between *in vitro* and *in vivo* results. The physiological significance of the recurrent inhibitory or facilitatory neural circuits is still unknown but they may play a very important role in the modulation of LHRH release into the portal vessels [10]. On the other hand, in ovariectomized rats norepinephrine has a biphasic effect on the stalk-median eminence, inhibiting LHRH release at low doses and stimulating at larger doses.

Several reports have previously shown that the stimulatory action of LHRH release involved the action of an α-adrenergic receptor [2, 10, 13]. It has been known for many years that α-adrenergic receptor blocking in monkeys [4] results in an inhibition of pulsatile LH secretion. It was also suggested that the increase in norepinephrine release during puberty contributes to the developmental increase in LHRH release [8]. In rats one of the mechanisms underlying the increase in the noradrenergic excitatory drive at the time of the preovulatory surge of LH is an increase in the neuronal response to α_1-adrenergic stimulation [6]. A castration of adult or prepubertal female releases a negative component of estrogen feedback, resulting in a chronic elevation of serum LH and FSH. A single injection of estrogen causes temporary inhibition and then elicits a surge release of gonadotropins. In pigs, estrogen acts on the hypothalamus to inhibit the release of LHRH for 54 h [5]. Our study has shown that the porcine stalk-median eminence terminals of LHRH neurones responded similarly *in vitro* to norepinephrine stimulus before estrogen administration as well as during the positive and negative feedback phases. On the other hand, we found an estrogen-dependent action of α_1-antagonist (urapidil). Urapidil did not affect LHRH release from the stalk-median eminence of ovariectomized gilts not primed with

oestradiol benzoate but considerably increased LHRH output from the stalk-median eminence collected 68 h after estrogen administration i.e. during the positive feedback phase of LH secretion in pigs. Collectively, the results presented in this paper suggest that the adrenergic system has influence on LHRH release from the stalk-median eminence of neonatal and prepubertal pigs mainly through a-receptors and this effect can be modulated by estrogens in the prepubertal gilts. The role of α_1- and α_2-receptors remains however to be elucidated.

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Basal and stress induced concentrations of adrenal gland catecholamines and plasma ACTH during aging

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Basal and stress levels of catecholamines (CA) in the adrenal glands, and circulatory levels of adrenocorticotropic hormone (ACTH) were examined in female Wistar rats aged 1, 3, 10 and 24 months. Our data showed reduction in basal dopamine (DA) concentration in adrenal glands and an increase in this catecholamine in response to stress at all ages (1, 3, 10, 24 months).

The greatest levels of basal norepinephrine (NE) and epinephrine (E) concentrations in the adrenal glands were noted in intact rats at the age of 24 months. On the other hand, the stress response of NE and DA had a tendency to fall, reaching basal values at the age of 10 and 24 months of age. Basal circulatory levels of ACTH showed a reduction with age. The stress response of ACTH was reduced in animals aged 10 and 24 months.

Reduced basal values of adrenal DA and increased NE and E values, suggest that there is increased adrenomedullar activity at the age of 24 months. On the other hand, the reduced or even absent stress response of NE and E observed in the adrenals, in 10 and 24 months old rats, may be of interest in considering the ability of these animals for adaptation. Basal and stress values of plasma ACTH are significantly reduced with the onset of senescence in female rats.

Keywords: aging, adrenal glands, catecholamines, ACTH

An increased amount of data in the literature indicates that normal processes of aging are a result of changes of CA levels in the hypothalamus both in animals and humans [17, 31], reflected through changes in the synthesis and release of pituitary hormones which bring about significant alterations in the functioning of peripheral endocrine organs under its control. The reduction of CA in the hypothalamus results in

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increased plasma prolactin concentration [8, 10, 13, 29, 30] as well as reduced reproductive functions manifested through irregular estrous cycles [9–11]. On the other hand, it has been shown that adrenomedullar activity, measured from the activity of tyrosine hydroxylase and dopamine beta-hydroxylase, increases with age [4, 18]. However, in studies performed on rats, there have been conflicting reports on the basal circulatory level of NE. No changes in NE values during aging are detectable according to Avakian and Horvath and McCarty [1, 25]. However, it has been also reported that the basal level is increased in old rats [7].

In this study, our intention was to analyze the levels of DA, NE and E in adrenals as well as the basal and stress levels of circulatory ACTH during aging by monitoring the basal and stress induced adrenomedullar activity.

Materials and Methods

Experiments were performed on female Wistar rats. The animals were kept under standard laboratory conditions (lights on 07:00–19:00 h) with food and water accessible *ad libitum*. The animals were divided into the following age groups: 1. infantile 1 month old, 2. mature young aged 3 months, 3. middle aged of 10 months and 4.) old rats aged 24 months. All animals were sacrificed between 9:00 and 10:00 h in the diestrous phase of the estrus cycle. Animals subjected to acute stress were injected with 1 ml of saline per 200 g body mass im. in the hind leg and sacrificed 5 minutes later. All animals were sacrificed by decapitation with a small animal guillotine and blood was collected in plastic tubes with EDTA (1 mg per ml of blood). Plasma was separated after centrifugation at 3000 g. Trasylol was added (250 IU/ml) and the plasma was stored at –20 °C until assayed for ACTH.

The adrenal glands were isolated, cleaned of surrounding tissue at 4 °C and stored at –70 °C until analyzed for catecholamines.

ACTH radioimmunoassay. Samples were assayed in duplicate using 100 µl of unextracted plasma. The ACTH RIA kit was purchased from the Laboratory for Isotopes of the "Vinča" Institute for Nuclear Sciences, Beograd. This RIA system uses porcine $ACTH_{1-39}$ labeled with I^{125}. The first antibody was raised in rabbits by injection of porcine $ACTH_{1-39}$. The period of incubation is 21 hours at 4 °C. Separation of the bound and free fractions is performed with a second antibody, ovine anti-rabbit gammaglobulins and polyethylene glycol. The minimum detectable level of hormone is 18 pg/ml. The nonspecific binding was 2.3% and the specific binding 24.1%. The intraassay and interassay coefficients of variation as determined by the analysis of replicate pool samples were 4.9% (n=8) and 8.9% (n=5), respectively.

Catecholamine analysis. For quantitative determination of the content of Ca in adrenal tissues, the adrenal glands were isolated, placed on ice at 4 °C for removal of excess tissue, weighed on an analytical balance, immediately cooled and stored at –70 °C until homogenized in acidified (pH 7.4) n-butanol. The catecholamine content of individual adrenals was determined by the fluorometric method on an Aminco-Bowman spectrophotometer [19] and expressed as per gram wet tissue.

Statistics. One way ANOVA and Student's *t*-test for between group comparisons were used. The level of significance was set at $p<0.05$.

Results

Body mass. Body mass of our laboratory rats increased up to the age of 10-months. The greatest body mass increase, about fourfold, was between 1-month and 3-months of age ($p<0.001$). The increase of body weight in 10-month old compared to 3-month old rats and also between 24-month old rats compared to 3-month old was also very significant ($p<0.001$). No significant difference between 10-month and 24-month old rats was observed (Table I).

Table I

Changes with aging of the body mass, pituitary and adrenal gland masses in female rats[1]

Age (months)	Body mass (g)	Pituitary gland (mg)	Adrenal glands (mg)
1	48.11±1.70 (26)[2]	2.67±0.09 (26)	15.58±0.66 (26)
3	191.27±8.44 (22)	9.12±0.39 (22)	46.61±1.92 (21)
10	290.15±7.94 (20)	16.57±0.67 (20)	64.02±2.54 (20)
24	305.40±7.24 (40)	18.14±0.81 (33)	71.66±1.72 (40)

[1] Values are the mean ±SEM

[2] ( ) N° of rats

Pituitary mass. Pituitary mass increased with age up to the 10th month. The increase was clearly evident and significant between 1-month and 3-month old rats and between 3-month and 10-month old rats ($p<0.001$). There was no significant difference between 10-month and 24-month old rats (Table 1).

Adrenal mass. Significant increases in adrenal mass in 3-month old compared to the 1-month old rats and also of 10-month old as compared to 3-month old rats were observed ($p<0.001$). The mass of the adrenals from 24-month old rats was also significantly greater than in 10-month old rats ($p<0.01$) (Table I).

Basal and stress levels of catecholamines in adrenal glands of rats during aging

Concentration of DA in adrenal glands. Basal levels of adrenal DA showed changes during aging (ANOVA, $F_{3,54}=10.461$, $p<0.0001$). No significant changes were observed between the basal DA level of 1 (23.66±1.96 nmol/g) and 3 month old rats (18.75±2.0 nmol/g). However, the basal level of adrenal DA in 1-month old compared to 10-month old rats (12.53±0.89 nmol/g) and in relation to 24-month old rats (14.55±0.71 nmol/g) was significantly lower ($p<0.001$). Basal levels of DA in 3-month old rats were significantly higher than in rats aged 10 and 24 months ($p<0.05$) (Fig. 1).

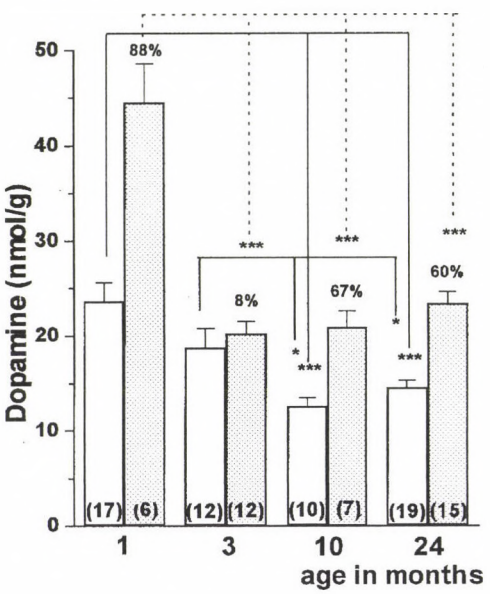

Fig. 1. Basal (——) and stress (----) levels of adrenal dopamine in female rats during aging
Bars represent mean ± SEM
Numbers above bars represent % of increase after stress
( ) N° of rats
*$p<0.05$
***$p<0.001$

There were also variations in stress levels of DA during aging (ANOVA, $F_{3,36}$=28.000, p<0.0001). The stress increase of DA in adrenal glands was significant compared to the basal level in all age groups studied. Comparing the levels of DA it may be seen that the greatest increase of DA levels was in 1-month old rats (44.54±4.04 nmol/g) (88%) compared to 3-month (20.21±1.29 nmol/g) (8%), 10-month (20.89±1.72 nmol/g) (66%) and 24-month old rats (23.34±1.27 nmol/g) (60%) (Fig. 1).

Concentration of NE in adrenal glands. Basal levels of NE varied significantly during aging (ANOVA, $F_{3,54}$=32.882, p<0.0001). Levels of NE in 3-month old rats (237.59±16.30 nmol/g) were significantly higher in relation to 1-month old rats (143.58±12.67 nmol/g) (p<0.001). There was no significant difference between 1-month and 10-month old rats (125.91±8.55 nmol/g). In 10-month old rats, the level of NE was significantly reduced in the adrenals compared to the 3-month old rats (p<0.001). The highest level of adrenal NE was observed in 24-month old rats

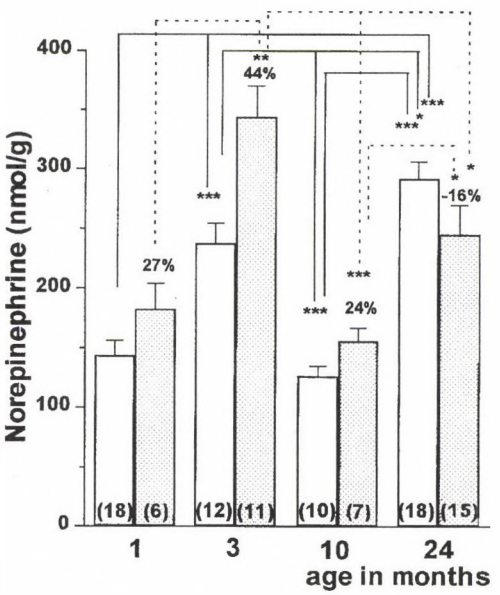

Fig. 2. Basal (——) and stress (----) levels of adrenal norepinephrine in female rats during aging
Bars represent mean ± SEM
Numbers above bars represent % of increase after stress
( ) N° of rats
*p<0.05
**p<0.01
***p<0.001

(292.11±14.27 nmol/g) and this difference was significant when compared to all three younger animal groups (Fig. 2).

NE stress values also varied significantly during aging ($F_{3,35}$=9.962, p<0.001). The stress increase of adrenal NE in relation to the basal level was significantly higher in 1-month (182.42±21.63 nmol/g) (27%) and in 3-month old rats (343.43±26.56 nmol/g) (44%). The stress response of NE in rats at 10 month old was 23% higher compared to the basal level, while in 24-month old rats there was no stress response (–16%) (Fig. 2).

Concentrations of E in adrenal glands. Patterns of basal E levels showed significant variation with age (ANOVA, $F_{3,54}$=62.654, p<0.0001). The basal concentration of E in 1-month old rats (1896.95±113.29 nmol/g) was lower (p<0.001) than the concentration of basal E of 3-month old rats (2775.16±192.61 nmol/g). There was no significant difference between E of 1-month (1896.95±113.29 nmol/g) and 10-month old rats (1880.04±136.90 nmol/g). The basal level of E in the adrenals was the greatest in 24-month old rats (4541.21±196.49 nmol/g) when compared to all younger animal groups (Fig. 3).

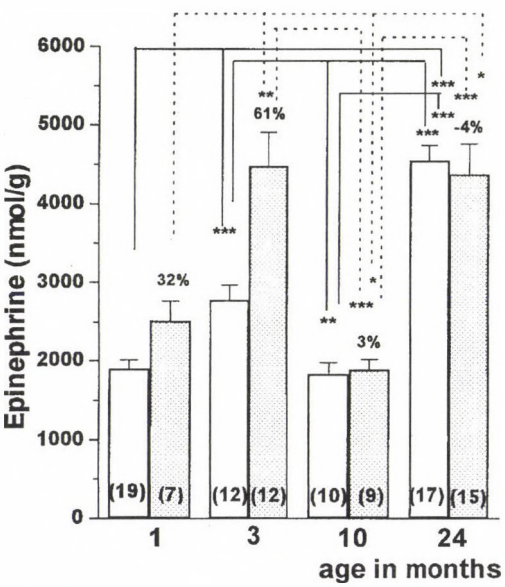

Fig. 3. Basal (——) and stress (----) levels of adrenal epinephrine in female rats during aging
Bars represent mean ± SEM
Numbers above bars represent % of increase after stress
( ) N° of rats
*p<0.05
**p<0.01
***p<0.001

The stress levels of E also showed significant variations during aging (ANOVA, $F_{3,39}$=11.761, p<0.0001). An increase of adrenal E in stressed animals compared to basal levels is observed in 1-month (2511.94±253.91 nmol/g) (32%) and in 3-month old rats (4474.16±427.54 nmol/g) (61%). There was no evident difference between basal and stress levels in 10-month old (1880.04±136.90 nmol/g) (3%) and in 24-month old rats (4374.62±380.88 nmol/g) (–4%) (Fig. 3).

Concentration of plasma ACTH during aging

Basal levels of plasma ACTH. Basal plasma ACTH concentrations showed significant changes as a function of age (ANOVA, $F_{3,45}$=5·893, p<0.01). Significant differences were not observed between basal plasma ACTH levels in 1-month (53.57±8.49 pg/ml) and 3-month old rats (64.54±7.69 pg/ml) not between the 1-month and 10-month old rats (36.65±4.42 pg/ml). However, a significant decrease of plasma ACTH was observed between 1-month and 24-month old rats (35.72±3.85 pg/ml) (p<0.05) A significantly higher basal level of ACTH was evident in 3-month old

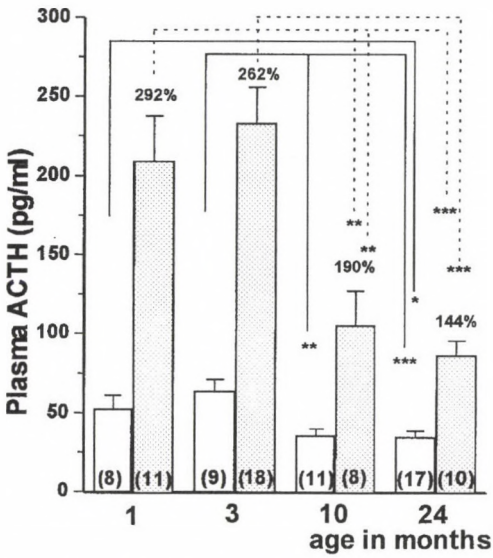

Fig. 4. Basal (——) and stress (----) levels of plasma ACTH in female rats during aging.
Bars represent mean ± SEM
Numbers above bars represent % of increase after stress
( ) N° of rats
*p<0.05
**p<0.01
***p<0.001

(64.54±7.69 pg/ml) when compared to 10-month ($p<0.01$) and 24-month old rats (35.72±3.85 pg/ml) ($p<0.001$). On the contrary, there were no significant differences of the basal ACTH levels, between the 10-month and 24-month old rats (Fig. 4).

Stress values of ACTH plasma levels as a function of age. Significant differences were observed in plasma ACTH stress values as a function of age (ANOVA; $F_{3,47}=9.761$, $p<0.001$). The stress levels of plasma ACTH of 1-month (210.05±28.36 pg/ml) and 3-month old rats (233.95±22.72 pg/ml) compared to basal levels showed a 292% and a 262% increase respectively. However, the stress levels in 10-month (106.23±21.66 pg/ml) and 24-month old rats (87.25±9.21 pg/ml) were increased by 190% and 144% respectively, compared to their basal levels, but this stress increase of ACTH in these animals was much lower than in the 1- and 3-month old groups (Fig. 4).

Discussion

Although there is considerable literature on aging, many details of the mechanisms involved in aging, especially those involving catecholamines, are still not clear. Trying to determine adrenal activity during aging, we examined the basal and stress levels of DA, NE and E in the adrenal glands, as well as the basal and stress circulatory levels of ACTH in female rats at four ages: 1, 3, 10 and 24 months.

An increased medullar content of NE and E in the adrenals of Wistar rats as a function of age [18] has been recorded. On the other hand, there was no change in the concentration of adrenal CA [6] in the mouse. Different species of animals, organ systems and types of tissues, seem to have their own aging characteristics [6], which must be taken into account when aging is generalized.

Analyzing the relationship of basal and stress NE and E levels in the adrenals of female rats aged 1, 3, 10 and 24 months, we recorded similar patterns of basal and stress values of NE (Fig. 2) and E (Fig. 3) in the adrenals of 3-month old rats and their significant increase (for NE $p<0.001$, for E $p<0.01$) in relation to 1 month old rats. The smaller amount of NE and E suggests reduced activity and amount of enzymes synthesizing these CA in the adrenals of 1-month old rats, compared to 3-month old animals, as confirmed by the increased basal and stress levels of DA in the adrenals of 1-month old rats (Fig. 1).

The results of Reis et al. [27] describing the importance, elevation, activity and amount of enzymes synthesizing CA in the adrenal glands which they studied in old (24–26 m) rats and mice in relation to young (4 m) point to the etiology of increased basal values of NE and E in the adrenals of our 24-month old rats in relation to the basal levels of NE and E of 3 month-old rats. It is of interest to note that almost

identical patterns of basal NE and E (Fig. 2, Fig. 3) are recorded in our 10-month old group, which were significantly lower in relation to other age groups. Moreover the basal level of DA is also much lower in 10-month old rats in relation to the 1-month old and 3-month old rats. It remains unclear why all three catecholamines showed the same trend in the age group of 10 months. One explanation may be that this is a transitory period from an intensive reproductive phase to the second half of the reproductive phase, at least when our Wistar rat strain is concerned. The capability for intact animals of this age to become pregnant is reduced, while in pregnant animals there is an increased number of resorptions in the uterus and reduced litter number [23, 24]. Our results concerning CA during aging are in agreement with the results of Avakian et al. [2] who have shown that the arterial plasma E concentration in old rats is significantly increased. The same author suggested that the basal and cold induced stress levels of CA indicate an increased adrenal medullary activity with the advent of old age. It should be noted that in our study, CA which were measured in whole adrenal glands, derived from catecholamine-producing chromaffin cells in the medulla [3]. Having in mind the aging characteristics of different tissues [6], our results suggest that the increase of E concentrations is an aging characteristic of the medulla tissue in our strain of rats.

In one of a series of papers showing amino acid levels in specific brain structures during aging, Branay-Schwartz et al. [5] showed that the levels of phenylalanine and tyrosine are significantly reduced in all structures of the lower brain stem and nuclei of the middle and posterior hypothalamus in old rats in relation to the young, which may explain the reduced CA concentrations in the hypothalamus correlated with age, both in animals and humans [31].

Plasma ACTH is multifactorially regulated, since ACTH, beside CRF, may be regulated by the activity of CA from the CNS, opiates and by some pituitary hormones. A significant reduction of the basal plasma ACTH level in 10-month old ($p<0.01$) and 24-month old ($p<0.001$) compared to 3-month old rats indicates changes in pituitary function as a reflection of reduced CA concentration during aging, as confirmed by many authors [9, 12, 14, 22, 28, 31, 34, 35]. On the other hand, this reduction of ACTH with age is in agreement with the known fact that prolactin increases with age [9, 11, 12, 14, 22, 28, 32, 35], suggesting that there is an inverse relationship between ACTH and prolactin. We confirmed such a relationship between ACTH and prolactin in our earlier studies in animal models bearing hyperluteinized ovaries in relation to corresponding intact controls [15, 16]. This reduction of basal ACTH levels as well as the increased adrenal activity recorded in our 24-month old rats compared to 3-month old rats ($p<0.01$) is in agreement with the results of several authors [2, 5, 14] who showed that the reduction of ACTH secretion is related to increased levels of plasma prolactin, as well as because of increased secretory activity of the adrenal glands in

hyperprolactinemic rats. The stress induced increase of plasma ACTH level when compared to basal levels in all age groups is significant (Fig. 4).

Our results show a reduced basal value of DA and increased NE and E, as well as a reduced stress response during aging, suggesting an increased adreno-medullar activity with the onset of senescence, possibly due to an increased activity of enzymes involved in the synthesis of NE and E in rat adrenal glands.

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Effects of Lormetazepam on glycemia and serum lipids in hyperlipidemic rats

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Lormetazepam (N-methyllorazepam) administered intraperitoneally to rats rendered hyperlipidemic by Triton WR-1339 induced an elevation of blood glucose level at all investigated doses. It brought about significant reduction of serum total lipids, total cholesterol and triglycerides. The dose-response relationship was bell-shaped. However, it presented two peaks, differing from the responses to other benzodiazepines (BZD) which were characterized by only one peak.

Keywords: benzodiazepine, hyperlipidemic, lormetazepam

In previous papers [for reviews see 2, 8] it was shown that lorazepam, similarly to other BZD, had the property to lower the serum lipids when they were increased in experimental conditions. Later, it was demonstrated that delorazepam, a compound closely related to lorazepam, possessed similar effects [3].

In this paper the results obtained in an investigation devoted to the effects of another lorazepam derivative, N-methyllorazepam (lormetazepam) on blood glucose levels and serum lipids in hyperdyslipidemic rats are presented. The investigations were prompted by the previous results obtained with other BZD which indicated that both in animals and humans BZD can attenuate the deposition of serum lipids on arterial walls providing protection in experimental and human atherosclerosis [2].

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Materials and Methods

The experiment was carried out on male Wistar rats, weighing 150–200 g and fed normal rat chow. The hyperdyslipidemia was induced by an intraperitoneal (ip.) injection of 10% aqueous solution of Triton WR-1339 (200 mg/kg). After Triton WR-1339 administration, the food was withdrawn, but the water was allowed ad libitum. Eighteen hours later, the blood was sampled from the retroorbital sinus. From the whole blood, blood glucose level was measured by the enzymatic procedure (Merck's test); from the serum, the total lipids (TL), the total cholesterol (Tchol) and triglycerides were determined according to Zöllner and Kirsch and the enzymatic techniques respectively (Merck's test). All these determination were described in [11]. The results were expressed as mmol/l with the exception of TL which were expressed as mg%.

The rats were randomized in the following groups:

1. Control hyperdyslipidemic (Triton WR-1339 treated) rats. Two hours prior to Triton WR-1339 administration they received ip. the mixture used for drugs suspension (methylcellulose 0.3% + glycerin 1%) in the same volume as in the experimental groups.
2. Reference group. An ip. injection of fenofibrate (200 mg/kg) was administered. The remainder of the procedure was indentical to that already described one [4].
3. N-methyllorazepam administered groups. The following doses were used: 2.5 – 5.0 – 7.5 – 10 and 15.0 mg/kg.

The statistical evaluation was performed by the non-paired "t"-test [13]. The results obtained in N-methyllorazepam and fenofibrate treated animals were compared with those seen in control hyperdyslipidemic rats (p) and in the reference group (p1).

Results

The reference compound induced a highly significant increase of glycemia and highly significant reduction of the serum lipids. The most sensitive parameter was the level of TG. N-methyllorazepam induced a hyperglycemic response at all doses. TL were diminished by all doses with the exception of the highest one (15 mg/kg). The same was valid for TChol and TG. Again as in the case of fenofibrate, TG were affected the most. The doses of 2.5 mg/kg and especially 10.0 mg/kg were the most active (Table I). Lormetazepam was generally a weaker lipid lowering agent than fenofibrate, with the notable exception of the doses of 2.5 and 10.0 mg/kg which exerted similar effect in term of most parameters.

Table I

Effects of N-methyllorazepam on blood glucose level and serum lipids in Triton WR-1339 administered rats

Group/Dose	N	Blood glucose level, mmol/l	Total lipids (TL) mg%	Total cholesterol (Tchol) mmol/l	Triglycerides (TG), mmol/l
Control hyperlipidemic (Triton WR-1339, 200 mg/kg)	20	3.60±0.12	3446±349.54	7.44±0.42	17.28±1.318
Reference (fenofibrate 200 mg/kg)	10	4.96±0.30 p<0.001↑	601.0±27.0 p<0.001↓	3.14±0.147 p<0.001↓	1.039±0.11 p<0.001↓
N-methyllorazepam (2.5 mg/kg)	20	4.83±0.16 p<0.001↑ p1 N.s.	708.8±51.25 p<0.001 p1<0.001↑	3.175±0.33 p<0.001↓ p1 n.s.	3.12±0.39 p<0.001↓ p1 n.s.
N-methyllorazepam (5.0 mg/kg)	10	5.58±0.24 p<0.001↑ p1 n.s.	1273.6±119 p<0.001 p1<0.001↑	4.19±0.265 p<0.001↓ p1<0.001↑	7.48±0.98 p<0.001↑ p1<0.001↓
N-methyllorazepam (7.5 mg/kg)	10	4.56±0.25 p<0.001↑ p1 n.s.	1071.42±176 p<0.001 p1<0.001↑	4.81±0.54 p<0.001↓ p1<0.001↑	5.58±0.87 p<0.001↓ p1<0.001↑
N-methyllorazepam (10 mg/kg)	10	5.83±0.28 p<0.001↑ p1<0.001↑	667.6±99.0 p<0.001↓ p1 n.s.	2.47±0.4 p<0.001↓ p1 n.s.	2.42±0.16 p<0.001↓ p1 n.s.
N-methyllorazepam (15 mg/kg)	10	5.22±0.31 p<0.001↑ p1 n.s.	4301.0±358 p n.s. p1<0.001↑	8.58±0.48 p n.s. p1<0.001↑	18.47±1.43 p n.s. p1<0.001↑

N: number of rats/group
p: compared with control hyperlipidemic group
p1: compared with the reference group
n.s.: non significant
↑: increase
↓: decrease

Discussion

Our results have shown that lormetazepam was similar in many aspects to other BZD with regard to its metabolic effects. So, it induced an increase of blood glucose level, as did many BZD administered in the same experimental model [4] and it reduced markedly serum lipids. The dose-response relationship was bell-shaped as for other BZD. However, varying from other BZD, it presented two peaks. It is noteworthy

that delorazem had a similar characteristic [3]. The cause of this difference is not known. One of the possible explanation of this phenomenon will be mentioned later.

As other BZD, lormetazepam was more active on TChol level than on TG level. It was akin in this respect to the fibrates [6].

A special attention is deserved by the relationships between lormetazepam, lorazepam and delorazepam. Both lorazepam and lormetazepam are metabolized to lorazepam [12] (Fig. 1). This compound is a very potent lipid-lowering [5, 9] agent.

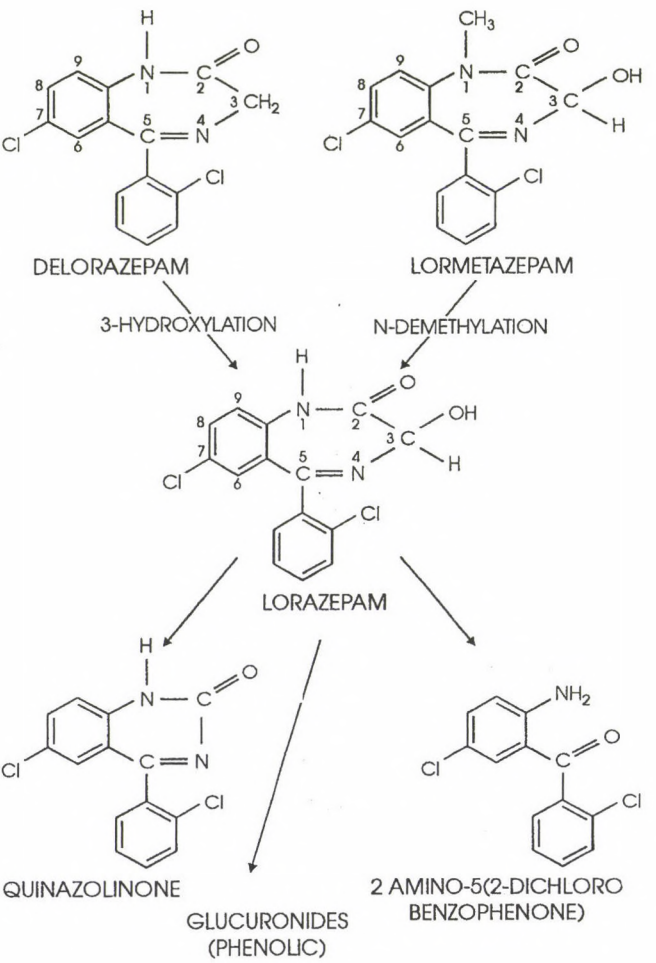

Fig. 1

It induced a reduction of the serum lipids even in normolipidemic rats, when administered ip. [10]. The same was true for delorazepam [3]. It is not excluded that lormetazepam and delorazepam would act as prodrugs. However, this hypothesis was not supported by the following circumstances:

1. Lorazepam inhibited fibrinolysis [1], while delorazepam enhanced it [3]. The effects of lormetazepam on fibrinolysis have been not investigated.
2. The half-life of delorazepam is 70–80 hours, while lorazepam has a half-life only of 12 hours [12]. Very probably during the 20-hour duration of our experiments, only a part of delorazepam was converted into lorazepam. We do not possess similar data with regard to lormetazepam.
3. The effective doses of delorazepam were much lower than the dose of lorazepam. On the other hand, the most effective doses of lormetazepam induced a larger reduction of TL and TChol than lorazepam.

The above considerations do not mean that we deny completely the possible participation of lorazepam in the metabolic effects of delorazepam and lormetazepam. We want to emphasize only that the conversion of these drugs in lorazepam cannot explain their effect entirely. Moreover, it is quite likely that the conversion of lormetazepam and delorazepam into lorazepam could afford an explanation for the appearance of the second peak (at 10.0 mg/kg) of the dose-response relationship. In this case, this higher dose would furnish a larger amount of lormetazepam and delorazepam to be metabolized. The summation of the nonmetabolized and the active metabolite would ensure a higher effect. No correlation can be established between serum lipid changes and the modifications of blood glucose level since other BZD induced a hypoglycemic or no response despite the fact all BZD induced a decrease of serum lipids.

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The effects of the intraperitoneal administration of midazolam on blood glucose level and serum lipids in streptozotocin-induced diabetes in rats

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In male Wistar rats rendered diabetic by streptozotocin administration, the intraperitoneal (i.p.) injection of midazolam (2.5–5.0 and 10 mg/kg) induced a significant reduction of hyperglycemia and hyperlipidemia. The plasma immunoreactive insulin level was slightly, but significantly increased. The lethality was diminished.

Keywords: rats, diabetes, streptozotocin, midazolam

Midazolam is a relatively recent development in the series of benzodiazepines (BZD.). From a pharmacological point of view it is akin to diazepam, the most used bzd. However, its shorter duration of action and its hydrosolubility (as hydrochloride formed in vivo) rendered it as a possible substitute of diazepam in medical practice. It is already preferred to diazepam in anesthesiology [12].

Previous investigations have shown that diazepam, as well as many other bzd, has the property to diminish the serum lipids when they are elevated [1, 4, 7]. In particular, diazepam reduced the serum lipids elevated in streptozotocin-induced diabetes mellitus in albino rats. Moreover, diazepam had a strong trend to blood glucose level normalization Cuparencu et al. [1].

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In the paper, we will present the result of an investigation which was carried out with the purpose to see whether midazolam has an antidiabetic effect similar to diazepam.

Materials and Methods

The experiments were performed on male Wistar albino rats, weighing about 120 g. The age was 45 days. The animals were fed a normal rat chow. They were kept at a constant temperature (20–22 °C) and humidity. The diabetes was induced by an intravenous (iv.) injection of streptozotocin (50 mg/kg), freshly dissolved in a 0.03 M citrate buffer (pH=4.5). In order to stabilize the glucose blood level which two hours after streptozotocin administration fell abruptly, an i.p. injection of 1 ml of 10% glucose solution/animal was administered. Immediately after the administration of streptozotocin, the rats were given an i.p. injection of midazolam (2.5–5–10 mg/kg, Dormicum^{R}). The administration of the drugs was repeated daily, for four days. In the fifth day after the streptozotocin injection, the fasted rats that were not fed that day, were killed in slight ether anesthesia, by decapitation. From the carotidal blood, the following determinations were carried out: blood glucose, by the enzymatic method (Merck's test); total serum lipids (TL), with the procedure of Zöllner and Kirsch; total serum cholesterol (TChol), with the enzymatic method (Reanal test); serum triglycerides (TG) with the enzymatic method (Merch's test). All the above-mentioned techniques were described by Manta et al. [8], the plasma immunoreactive insulin levels, by the method of Simionescu [9]. The reagent needed for these determinations were purchased from Sigma Co, Saint Louis, USA.

Lethality, expressed as percent, was also recorded.

Two control groups were used: 1. Normal rats, that were given i.p. the solution used for the dissolution of midazolam (0.8% sodium chloride, 0.01% disodium, EDTA 1%) and the glucose solution; 2. Diabetic rats, similarly treated.

The comparison of the results was made between diabetic rats (p1) and diabetic rats given midazolam (p).

The statistical evaluation was performed by means of unpaired Student's *t*-test Snedecor and Cochran [10].

Results

As seen from Table I, lethality was diminished from 45.46% (diabetic rats) to 36.45% by the dose of 10 mg and to 18.19% by the dose of 5 mg. The dose of 2.5 mg was ineffective.

The blood glucose level was very significantly raised in diabetic rats when compared to the normal rats.

Midazolam, excepting the dose of 10 mg/kg, which was inactive, reduced very significantly hyperglycemia. The most active was the dose of 5 mg.

Table I

The effects of various doses of midazolam on lethality, blood glucose level, serum lipids and plasma immunoreactive insulin levels in diabetic rats

Group/Dose	n	Lethality (%)	Blood glucose level (mMol/l)	Total lipids (mg%)	Total Chol (mMol/l)	Triglycerides (mMol/l)	Immunoreactive insuline (μIU/ml)
Normal control	10	–	2.97±1.18	3996.54±11.84	1.88±0.09	0.75±0.12	17.0±0.8
Diabetic control (streptozotocin 50 mg)	22 12	45.5	18.03±0.73 p<0.001↑	1389.3±123.0 5 p<0.001↑	5.49±0.56 p<0.001↑	8.97±0.12 p<0.001↑	4.34±0.32 p<0.001↓
Diabetic + midazolam (2.5 mg)	11 6	45.5	10.23±0.42 p<0.001↓ p1<0.001↑	384.6±26.6 p<0.01↓ p1 n.s.	2.27±0.12 p<0.001↓ p1<0.001↑	1.1±0.11 p<0.001↓ p1<0.01↑	4.4±0.5 p n.s. p1<0.01↓
Diabetic + midazolam (5.0 mg)	11 9	18.2	5.57±0.5 p<0.001↓ p1<0.01↑	285.36±23.2 p<0.001↓ p1<0.001↓	1.58±0.07 p<0.001↓ p1<0.001↓	0.85±0.04 p<0.001↓ p1 n.s.	6.17±0.41 p<0.001↑ p1<0.001↓
Diabetic + midazolam (10 mg)	11 7	36.45	16.14±0.82 p n.s. p1<0.001↑	844.12±26.4 p<0.001↓ p1<0.001↑	3.93±0.5 p<0.001↓ p1<0.001↑	6.27±0.5 p<0.001↓ p1<0.001↑	7.38±0.4 p<0.001↑ p1<0.001↓

p: compared with diabetic control

p1: compared with the normal control

n: number of rats/group. The numerator: the initial number of rats. The denominator: the final number of rats.

n.s.: nonsignificant

TL were also greatly augmented in diabetic rats, when compared to normal animals. All doses of midazolam induced very significant reductions. Moreover, the dose of 5 mg elicited even a diminution of this parameter, when compared to normal control. The same picture was observed with TChol. TG were also reduced, but in no instance did they reach levels bellow that seen in normal rats.

The plasma immunoreactive insulin level was very much reduced in diabetic rats. The lowest dose of midazolam failed to affect the diabetic plasma insulin level. Other doses of midazolam raised very significantly from a statistical point of view the plasma insulin level. However, these increments were very small from a biological point of view (see discussion).

Discussion

In our experiments, midazolam had an obvious antidiabetic activity. It diminished the lethality, the blood glucose level, the serum lipids of diabetic rats. It increased the plasma immunoreactive insulin level, although the last phenomenon has doubtful biological significance.

As in the case of other experimental models (Triton-induced hyperlipidemia, margarine-induced increases of serum lipids, partly normolipidemic rats [1, 4, 7] and as well as with other bzd, the dose-response relationship had a bell-shape. That means that the lowest and the highest doses had a lower activity than the medium dose.

Another characteristic of changes induced by midazolam and other bzd was the fact that the most affected parameter was the TG [1, 4, 7].

The cause(s) of the anthyperglicemic activity of midazolam in diabetic rats is (are) not elucidated. It is true that midazolam induced an elevation of the plasma immunoreactivite insulin levels. Despite the fact that this increase was very significant from a statistical point of view in comparison with the value recorded in control diabetic animals (with the exception of the lowest dose –2.5 mg/kg).

However, the biological significance of this phenomenon is doubtful, since the increases were very small and very far from the control normal values. Moreover, a disagreement could be observed between the level of the blood glucose, which was the lowest at the dose of 5 mg, than that seen at the dose of 10 mg, which had a higher blood glucose level (not significantly different from the observed in control diabetic rats) and the level of the immunoreactive insulin which was higher at the dose of 10 mg.

It is noteworthy that in a previous paper Cuparencu et al. [2] noted that diazepam administered in similar (but not identical) doses and experimental conditions, failed to have any significant influence on the plasma immunoreactive insulin level. Corroborating the results obtained in both investigations, it may be concluded that the blood glucose level raising activity of bzd was not due to the release of insulin from the beta cells of pancreas, which were not destroyed by streptozotocin.

We have also shown that diazepam enhanced glucose uptake by the rats [3]. If this finding will be confirmed in the case of midazolam, the conclusion could be drawn

that bzd have an antihyperglycemic activity mainly by favoring the uptake of glucose by peripheral tissue, i.e. they would possess an insulin-like action. When comparing the dose-response curves of Diazepam and Midazolam, it results that the dose-response curves for serum lipids were quite similar. The dose-response relationship for blood glucose level of Midazolam was slightly different from that of Diazepam. Indeed, the highest dose of Midazolam was ineffective, whereas the highest dose of Diazepam had a moderate, but significant blood glucose level lowering activity. The causes of this discrepancy are unknown.

It seems that the decrease of the serum lipids elicited by midazolam (as well as by other bzd) and the effects of blood glucose level, are independent phenomena. Indeed, midazolam induced very significant decrease of glycemia in hyperlipidemic rats (hyperlipidemia induced by Triton WR-1339) parallel with the reductions of the serum lipids [5]. On the contrary, in normoglycemic and nomolipidemic rats, it evoked a hyperglycemic reaction, accompanied by decrease of the serum lipids Cuparencu et al. [6]. It seems that the nutritional status of the rats would have a profound influence on the type of the changes of blood glucose level.

In conclusion, our experiments have shown that diazepam may be useful in the treatment of the streptozotocin-induced diabetes mellitus in albino rats. Due to the close similitude between this experimental model and the human diabetes [11], it may be suggested that the investigation of midazolam on the human diabetes would be of interest.

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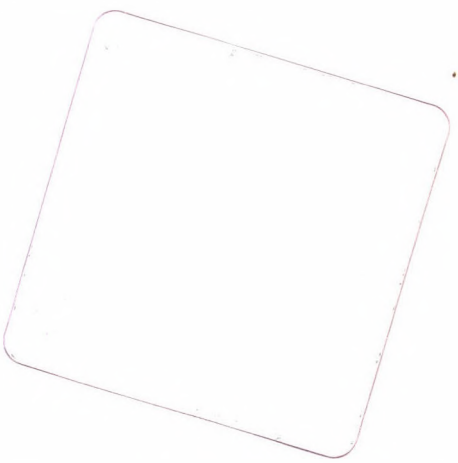

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The effect of substance P on the arachidonate cascade of rat platelets*

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The role of substance P (SP) in neurogenic inflammation is well known. Through neurokinin receptors, SP activates cells, including the arachidonate cascade of platelets. Our *in vitro* experiments were carried out to determine the effect of SP on the arachidonate cascade of rat platelets. The platelets were labelled with ^{14}C-arachidonic acid, and the ^{14}C-eicosanoids were then separated by means of overpressure thin-layer chromatography or high-performance liquid chromatography and were quantitatively determined. SP (10^{-9} and 10^{-8}) mol/L significantly increased the rate of the arachidonate cascade. The lipoxygenase pathway of platelets was stimulated by SP, which can result in the activation of protein kinase C mediated intracellular events. The cyclooxygenase system was inhibited by 10^{-12} mol/L, and stimulated by 10^{-9} mol/L SP. In our experiments SP in the physiological range of plasma concentration (10^{-12} mol/L) decreased the synthesis of vasoconstrictor arachidonate metabolites (TxA_2 and $PGF_{2\alpha}$). These data suggest that in physiologic conditions the arachidonate cascade of platelets may play role in the vasodilator effect of SP. The formation of thromboxane in rat platelets was stimulated by higher concentration of SP (10^{-9} mol/L), and therefore the SP-induced cytotoxicity against parasites might be mediated by the stimulation of thromboxane A_2 synthesis.

Keywords: substance P, platelet, prostaglandin, thromboxane

Many of the details of the mechanism of neurogenic inflammation have been well known for many years [2]. During this process, neuropeptides (substance P, calcitonin gene-related peptide and neurokinin A) are released from nerve endings, and initiate inflammatory reactions in the tissues. Substance P (SP) dominates the process of neurogenic inflammation [10]. SP is a member of the tachykinin family. The actions of tachykinins in mammals are mediated by at least three receptors, neurokinin-1

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(NK-1), NK-2 and NK-3 [19]. Via these receptors, SP can activate lymphocytes [19], monocytes [13], neutrophils [26] and fibroblasts [21], induce cytokine production (IL-1, IL-6, TNF-α, etc.) [14], modulate eosinophil chemotaxis [22], and mobilise histamine from mastocytes [15]. SP might influence both immune and inflammatory processes, modifying various cell functions, haemostasis and microcirculation locally.

Towards haemostasis platelets participate directly in immunological processes also, e.g. they exert cytotoxic activity against tumour cell lines [24] and parasites [1, 6, 29]. Symer et al. [28] state that the cytotoxic activity of platelets requires the membrane-associated activation of phospholipase A_2 (PLA_2), the rate-limiting enzyme of the arachidonate cascade.

A number of peptide receptors is expressed on the surface of platelets, including NK-1 receptor [25]. NK-1 is a member of the G-protein coupled receptor family, and its intracellular second-messenger system is connected to the activation of the phosphoinositide pathway [12]. Through this signal transduction mechanism SP might influence platelet functions including their arachidonate cascade.

The arachidonate cascade of platelets is initiated by a receptor mediated liberation of arachidonic acid from phospholipids by the action of phospholipases. Arachidonate at sn-2 position of membrane phospholipids is cleaved mainly by the direct action of PLA_2, but phospholipase C (PLC) also play a role in the release of free arachidonic acid. The phosphatidil-inositol specific PLC catalyzes the formation of diacylglycerol. The release of arachidonate from diacylglycerol requires the sequential action of two enzymes, a diglyceride lipase and a monoglyceride lipase. Diglyceride lipase catalyzes specifically the deacylation of the sn-1 fatty acyl residue of a diacylglycerol, whereas the monoglyceride lipase catalyzes the hydrolysis of the resulting 2-arachidonyl-monoacylglycerol to arachidonate and glycerol [3].

The free arachidonic acid is metabolised to lipoxygenase products (12-hydroperoxy-5,8,10,14-eicosatetraenoic acid [12-HPETE] and 12-hydroxy-5,8,10,14-eicosatetraenoic acid [12-HETE]) and also to cyclooxygenase metabolites (thromboxane A_2 [TxA_2], prostaglandin [PG]D_2 PGE_2, $PGF_{2\alpha}$ and PGI_2). The formation of TxA_2 is accompanied by the production of 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT), with the release of malondialdehyde in an amount equivalent to the TxA_2. The cyclooxygenase and lipoxygenase metabolites of the arachidonate cascade might play a regulatory role in the inter- and intracellular functions of platelets [5].

The present *in vitro* study was carried out to determine the effect of SP on the arachidonate cascade in rat platelets.

Materials and Methods

Arachidonic acid (grade I), HHT and 12-HETE standards, Medium 199 (HEPES modification) and substance P were purchased from Sigma Chemical Co., St. Louis, MO (USA). 1-^{14}C-Arachidonic acid (spec. act. 2035 MBq/mM) was obtained from Amersham (England). Silica gel thin-layer plates (0.25 mm) were obtained from Merck AG, Darmstadt (FRG). PGE_2, PGD_2, TxA_2, TxB_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ were generously provided by Upjohn Co., Kalamazoo (USA).

Isolation of washed blood platelets

Under light ether anesthesia, blood was drawn from the abdominal aorta of male rats of the Wistar strain (body weight: 185±15 g). Samples were diluted with phosphate buffer (pH 7.4) containing EDTA (5.8 mM) and glucose (5.55 mM). The platelet-rich plasma was collected after the whole blood had been centrifuged at 200 g for 10 min at room temperature, and after collection, the platelet-rich plasma was centrifuged at 1000 g for 10 min at room temperature. The platelets in the pellet were contaminated with red blood cells which were hemolysed with hypotonic solution (which contained 9 parts of 0.83% NH_4Cl solution and 1 part of 0.02% EDTA solution) for 15 min at 4 °C. After hemolysis, the platelet suspension was diluted with phosphate buffer and centrifuged at 1000 g for 10 min at room temperature. Platelets free from leukocytes, lymphocytes, red blood cells and plasma proteins were resuspended in Medium 199 cell culture reagent buffered with HEPES solution. The pH of the incubation mixture was 7.4. The platelet count was determined with a hematocytometer.

Animal experiments were performed with the permission of the Ethical Committee for the Protection of Animals in Research of Albert Szent-Györgyi Medical University, Szeged, Hungary.

Analysis of eicosanoids

From the platelets of each animal 8 incubation samples (SP 10^{-13}–10^{-7} mol/L and the control) were prepared. Each incubation mixture cointained 10^8 cells in 1 ml buffered Medium 199. All samples were then incubated at 37 °C for 5 min. The enzyme reaction was started by the introduction of a tracer substrate, 1–^{14}C-arachidonic acid (0.172 pmol, 3.7 kBq), into the incubation mixture. Ten minutes later, the enzyme reaction was stopped by bringing the pH of the incubation mixture to 3 with formic acid. (A period of 5–10 min has been found to be appropriate for the labelling of platelets [7, 20].) The samples were then extracted with ethyl acetate (2 × 3 ml), and the organic phases were pooled and evaporated to dryness under nitrogen. The residues

were reconstituted in 150 µl ethyl acetate and quantitatively applied to silica gel G thin-layer plates. The plates were developed to a distance of 15 cm in an organic phase of ethyl acetate : acetic acid : 2,2,4-trimethylpentane : water (110:20:30:100) by means of overpressure thin-layer chromatography. Each 3 mm band of the chromatograms was scraped off and the radioactivity was determined in a Beckman LS 1800 liquid scintillation counter, using 5 ml toluene containing 0.44% w/v PPO, 0.02% w/v POPOP and 10% v/v ethanol. The radiolabelled products of arachidonic acid were identified with unlabelled authentic standards, which were detected with anisaldehyde reagent [17]. For the separation of lipoxygenase products of the arachidonate cascade, high-performance liquid chromatography (HPLC) was applied, by using a reversed phase column (4.6 × 250 mm), connected to a guard column (4.6 × 25 mm), both packed with Nucleosil C18 (5 µm particles). The eluent consisted of acetonitrile : methanol (750:250), and phosphoric acid (150 µl) was added to adjust the pH to 4. Lipoxygenase products were monitored at 235 nm with a Hewlett-Packard 1050 UV detector and their amounts were measured with a Beckman LS 1800 liquid scintillation analyzer.

Statistical analysis was carried out by using SPSS for Windows (version 6.1.2). Analysis of variance was performed, followed by the Duncan multiple comparison *post hoc* test.

Results

The lipoxygenase pathway is quantitatively the most important route of the arachidonate cascade in platelets. This pathway is characterized by the synthesis of 12-HETE. SP (10^{-11}, 10^{-9} and 10^{-8} mol/L) significantly increased the synthesis of 12-HETE (Table I).

The other metabolic route of the arachidonate cascade in platelets is the cyclooxygenase pathway. 10^{-12} SP inhibited, whereas 10^{-9} mol/L SP activated the cyclooxygenase pathway (Table I). The main cyclooxygenase metabolite of platelets is TxA_2, which induces platelet aggregation and vasoconstriction. The dose response curve of TxA_2 (the stable metabolite of which, TxB_2, was determined) to SP exhibited a similar pattern to that detected for the cyclooxygenase pathway. The synthesis of TxA_2 is accompanied by the parallel production of HHT directly from PGH_2 with the release of malondialdehyde [8]. The synthesis of HHT was also inhibited by SP at 10^{-12} mol/L concentrations. An other vasoconstrictor PG metabolite of platelets is $PGF_{2\alpha}$. The dose-response curve of $PGF_{2\alpha}$ to SP exhibited a similar pattern to that detected for TxB_2 formation but the inhibition at concentration of 10^{-12} mol/L was not significant at the significance level $p<0.05$. Among the production of the vasodilator cyclooxygenase metabolites (PGD_2, PGE_2 and PGI_2, the stable metabolite of which, 6-keto-$PGF_{1\alpha}$, was detected) only the synthesis of PGE_2 was significantly influenced by SP. The production of PGE_2 was elevated by 10^{-9} mol/L SP (Table II).

SP increased the total metabolite formation in the arachidonate cascade of washed rat platelets at the concentration of 10^{-9} and 10^{-8} mol/L, respectively (Table I).

Discussion

SP is one of the most potent vasodilators so far known [18]. Results indicate that PGs might participate in some of the vascular effects of SP [9, 23]. In our experiments SP in the physiological range of plasma concentration (10^{-12} mol/L) decreased the synthesis of vasoconstrictor arachidonate metabolites (TxA_2 and $PGF_{2\alpha}$). This suggests that in physiologic conditions the arachidonate cascade of platelets may play some role in the vasodilator effect of SP.

Yong et al. [29] demonstrated a novel cytotoxic effect of platelets against *Toxoplasma gondii*: the interaction of *Toxoplasma gondii* with platelets resulted in a marked increase in TxB_2 production. Damonneville et al. [6] reported that SP induces the cytotoxic activity of platelets against the larvae of *Schistosoma mansoni.* They found that SP was most effective in the range of 10^{-10}–10^{-9} mol/L. Our findings may present one possible link between the results of Yong et al. and Damonneville et al. In our experiments, the formation of Tx in rat platelets was increased with 30.4% by higher concentration of SP (10^{-9} mol/L), and therefore the SP-induced platelet cytotoxicity against parasites might be mediated by an elevated Tx synthesis.

In our experiments we found that 10^{-11}, 10^{-9} and 10^{-8} mol/L SP stimulated the lipoxygenase pathway of platelets, resulting in an increased formation of 12-HETE. 12-HETE induces chemokinesis, chemotaxis in leukocytes [11] and degranulation in neutrophils [27], and its precursor 12-HPETE activates 5-lipoxygenase and consequently leukotriene synthesis in neutrophils [16]. 12-HETE can activate protein kinase C, increase the cell surface expression of integrins, enhance adhesion and induce endothelial cell retraction [4]. The elevated synthesis of 12-HPETE and 12-HETE of rat platelets induced by SP might be involved in the process of neurogenic inflammation.

10^{-9} and 10^{-8} mol/L SP significantly increased the total metabolite formation in the arachidonate cascade of rat platelets with 40%, which suggest that these concentrations of the peptide increase the arachidonate liberation from phospholipids via activation the PLA_2 and PLC.

Our data demonstrate that SP has an effect on the arachidonate cascade of rat platelets *in vitro.* The metabolites of the arachidonate cascade in the platelets might participate in the action of SP in the process of neurogenic inflammation and the regulation of local haemostasis and immunological processes against parasites.

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Table I

The effect of SP on the arachidonate cascade of rat platelets

	control		10^{-13}		10^{-12}		10^{-11}		10^{-10}		10^{-9}		10^{-8}		10^{-7}	
	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%
Cyclooxy.	7180	100	5707	79.5	5310*	73.9	6711	93.5	7386	102.9	9524*	132.6	6867	95.6	6507	90.6
±S.E.	±352		±743		±419		±387		±515		±497		±482		±465	
12-HETE	45 706	100	49 902	109.2	53 106	116.2	60 228*	131.8	50 958	111.5	65 738*	143.8	68 560*	150	59 257	129.6
±S.E.	±5079		±7341		±8537		±6033		±3596		±5108		±8268		±6706	
Total	52 887	100	55 609	105.1	55 476	104.9	66 939	126.6	58 371	110.4	75 261*	142.3	75 427*	142.6	65 763	124.3
±S.E.	±5331		±7693		±8848		±6177		±3573		±4977		±8819		±6979	
n	6		6		6		6		6		6		6		6	

The effect of substance P on the arachidonate cascade of isolated rat platelets was investigated. The platelets were labelled with ^{14}C-arachidonic acid, and the ^{14}C-eicosanoids were then separated by means of overpressure thin-layer chromatography or high-performance liquid chromatography and were quantitatively determined in a liquid scintillation counter. The first line of the Table shows the different concentrations of SP in mol/L, while the last line indicates the numbers of experiments performed (n). The tabulated data are the mean values and the standard errors in DPM (desintegration per minute), and the mean values in the percent of the control. *$p<0.05$, compared to control. Cyclooxy.: the total amount of the cyclooxygenase metabolites, 12-HETE: 12-hydroxy-5,8,10,14-eicosatetraenoic acid, Total: the total amount of eicosanoids synthesized by platelets (Total = cyclooxy. + 12-HETE).

Table II

The effect of SP on the cyclooxygenase pathway of rat platelets

	control		10^{-13}		10^{-12}		10^{-11}		10^{-10}		10^{-9}		10^{-8}		10^{-7}	
	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%	dpm	%
PGD_2	1399	100	946	67.6	1128	80.6	1191	85.1	1580	112.9	1201	85.8	1172	83.8	1007	71.9
±S.E.	±137		±152		±228		±151		±137		±221		±174		±158	
PGE_2	688	100	506	73.5	741	107.7	962	139.8	788	114.5	1299*	188.8	694	100.8	689	100.1
±S.E.	±40		±101		±102		±158		±105		±158		±119		±154	
6-keto-$PGF_{1\alpha}$	350	100	278	79.4	326	93.1	325	92.8	404	115.4	463	132.3	469	134	436	124.6
±S.E.	±31		±55		±28		±47		±66		±63		±48		±51	
TxB_2	2410	100	1976	81.9	1224*	50.8	2087	86.6	2162	89.7	3142*	130.4	2080	86.3	2167	89.9
±S.E.	±195		±375		±106		±244		±264		±523		±247		±189	
HHT	1951	100	1553	79.6	1309*	67.1	1523	78.1	1650	84.6	2081	106.7	1718	88.1	1619	82.9
±S.E.	±134		±185		±102		±185		±177		±126		±94		±147	
$PGF_{2\alpha}$	789	100	698	88.5	527	66.8	676	85.7	801	101.5	1338*	169.6	734	93	849	107.6
±S.E.	±49		±24		±76		±75		±81		±140		±80		±59	
n	6		6		6		6		6		6		6		6	

The effect of substance P on the arachidonate cascade of isolated rat platelets was investigated. The platelets were labelled with ^{14}C-arachidonic acid, and the ^{14}C-eicosanoids were then separated by means of overpressure thin-layer chromatography or high-performance liquid chromatography and were quantitatively determined in a liquid scintillation counter. The first line of the Table shows the different concentrations of SP in mol/L, while the last line indicates the numbers of experiments performed (n). The tabulated data are the mean values and the standard errors in DPM (desintegration per minute), and the mean values in the percent of the control. *$p<0.05$, compared to control. PG: prostaglandin, Tx: thromboxane, HHT: 12L-hydroxy-5,8,10-heptadecatrienoic acid.

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Pro-, antioxidant and filtration changes in the blood of type 1 diabetic patients

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In our present work we attempt to clarify the pro-, antioxidant status (redox status) of blood and the red blood cell (RBC) filtration changes in type 1 (insulin dependent diabetes mellitus = IDDM) diabetic patients, broadening our biochemical knowledge about the mechanism of diasease. Further on we try to apply our observations in therapy. Our studies on enzymes and the pro- and antioxidant status in type 1 diabetes are closely related to earlier works. Our studies on antioxidants have been extended deeper on redox conditions for example on the reduced and oxidized glutathione (GSH and GSSG) and glutathione reductase activity. The properties and changes of antioxidant enzyme activities (superoxide dismutase, glutathione peroxidase and catalase) as well as lipid peroxidation (LP) have been studied earlier without selecting the different type of human diabetics.

At the same time the red blood cell filtration characteristics are compared also with normal values.

The results of our studies confirmed the earlier findings that human diabetes is accompanied by a strong oxidative predominance (oxidative stress) in blood.

Keywords: antioxidant status, diabetes, filtration, red blood cell

Studying and comparing the metabolic changes and differences in human diabetes are "evergreen" issues because of their results could be useful in therapy.

The changing blood glucose and other oxidative changes resulted early cellular damages and complications in human diabetics and in experimental diabetes, too [16, 19, 20].

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Phenomena have been frequently dealt with and it has been proven that diabetes is accompanied by increased LP which unambiguosly means membrane damage. In such cases, filtration behavior of red blood cells (RBCs) also changes. RBCs become more rigid which results in increased filtration rate [5, 14].

In the present study we measured filtration characteristics and glycohemoglobin content, plasma LP, GSH and GSSG content of children with type 1 diabetes. From RBC hemolysates, besides measurement of quantitative values mentioned in case of plasma, antioxidant enzyme activities were compared. Superoxide dismutase SOD, glutathione peroxidase (GPx-ase) and reductase (GR-ase), and catalase (C-ase) activities were measured. With the studies of pro- and antioxidant changes and demonstration of prooxidant dominance in type 1 diabetes, continuous prevalence and effect of "oxidative stress" was to be confirmed with further data.

Materials and Methods

Blood of 41 patients suffering from clinically diagnosed type 1 diabetes (IDDM) was examined (23 females and 18 males, age group 4–22 years, average 13 years). Patients were informed about the purposes of the investigations and our study was performed with their approval.

Blood for control measurement was obtained from the Blood Transfusion Center of Szent-Györgyi Albert Medical University.

Blood taken from cubital vein into a tube with heparin, was separated into two parts with centrifugation at low rpm and RBCs and plasma (supernatant) were separately used for measurements. RBCs were washed with physiological saline three times and the packed RBCs hemolysed with distilled water and then the centrifuged hemolysate supernatants were used for quantitative determination of LP and antioxidant enzyme activities.

For rheological (deformability) tests 5% hematocrit suspensions were prepared with prefiltered PBS buffer (serum albumin phosphate buffer) at pH 7.4 (290 mOsm/kg). Deformability measurements were performed with an M-100 filtrometer (Mikron, Budapest, Hungary) (filtrometer was constructed on the basis of the St. George's filtrometer) using a Nucleopore filter (Nucleopor Corp., USA) with a diameter of 5 micron. Measurements were performed within 2 hours after the blood samples were taken. The relative filtration rate (F_i), the average cell transit time (Tc) and the clogging rate (CR) by a computer coupled to the filtrometer were calculated.

LP, reduced and oxidized glutathione (GSH and GSSG), protein were determined in plasma. Antioxidant enzyme activities, namely the activities of superoxide dismutase (SOD; EC 1.15.1.1), glutathione peroxidase (GPx-ase; EC 1.11.1.9), glutathione

reductase (GR-ase; EC 1.6.4.2) and catalase (C-ase; EC 1.11.1.6) were measured simultaneously with the above-mentioned components from the aliquots of the supernatants of washed RBC hemolysates.

Blood glucose was determined using o-toluidine method [11]. Glycohemoglobin was estimated by a rapid and simple method [6].

Protein content both in plasma and hemolysate was determined with the method of Lowry et al. [15]. Determination of the other components was performed as follows:

(i) Lipid peroxidation (LP) was measured by the thiobarbituric acid method described by Placer et al. [23].

(ii) Reduced glutathione (GSH) was determined by the method of Sedlak et al., with Ellman's reagent [24].

(iii) Oxidized glutathione (GSSG) was measured by the method of Tietze [25].

(iv) Measurement of SOD activity was done through the enzyme dependent inhibition of epinephrine – adrenochrome autooxidative transformation [18, 21].

(v) GPx-ase activity was determined with spectrophotometric, so-called "chemical" activity measurement [22].

(vi) Glutathione reductase was measured by NADPH extinction decrease at 340 nm, 30 °C and pH 7.4 [22], 1 Unit of GR-ase was defined as 1 micromole of GSSG reduced/min.

(vii) C-ase activity was measured with the method of Beers et al. and Matkovics et al. [3, 18].

Statistical evaluation: the reported data are means of measurements and their SEM values. For statistical evalution Student's *t*-test was used. Differences were considered significant if $p<0.05$.

Results

The average blood glucose level of IDDM patients was 12.09±4.69 mMol/L and the amount of glycohemoglobin (hemoglobin A_{1C}) concentration was 9.86±1.64%.

Age of patients ranged between 4–22 years, their average age was 13 years. Sex distribution was 23 females and 18 males. The rest of the results are summarized in Tables I–III.

(i) Deformability characteristics of RBCs decrease, first of all the transit time increases (Table I).

(ii) All the components tested increases significantly in the plasma, LP, GSH and GSSG respectively, which unequivocally reflecting the change of plasma into prooxidant direction (Table II).

Table I

Filtration values

Parameters	F_i	Tc	CR
Control (n=40)	0.75±0.04	7.64±1.29	2.92×10^{-3}±0.78
IDDM (n=41)	0.63±0.02	12.72±1.29	2.51×10^{-3}±0.95
	p<0.01	p<0.01	p<0.02

Table II

Plasma pro- and antioxidants in type 1 diabetics

Parameters	LP	GSH	GSSG
	nMol MDA/mg protein $\times10^{-2}$	micromol/mg protein $\times10^{-3}$	micromol/mg protein $\times10^{-3}$
Control, (n=40)	9.3±0.03	1.61±0.3	5.1±0.3
IDDM (n=41)	21.0±6.2	2.15±0.47	17.0±5.1
	p<0.01	p<0.01	p<0.05

(iii) The pro-, antioxidant change of hemolysate can be characterized as follows: LP decreases significantly, the glutathione components are (GSH, GSSG) significantly higher in diabetic hemolysate, GPx-ase activity is significantly higher and GR-ase activity significantly decreases. GR-ase is very sensitive to oxidative damage [1] (Table III).

(iv) Increase of SOD activity and significant decrease of C-ase have also important role in the development of prooxidant predominance.

Discussion

In a work published in 1990 Mandrup-Poulsen et al. summarized "that non-specific pharmacological inhibition of the immune system is able to intervene of the pathogenic process of IDDM". The results hopefully provide further broadening of the therapeutic possibilities in IDDM [16]. The dangerous effect of oxygen radicals depends on several cellular mechanism, e.g.:

(i) The amount of polyunsaturated fatty acids in cell membranes. If they are peroxidised in general it disrupts the cell membranes and the lysosomal membranes also.

(ii) Damaging influence on the DNA and proteins.

(iii) Activating the phospholipases and enhancing the eicosanoid synthesis.

(iv) The extracellularly released LP products increased vascular permeability and the leucocyte chemotaxis.

Table III

RBC (hemolysate) pro- and antioxidants in type 1 diabetics

Parameters	LP	GSH	GSSG	GPX-ase	GR-ase	SOD	C-ase
	nMol MDA/ mg prot.	microMol/ mg prot.$\times 10^{-3}$	microMol/ mg prot.$\times 10^{-3}$	U/mg prot. $\times 10^{-3}$	U/mg prot. $\times 10^{-3}$	U/mg prot.	BU/mg prot. $\times 10^{-3}$
Control	0.71 ± 0.06 (n=40)	6.71 ± 0.73 (n=40)	0.19 ± 0.01 (n=10)	9.10 ± 0.65 (n=40)	61.4 ± 5.5 (n=40)	1.32 ± 0.40 (n=40)	2.85 ± 0.31 (n=40)
IDDM	0.55 ± 0.17 (n=41)	8.63 ± 3.58 (n=41)	0.61 ± 0.06 (n=23)	10.9 ± 3.13 (n=41)	5.43 ± 1.80 (n=41)	2.49 ± 0.83 (n=41)	4.69 ± 1.30 (n=41)
	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$

More recent observation have proved that the human pancreatic islets are more resistant against toxic agents or endogen free radicals, nitric oxide and cytokines as their rodent counterpart.

The main defending system of human pancreatic islets are the HSP 70, SODs (mainly inducible Mn-SOD) and the C-ase activity increase [7].

Three other works must be mentioned here, before explaining our findings considering of therapy.

In 1993 in a lecture E. Lilly summarized the connection between "glycation and diabetic complication". However, advanced glycation end products (AGEs) are influences:

(i) the signal transduction pathways, they may alter the level of soluble signals.

(ii) Intracellular glycation directly alters the tissue proteins and enzymes.

The glycoseaminoglycans appear in the urine and their amount correlates well with the duration and complications in IDDM [4, 8, 10, 17].

In the brain it is known that the GR-ase is very sensitive against oxidative damage and the main antioxidant is the GSH and the GPx-ase protects the brain tissue agains the accumulation of hydrogen peroxide and other oxygen species [1].

In our earlier publications [20] we proved that in the blood of not selected human diabetics significant LP, GPx-ase activity increases were detectable with a minimal C-ase and significant Cu, Zn-SOD activity decrease. In our present work we try to prove in type 1 human diabetics the prooxidant dominance and the antioxidant systems' imbalance.

The finding of the present work based on blood analysis data has been that there is a prooxidant predominance (oxidative stress) in type 1 diabetic cases.

It has been proposed earlier and nowadays too that some of thediabetic complications are bound together with the enhanced activity of reactive oxygen species and the accumulation of lipid peroxidation and advanced glycation end products [2, 9] could correct or stop the permanent use of antioxidant, e.g. Vitamin E or ascorbic acid the later in a lower concentration [12, 13, 26].

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Pro-, antioxidant and rheologic studies in the blood of type 2 diabetic patients

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This study was conducted on type 2 non-insulin-dependent diabetes mellitus (NIDDM) cases and healthy blood donors. Lipid peroxidation (LP) products in plasma and red blood cell (RBC) hemolysates were estimated as total thiobarbituric acid reactive substances (TTBARS). The plasma and hemolysate reduced and oxidized glutathione (GSH and *GSSG)* levels are compared.

In the hemolysates the antioxidant enzymes namely superoxide dismutase (SOD), glutathione peroxidase (GPx-ase), glutathione reductase (GR-ase) and catalase (C-ase) are also compared.

The RBC filtration characteristics are determined and compared with controls:

1. LP and GSH in diabetic plasma were significantly higher, but in the hemolysate the GSH raised but the LP was significantly lower in diabetics than in healthy controls.

2. Superoxide dismutase and C-ase were significantly higher in NIDDM hemolysate. Contrary the GPx-ase activity was significantly lower in diabetics.

3. The diabetic RBCs filtration characteristics are changed in respects significantly namely the F_i was lower, the Tc and CR were higher. It means higher rigidity and oxidative damage of the membrane of diabetic RBCs.

Keywords: NIDDM, blood, oxidative stress, proved

The oxygen stress in the cells are common in diabetes mellitus (DM) [8] and other disorders. The aerob cells produce oxygen radicals and during lipid peroxidation a lot of other alkoxy radicals were present and they are toxic, they damaging DNA,

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inactivate enzymes, oxidize or react with proteins and hormones, damaging the membranes [16].

Reactive oxygen radicals [superoxide (O_2^-), hydroxyl (HO·) radicals and hydrogen peroxide (H_2O_2)] are increasingly formed in DM by the autoxidation of glucose (protein glycation). Hyperglycemia leads to the activation of sorbitol pathway and inhibits the formation of triose phosphate and its autoxidation results in alfa-oxoaldehyde and H_2O_2. Oxygen and other radicals and the tissue oxidative stress are the main reason of the later damages in diabetes. In the blood and tissues are existing defending systems. If the defending systems are incomplete it leads to the oxidation of cellular components and glycoconjugation. Chemically the main stream of changes in observed oxidation, fragmentation and cross-linking. Beside reactive oxygen species, the LP products are also the reasons of a number of secondary complications in DM [1].

The redox substances and the antioxidant enzymes are the most important endogenous antioxidants.

In our present study it was determined and compared the LP, GSH, GSSG quantities (the antioxidant status) of plasma and the pro- and antioxidant systems in hemolysate and filtration properties in type 2 (NIDDM) human diabetic cases.

The enzymes SOD (EC 1.15.1.1), glutathione peroxidase (EC 1.11.1.9), glutathione reductase (EC 1.6.4.2) and C-ase (EC 1.11.1.6) activities are measured and compared with healthy controls.

Materials and Methods

Chemicals used for enzyme measurements and other determinations, were of the highest purity and were purchased from the most reliable companies (Sigma, Merck, Boehringer, Calbiochem, Reanal).

The venous blood was taken from the cubital vein after fasting overnight. 0.2 ml heparin injection was used as anticoagulant (1000 IU). All patients were under a suitable therapeutic regimen at the time of blood sampling. Informed consent was obtained from all the participants of the study and the protocol was approved by the ethical committee of the hospital. The control group comprised 10 or 40 healthy blood donors.

Blood glucose was determined using o-toluidine method [5].

The amount of TTBARS (or with other word LP) was measured from plasma and hemolysed RBC aliquots by the method of Placer et al. [14].

Before enzyme activity measurements and determination of glutathione, the hemolysates were centrifuged at 3000 g for 10 min and supernatants were used for measurements.

SOD activity was determined on the basis of inhibition of epinephrine-adrenochrome autoxidation [9, 10].

C-ase activity was measured with a spectrophotometric method at 240 nm [2]. C-ase activites are given in Bergmeyer Unit (BU; 1 BU means 1 g H_2O_2 decomposition/min at 25 °C).

GPx-ase activity was determined with so-called "chemical" method. In these case cumene-hydroperoxide and GSH were the substrates of GPx-ase [13].

Glutahione reductase (GR-ase) was measured by NADPH extinction decrease at 340 nm, 30 °C, pH 7.4 [13].

Amount of GSH was determined by Ellman's reagent with spectrophotometry [15] while GSSG with the method of Tietze et al. [17].

Protein content was measured with Folin reagent using plasma albumin for standard [8].

For rheological (deformability) tests 5% hematocrit suspensions were prepared with prefiltered PBS buffer (serum albumin phosphate buffer) at pH 7.4 (290 mOsm/L). Deformability measurements were performed with an M-100 filtrometer (Mikron, Budapest, Hungary) (filtrometer is constructed on the basis of the St. George filtrometer) using a Nucleopore filter (Nucleopore Corp., USA) with a diameter 5 micrometer. Measurements were made within 2 hours after the blood samples were taken. A computer coupled to the filtrometer calculated the relative filtration rate (F_i) the average cell transit time (Tc) and the clogging rate (CR) [11, 12].

Statistical evaluation: Our data indicate means of measurements and their SEM values. For statistical evaluation Student *t*-test was used and was considered significant if $p<0.05$ or lower.

Results

The blood glucose average of the type 2 diabetics was: 11±3.44 mmol/L (n=76) and glycohemoglobin A_{1c} level: 8.23±1.98 (n=74).

In the experiments 77 patients participated. Age group was between 20–84 years (average 53 years, 38 females and 39 males).

Evaluating our results based on the series of Tables, it was stated as follows:

Table I summarizes the plasma values of LP, GSH and GSSG quantities. All of these parameters increased significantly in the cases of type 2 diabetics comparing them with normal plasma controls.

Table I

LP, GSH and GSSG plasma values in normal and NIDDM patients

Parameters / Cases	LP	GSH	GSSG
	mmol MDA/mg prot.	micromol/mg prot.	micromol/mg prot.
Control (n=40)	9.30×10^{-2} ±0.03	1.61×10^{-3} ±0.30	5.07×10^{-5} ±0.05
NIDDM	20.5×10^{-2} ±3.3 (n=90)	2.06×10^{-3} ±0.34 (n=104)	11.67×10^{-5} ±0.13 (n=10)
Significance	p<0.01	p<0.01	p<0.01

Table II

The pro- and antioxidant values in hemolysate of NIDDM cases comparing with controls (Mean± SEM)

Parameters / Cases	LP	GSH	GPx-ase	SOD	C-ase
	nmol MDA/mg prot.	micromol/mg prot.	U/mg prot.	U/mg prot.	BU/mg prot.
Control (n=10)	0.71 ±0.06	6.71×10^{-3} ±0.73	9.1×10^{-3} ±0.65	1.32 ±0.40	3.54×10^{-3} ±0.03
NIDDM (n=85)	0.410 ±0.105	7.45×10^{-3} ±3.10	5.81×10^{-3} ±2.46	2.70 ±1.15	4.36×10^{-3} ±0.03
Signficance	p<0.01	n.s.	p<0.01	p<0.01	p<0.05

n.s. = non significant

In Table II we compared the hemolysate pro- and antioxidant values and enzyme activities. The measured diabetic hemolysate values of LP and GPx-ase activity were significantly lower but the other parameters were significantly higher than in the normal hemolysates.

The RBC filterability results are summarized in Table III. The F_i is significantly decreased in the NIDDM blood cells, however, the transit time (Tc) and clogging rate (CR) are significantly higher.

Table III

Comparison of RBCs calculated values

Filtration parameters / Cases	F_i	Tc	CR
Control (n=40)	0.754 ±0.039	7.640 ±1.294	2.918×10^{-3} ±0.783
NIDDM (n=84)	0.656 ±0.055	11.700 ±2.908	7.277×10^{-3} ±3.907
Significance	p<0.01	p<0.01	p<0.01

Discussion

In the discussion we focus on and comparing our results with the publication of Sundaram et al. That is the last pro-, antioxidant and vitamins survey with "large sample size" on the Indian Asian ethnic group. It was published in 1996 [16]. The authors stated also that their study "is unique".

If we compare our and the mentioned plasma LP values both are significantly higher in the diabetic groups. Similar results have been published by other authors too [3, 4]. However, in our measurements the GSH and GSSG in plasma are significantly higher in the diabetics.

As to the results there is strong evidence that the plasma LP correlate with the duration and complication of NIDDM. Surprisingly in our cases the hemolysate LP values are significantly lower. However, we suppose that the higher SOD activity (in our cases) dismutates the superoxide (O_2^-) anion as well as the higher C-ase activity destroy the H_2O_2 excess. Therefore Haber-Weiss or Fenton reactions which initiate LP are not possible.

The GPx-ase activity is lower in our cases too. We suppose that the lower level of polyunsaturated fatty acids (PUFAs) and LP products in RBCs of human diabetics are the real reason of the lower GPx-ase activity.

The earlier supposition supports the values in Table III where the RBCs show greater transit time (Tc) and lower filtration rates (F_i). Our experiences are, that O_2^- radicals and H_2O_2 influence the RBC filtration parameters in a similar way [11, 12].

Our hypothesis after our results is that the antioxidant changes are compensatoric. The antioxidants try to compensate the prooxidant predominance ("oxidative stress").

Therefore it is clear that the antioxidant support in NIDDM suitable for prevent or slow down the diabetic complications [6].

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Immunohistochemical localization of the epidermal growth factor receptor in normal mouse tissues

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The levels of epidermal growth factor (EGF) receptors were investigated in normal mouse tissues. Two patterns of reactivity were recognized: strong membraneous or diffuse cytoplasmic staining.

The basal epithelial cells of the skin and the collecting tubule cells of the kidney showed membraneous reactivity whereas the bronchial epithelial cells, parietal cells of the stomach and tubular cells of the kidney showed cytoplasmic reactivity predominantly with the antibody.

The distribution of the epidermal growth factor receptors in different cell types and cellular compartments may implicate varying activation states of the growth factor and its interaction with a milieu available ligand.

Keywords: epidermal growth factor-receptor, mouse tissue, immunohistochemistry, expression

Epidermal growth factor (EGF) was originally described as a substance isolated from the mouse salivary glands which initiated premature eyelid opening and incisor eruption when injected into the neonatal mouse [1]. These physiological events are the net results of the proliferation and differentiation of the cells on EGF-specific receptor molecules at the cell surfaces [2, 3]. EGF–EGF repector interactions are multistep processes they can be controlled by the ligand which is biologically active in optimal

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concentration, and by expression of the receptor on the surface of some specific cell types [4].

Binding of EGF to the EGF receptor can initiate a mitogenic response [5, 6], but the mechanism of intracellular signalling is not yet clear. EGF binding activates the tyrosine protein kinase which results in the phosphorylation of the receptor and other cellular substrates [7, 8, 9].

Receptor ligand complexes cluster within clathrin-coated pits, undergo endocytosis accumulate in endosomes and can than be delivered to another subcelluler compartment, presumably the lysosome, where they are both degraded [10, 11, 12]. Before binding to the ligand, EGF receptor must be expressed on the cell surface and this process is controlled at various levels [4].

EGF receptor expression in normal tissue has been investigated by both analysis of the specific binding of labelled EGF and the receptor-specific monoclonal antibodies. By comparing the distributional disparities that arise from combined use of these methods, the location and activity of the receptor can be deduced. Analysis of the cellular site of receptor localization can also provide information regarding the activity of the receptor. In this study, we have examined the distribution of the EGF receptor expression immunohistochemically in the mouse tissues.

Material and Method

Preparation of tissue samples: Male Swiss albino mice (n=4), weighing approximately 30 gram were fixed with 4% paraformaldehyde by cardiac perfusion. After perfusion for 10 minutes the mice were further perfused with 10% neutral formaline for 5 minutes. Thereafter tissues were removed. The stomach, lung, liver, kidney, testis, thymus, lymph node and skin of the mice were sampled and postfixed in 10% neutral formaline for 24 hours. After processing the specimens, they were embedded in paraffin. 4–5 μm thick sections were used for stainings.

Antibodies and staining procedure: PAP method was used for immunohistochemical staining. The monoclonal antibody (Sigma Anti mouse EGF-R) was diluated (1:200) and performed for 60 minutes at room temperature. After washing in 0.01 M phosphate buffered saline (PBS, PH: 7, 4), slides were covered with rabbit anti-mouse Ig which was labeled with peroxidase and diluted (1:200)(CBL) in PBS containing 0.2% BSA (Bovine serum albumin) and normal human serum. After washing in PBS the slides wee stained with 3,3'-diaminobenzidine-tetrahydrochlorid (DAB, Sigma) containing 0.01% H_2O_2 for peroxidase activity. Counter staining was done with hematoxylin. Negative control staining was run by ommitting the first (primary antibody) step [13].

Intensity of immunohistochemical staining was subjectively estimated and expressed as negative (–), questionably or weakly positive (+/–), definitively positive (+) or very strongly positive (++).

Results

The distribution of the EGF-R in the mouse tissues is shown in Table I.

Two reaction patterns were recognized: membraneous or diffuse cytoplasmic staining obvious reactivity of the cell membrane. Membraneous staining pattern was observed in the basal layer of the skin. The epithelial cells of proximal and distal tubules of the kidney showed an intense diffuse cytoplasmic reactivity and glomerula were not stained (Figs 1, 2).

Diffuse cytoplasmic staining was seen in luminal half of the collecting tubules of the kidney (Fig. 2).

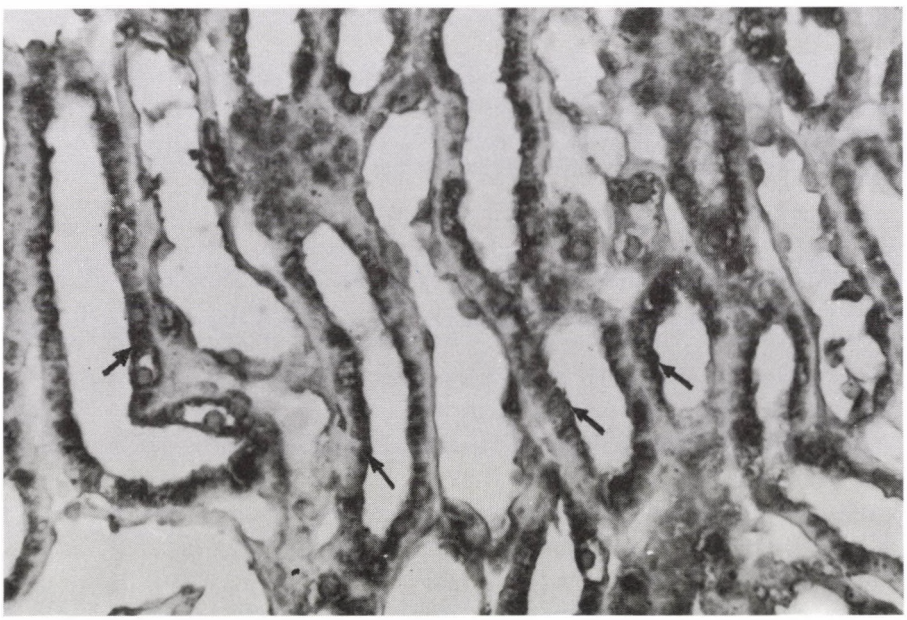

Fig. 1. Immunohistochemical staining for EGF-R in mouse kidney. The cells of proximal (thin arrows) and distal tubule (thick arrows) was stain diffuse cytoplasmic. Glomerule was not stain (*). (Immunoperoxidase; Hematoxylen, ×480)

Table I

Distribution of EGF receptors in normal mouse tissues

Tissue	Reactivity with EGF–R	
	Intensity	Pattern
Skin (Epidermis)	+	Membraneous in basal layer
Lung		
Branchial epithelium	++	Diffuse cytoplasmic
Alveolar epithelium	++	Diffuse cytoplasmic
Stomac (Fundus)		
Mucus secreted cells	–	–
Gastric glands	–	–
Parietal cells	+	Diffuse cytoplasmic
Kidney		
Glomerul	–	–
Proximal tubules	++	Diffuse cytoplasmic
Distal tubules	++	Diffuse cytoplasmic
Collecting tubules	++	Diffuse in luminal half of the cytoplasm
Liver		
Hepatocytes	–/+	Diffuse cytoplasmic
Bile duct cells	–/+	Membraneous
Testis	–	
Lymph node	–	
Thymus	–	

Diffuse cytoplasmic reactivity was observed in the bronchial epithelium (Fig. 3).

In the liver all hepatocytes and the bile duct cells showed weak positivity. Testis, thymus and lymph node cells were found to be negative.

In the negative control, no specific staining was observed.

Discussion

In the present study, EGF receptor expression in normal mouse tissues has been investigated by analysis of receptor-specific monoclonal antibodies.

We observed that epithelial and parietal cells of the stomach, the bronchial cells, the proximal, distal and collecting tubular cells, expressed receptor in their cytoplasm. Localization of EGF receptor in the cytoplasm may reflect internalization of the receptor, a rapid process that occurs after ligand binding [10, 12]. Rapid degradation of the internalized repector is shown in a mast cell culture system [10]. However it is suggested that some of the internalized receptor in EGF treated hepatocytes [11, 14] may recycle to the cell surface. Intracellular receptor localization also represent the newly synthesized molecules in the synthetic machinary of the cells prior to their

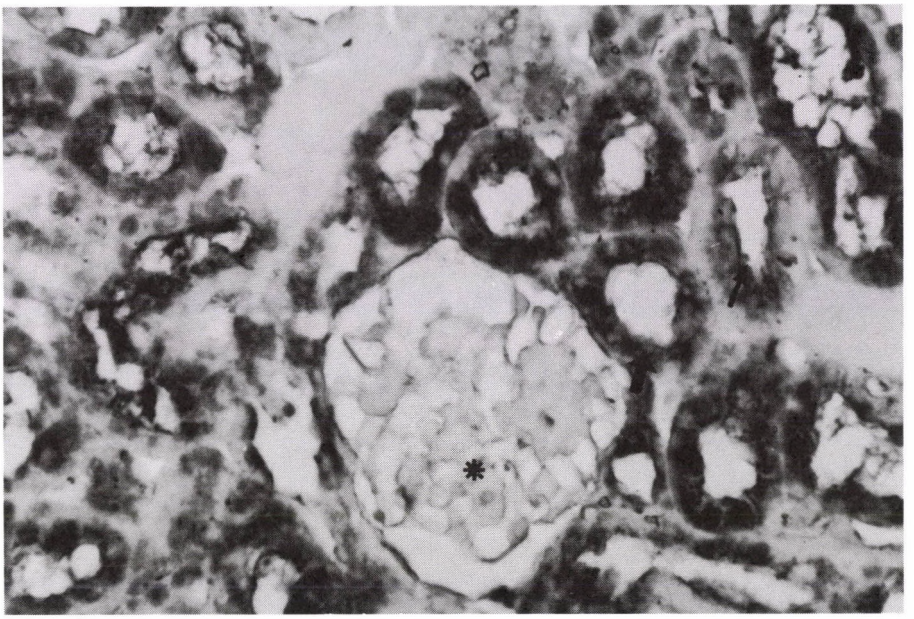

Fig. 2. Mouse kidney showing positive immunoreaction in the collector tubules following incubation with anti-mouse EGF-R antibody. In the supranuclear portion of the cytoplasm showed diffuse cytoplasmic reactivity (thin arrows). (Immunoperoxidase; Hematoxylen, ×480)

insertion into the cell membrane. Internalized receptor may also represent posttranslationally modified receptor; phorbol ester-treated cells reportly internalize receptor into a cellular compartment from which it may recycle to the cell surface [15, 16]. Investigation of the EGF receptor in the isolated perfused rat liver has revealed a large pool of latent receptor with low affinity for EGF [11, 14]. Although cell fractionation studies localized this low affinity class of receptors as a distinct population of intracellular vesicles, all of the immunohistochemically detected receptors were at the cell surface [11]. Since ligand affinity and cellular localization can be rapidly changed by receptor phosphorilation [15, 16, 17] one could hypothesize that a rapid flux between latent internalized and high-affinity membrane localized receptor could be induced by local stimulation. Damjanov et al. [4] reported that the majority of the receptors is in the internalized form in the cells of mice tissues.

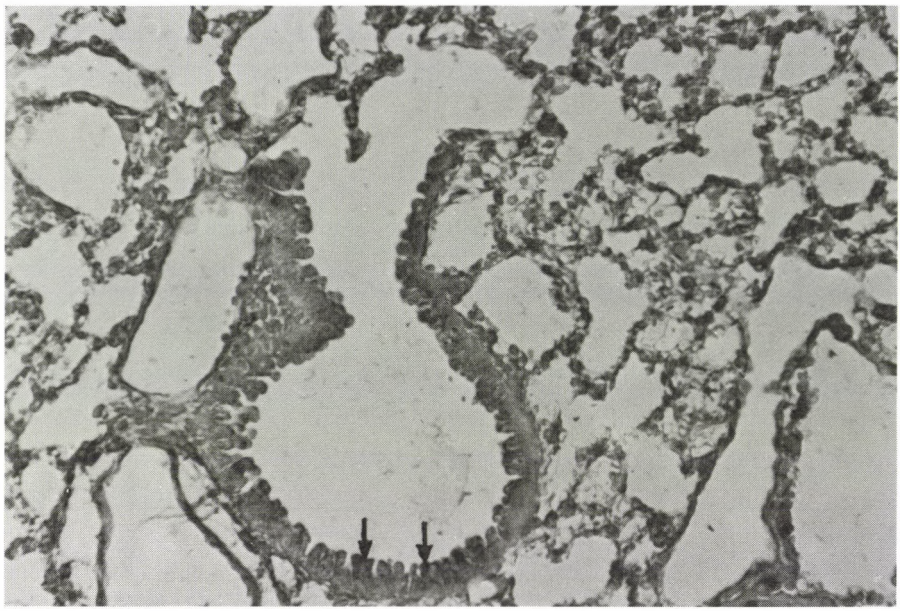

Fig. 3. Immunoperoxidase staining of lung by anti-mouse EGF-R. The branchial and alveolar epithelium are indicated. The branchial epithelium showed intense diffuse cytoplasmic immunoreactivity (arrows). (Immunoperoxidase; Hematoxylen, ×240).

In addition, we detect EGF receptor expression on the cell surface in the basal epithelial cells of the skin and the collector tubule cells of the kidney. Especially, in the skin, the cells that continue to proliferate, express the EGF receptor, while cells of the stratified cornified epithelium do not. Thus, we propose that receptor expression, on the cell surface as a consequence of differentiation, may be one of the mechanisms that regulates the EGF inducible proliferation of cells in a particular tissue that exposed to a constant source of growth factor.

Lack of detection of EGF receptor on the membrane by immunohistochemical methods cannot be interpreted as total absence of EGF receptor.

EGF is mainly synthesized in the submaxillary glands in rats and mice. But prepro EGF (the EGF presursor) messenger RNA (mRNA) is surprisingly abundant in the kidneys [18]. Thus, EGF plays a number of important roles in renal physiology. Recently, EGF was found to be important in initiating controlateral renal hyperthrophy in uninephrectomized mice [19, 20]. In this study, presence of EGF receptor is

determined at significantly high levels in the proximal, distal and collector tubules. In other words these finding reflects that EGF has ability to induce mitogenesis in the renal tubul cells in mouse kidney under normal conditions.

We observed three distinct patterns of EGF receptor in various epithelia. In the first group, cells were not expressing EGF receptor at probably immunohistochemically detectable level.

The second pattern, a strong membraneous and also cytoplasmic expression was observed in actively proliferating cells like the basal layers of stratified epithelia.

The third pattern, predominantly cytoplasmic expression, was observed in certain epithelia like kidney tubule reflecting the intensity of internalized receptor and/or newly synthesized molecules.

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Expression of epidermal growth factor receptor in normal colonic mucosa and in adenocarcinomas of the colon

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The levels of epidermal growth factor receptors (EGF-R) were investigated in normal colon mucosa and in adenocarcinomas of the colon. The frequency of expression and localization of epidermal growth factor receptors were examined by immunohistochemistry in 17 carcinomas (well differentiated) and adjacent non-involved normal colon mucosa.

EGF-R was observed that expression in carcinomas were significantly higher than in normal colon mucosa. EGF-R may be useful as a marker in malignant potential of adenocarcinomas. Furthermore, the detection of EGF-R expression in biopsy materials by immunohistochemistry staining offers precise diagnostic information of the involvement of adenocarcinomas.

Keywords: epidermal growth factor receptor, colon adenocarcinoma, immunohistochemistry

Epidermal growth factor (EGF) stimulates the growth and proliferation of a variety of cell types in vitro and in vivo through interaction with a specific cell surface receptor. EGF–EGF receptor interactions are considered as a multistep process that can be controlled by the availability of biologically active ligand in optimal concentration and by expression of the receptor on the surface of specific cell types [1, 2, 3].

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It has been found that EGF receptor is homologous to the product of the avian erythroblastosis virus oncogene v-erb and it has been suggested that the v-erb B oncogene product is a truncated form of EGF receptor which is produced from the host cell protooncogene v-erb B [3, 4, 5, 6]. Thus the relationship between the EGF/EGF receptor system and cancer has been a focus of attention.

EGF receptor levels of various cultured cells and tumor tissues have been measured by radioreceptor assay or immunohistochemical staining. Many squamous cell carcinoma lines and some cell lines derived from other tumors express a large number of EGF receptors [7, 8, 9, 10]. The expression of EGF receptors have been demonstrated in some glioma [11], breast tumors [4, 12, 13], bladder tumors [14], thyroid tumors [15], sarcomas [16], gynecological tumors [17], lung tumors [4, 18], esophageal tumors [4, 19] and gastric, colonic carcinomas [4, 20]. EGF receptor levels of established cell lines may not reflect those of the original individual cells in tumors because cell lines may suffer certain selection during the establishment of culture or the characteristics of cell lines may gradually change with repeated transfer. Thus the measurement of EGF receptor in tumors is significant.

In this study, the EGF receptor levels of 17 human colon adenocarcinomas and normal colon tissues were measured for comparison and cellular localizations of EGF receptors were examined in detail.

Material and Methods

The frequency of expression and localization of epidermal growth factor receptor (EGF-R) were examined by immunohistochemistry in 17 adenocarcinomas (well differentiated) and adjacent non-involved normal colon mucosa. Tissue specimens were obtained from patients who had undergone surgery at Gazi University Hospital. These patients had never received radiotherapy or anti-cancer chemotherapy.

All tissue specimen were fixed in 10% neutral formaline for 24 hours. Then, specimens were processed and embedded in paraffin. 4–5 μm thick sections were used for stainings.

EGF receptors were detected by PAP method. EGF receptor staining was performed as described previously [21]. The monoclonal antibody (Sigma anti-human EGF-R) was diluted (1:200) and performed for 60 minutes at room temperature. After washing in 0.01 M phosphate buffered saline (PBS), PH 7,4), slides were covered with a human anti-human Ig which was labeled with peroxidase and diluted (1:200)(CBL) in PBS containing 0.2% BSA normal human serum. After washing in RBS the slides were stained with 3.3'-diaminobenzidine-tetrahydrochlorid (DAB, Sigma) containing 0.01%

H_2O_2 for peroxidase activity. Counter staining was done with hematoxylin. Negative control staining was run by omitting the first (primary antibody) step [22].

Results

Expression of EGF-R in normal mucosa

The expression of the epidermal growth factor receptor was rather homogenous throughout the normal colon mucosa. In all of the cases, EGF-R could be detected in columnar cells and in goblet cells, except the mucinous compartment. The antigen was located on the cell surface predominantly and in the cytoplasm. The receptor was found to be equally distributed through the colonic epithelium (Fig. 1) and again, on the cell surface and within the cytoplasm.

The mucosal stroma and the authochthonous structures of the sut wall were nonreactive or reacted only slightly above background levels.

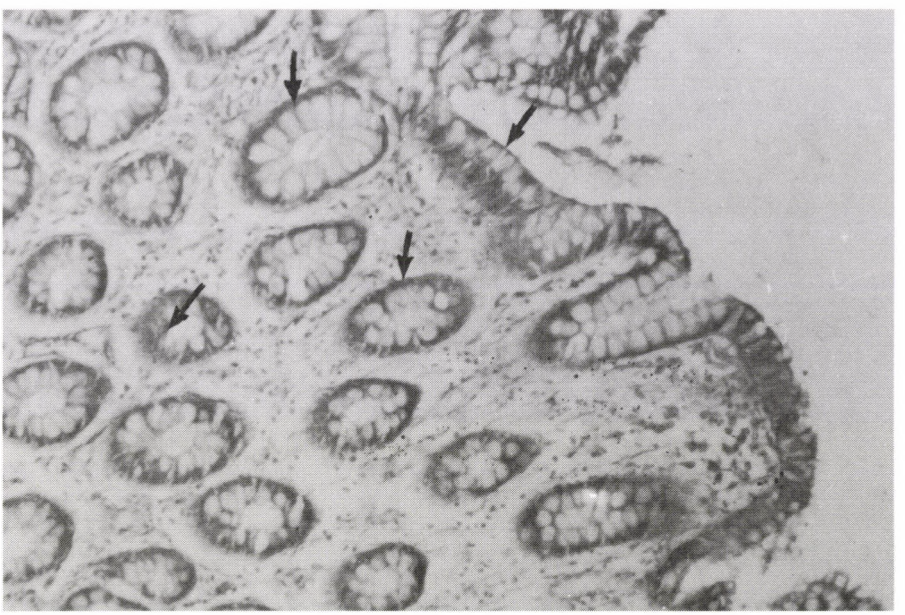

Fig. 1. EGF-R expression in normal colonic mucosa. The EGF-R are expressed throughout the epithelium; basal and apical parts of the crypts are equally positive (arrows) (Immunoperoxidase – HE) ×480

Expression of EGF-R in colon adenocarcinomas

Epidermal growth factor receptor was detectable in all the 17 cases. EGF-R was found over-expressed in cancer as compared to that in the non-cancerous tissue. In colon adenocarcinomas (well differentiated), cytoplasm of all the adenocarcinoma cells were stained. In addition, cell membranes were also stained (Fig. 2) but the stromas were not stained.

EGF-R were not found in fibroblast, smooth muscle cells, nerve fibers, veins, venules and capillary endothelium.

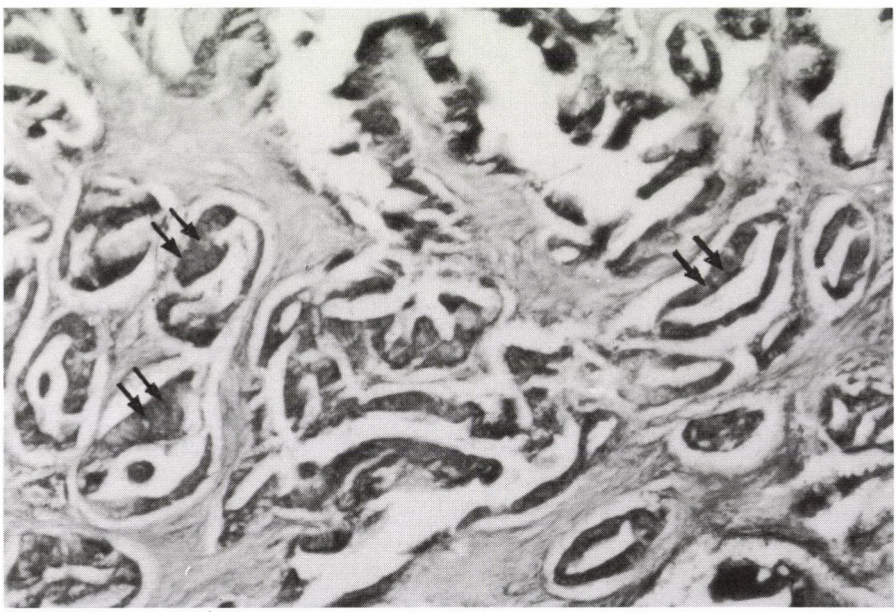

Fig. 2. EGF receptor expression in colonic adenocarcinoma. This adenoma was located in the close vicinity to a well-differentiated carcinoma that shows complete EGF-R expression. In this adenoma specimen, the antigen (receptor) located intracytoplasmically (arrows). (Immunoperoxidase – HE) ×480

Discussion

Results of recent studies indicate that some cultured human carcinoma cell lines are capable of proliferating autonomously in serum free medium as a result of the synthesis and secretion of transforming growth factor alpha (TGF α). TGF α interacts with epidermal growth factor receptor (EGF-R) and induces its activation. It has been suggested by Hirsch and colleagues that the proliferation of cells of the human SW 403 adenocarcinoma cell line is regulated by an activation TGF α loop and that this regulatory pathway can be interrupted by using anti-EGF-R mAb [23]. In a with limited number of samples, significant trends for coexpression of EGF-R and TGF-α were detected in carcinomas by using immunohistochemistry. In this study, it was suggested that TGF-α may play an important role in the development of colorectal carcinomas through an autocrin mechanism involving EGF-R [24].

EGF-EGF related molecules are potent mitogens that mediated their effect through the EGF-R. Rajagapol et al. [26] determined the efficacy of anti-sense EGF-R RNA in circumventing the EGF-R related pathway of proliferation. Anti-sense EGF-R RNA was found to be an anti-proliferative agent in both relatively non-aggressive and highly aggressive human colon cancer cells [25].

Thus, an elevated level of EGF receptor appears to be involved in the development of adenocarcinomas.

Elevated levels of EGF receptors have been reported in number of colon carcinoma cell lines [26, 27, 28] and in biopsy specimens of adenocarcinomas from the colon [29, 30, 31, 32, 33].

This study provides additional evidence to support these previous findings. In this study membrane extracts of surgically resected adenocarcinoma and nearby non-cancerous tissues were prepared and the expression of EGF-R was examined by using immunohistochemistry methods. The EGF-R tended to express more strongly in carcinoma cases than in the non-cancerous tissue.

A correlation between EGF receptor level and biological behavior has been found for various colon cancers. Karameris et al. [17] expressed the epidermal growth factor receptor in gastric and colorectal carcinomas. Their results suggested that gastrointestinal carcinomas expressing EGF-R display pathological features of more aggressive dissease [26]. Koike Y. [19] indicates that EGF-R positive cases in colorectal cancers have higher proliferative activity and more invasive stage, compared with EGF-R negative cases and suggested that EGF-R produced by tumos cells plays an important role in the progression and proliferation of colorectal cancers [29]. It has been postulated by Liu et al. [21] that EGF-R was found over expressed in cancer as compared to that in non-cancerous tissue [31].

Hayashi et al. [13] used immunohistochemical studies to clarify the significance of the expression or over expression of EGF-R in tubular adenoma and adenocarcinoma. This study suggests that EGF-R seems to have some role in the progression of colon neoplasms [31]. In another study, EGF-R was found over expressed in colorectal cancer as compared to that in the non-cancerous tissue. The result suggests an important role of an autocrine/paracrine loop of growth regulation in colorectal cancer [31].

We have also observed that EGF receptor expression in adenocarcinomas are significantly higher than in normal adjacent tissue. Thus, in investigating the malignant potential of adenocarcinoma, the EGF receptor appears to be a useful marker. Furthermore, the detection of EGF receptor expression in biopsy sections by immunohistochemical staining offers precise diagnostic information on the involvement of adenocarcinomas.

The above-mentioned approaches and the determination of prognosis via EGF receptor level measurement may allow a significant improvement in the treatment of patients with adenocarcinomas in the near future.

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Lipid peroxidation and antioxidant system changes in acute L-Arginine pancreatitis in rats

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The important role of oxygen radicals in acute experimental pancreatitis was demonstrated by study of the changes in the antioxidant system in the blood, liver, kidney and pancreas of rats after the administration of a large quantity of L-arginine (L-Arg). The changes in lipid peroxidation and in reduced and oxidized glutathione were followed as well as the activities of peroxide-decomposing enzymes (glutathione peroxidase and catalase) and H_2O_2-producing superoxide dismutases. The results demonstrated that acute pancreatitis and "oxidative stress" develop rapidly after L-Arg treatment. "Oxidative stress" symptoms are expressed 24 hours after the final treatment. Slow restitution of the studied antioxidant system can be demonstrated as early as after 48 hours.

Keywords: LP, antioxidant enzymes, L-Arg, acute pancreatitis

A number of authors have studied the consequences of acute inflammation of the pancreas following the administration of a large dose of L-arginine (L-Arg) and secretion of the enzymes produced by the acini [1, 2]. Besides the enzyme activities, the morphological changes in acute pancreatitis induced by L-Arg administration have also been studied [3].

The important role of oxygen radicals in acute pancreatitis has been described [4-7]. Nitric oxide (NO), the "molecule of 1992", is also presumed to participate in the affliction of the pancreas acini, blood vessels and hormone-producing beta-cells [8, 9].

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The aim of the present comparative studies was to establish the role of the changes in the antioxidant enzyme activities and lipid peroxidation (LP) after administration of a large dose of L-Arg in order to induce acute pancreatitis.

Besides the plasma and the red blood cell (RBC) hemolysate, the changes in the antioxidant system (AOS) in two major parenchymal organs and in the pancreas were also studied. Of the members of the antioxidant system, the amounts of oxidized and reduced glutathione (GSH and GSSG) and the activities of the antioxidant enzymes superoxide dismutase (SOD; EC 1.15.1.1), catalase (C-ase; EC 1.11.1.6) and glutathione peroxidase (GP-ase; EC 1.11.1.9) were determined.

Materials and Methods

The chemicals applied for enzyme measurements and other determinations were of the highest purity. They were purchased from Sigma, Merck, Boehringer, Calbiochem and Reanal.

L-Arg was administered i.p. to 10 male Wistar rats (body weight (b.w.) ranged between 240–270 g) on two consecutive days in a total amount of 2×125 mg L-Arg/100 g (b.w.). Controls (n=10) were injected i.p. with distilled water. Measurements were performed 24 and 48 hours after the second injection. Blood was taken from the hearts of the rats following nembutal anaesthesia and was immediately heparinized. The plasma glucose level and alpha-amylase were determined [10, 11]. The liver, kidney end pancreas were simultaneously removed and homogenized by a method earlier described [12].

LP was measured in the plasma, RBC haemolysate and whole cell homogenate.

Before enzyme activity measurements and glutathione determination, the homogenates were centrifuged at 3000 g for 10 minutes and the supernatants were used for measurements.

In LP measurements, the total amount of thiobarbituric acid (TBA) active substances was determined [13, 14].

SOD activity was determined on the basis of the inhibition of epinephrine-adrenochrome autoxidation [15, 16]. The measurements in supernatant aliquot Mn-SOD activity happened in the presence of 5×10^{-3} M KCN with the same method [17].

C-ase activity was measured spectrophotometrically at 240 nm [18]. In the Tables, C-ase activities are given in Bergmeyer units (BU) (1 BU = decomposition of 1 g H_2O_2/minute at 25 °C).

GP-ase activitiy was determined by a "chemical" method, with cumene hydroperoxide and GSH as the substrates of GP-ase [19].

GSH was determined spectrophotometrically with Ellman's reagent [19], and GSSG by the method of Tietze et al. [20].

Protein content was measured with the Folin reagent, using plasma albumin as standard [21].

Statistical evaluation: The reported data are means of measurements and their SEM values. For statistical evaluation, Student's *t*-test was used, differences were considered significant if $p<0.05$.

Results

The control fasting blood sugar level, 6.32±0.65 mM (n=5) was lowered to 5.55±0.67 mM (n=6) after 24 hour, but increased to 6.87±0.68 (n=6) mM 48 hours after the L-Arg treatment. Higher alpha-amylase activity values confirmed the diagnosis of pancreatitis [11]. (Control plasma values: 10 090.0±1 481.4 U/L and 12 hours after L-Arg administration: 11 022.0±3 832.0 U/L.)

The plasma LP (Table I) was unchanged 24 and 48 hours after L-Arg administration compared with the control. Lipid peroxidation of the hemolysates increased following treatment, but not significantly.

The GSH was significantly increased after 24 hours in hemolysate and after 48 hours in plasma, following treatment. GSSG decreased at both measurements but not significantly.

The significant changes in GP-ase activity in the hemolysate followed the changes in GSH.

The catalase activity like those of other H_2O_2-decomposing enzymes increased in the first 24 hours following treatment, and then they decreased.

Superoxide dismutase activity was increased 24 hours after L-Arg treatment and decreased at 48 hours, but not significantly.

Table II lists the antioxidant system data on the treated and control liver tissue samples. The liver samples similarly to the kidney and pancreas tissue samples, were randomly selected. The enzymatic antioxidant system measurements were complemented here and into the other organs with total-SOD (t-SOD) measurements, from which, by subtraction of the Mn-SOD activities measured in the presence of CN-, the Cu, Zn-SOD activities were obtained.

L-Arg treatment induced a significant decrease of lipid peroxidation in liver tissues. The GSH level first decreased and then increased, whereas, the level of GSSG was significantly increased at both times after treatment. The GP-ase activity followed the GSH level, changes were significant 24 hours after treatment. The C-ase activity was elevated at both measurement times. These changes were not significant, but activities of SODs were significantly lower at both times of measurement.

Table I

Changes in plasma lipid peroxidation (LP), red blood cell (RBC) hemolysate LP, reduced- (GSH) and oxidized glutathione (GSSG), antioxidant enzymes, namely superoxide dismutase (SOD), glutathione peroxidase (GP-ase) and catalase (C-ase) activities in random samples of rat blood 24 and 48 hours after treatment with 250 mg/100 g b.w. L-arginine (L-Arg). (Figures are mean values ± SEM. Probability levels of $p<0.05$ or lower were regarded significant.)

Hours after treatment		Plasma		Hemolysate					
		LP	GSH	LP	GSH	GSSG	Activity of antioxidant enzymes		
	Groups	nmol MDA/ mg prot.	nmol/ mg prot.	nmol MDA/ mg prot.	nmol/ mg prot.	nmol/ mg prot.	SOD	GP-ase	C-ase
							U/mg prot.	U/mg prot.$\times10^{-2}$	BU/mg prot.$\times10^{-2}$
		0.37	1.72	0.81	18.34	1.37	10.71	3.23	0.80
	C	±0.04	±0.04	±0.22	±3.4	±1.13	±3.71	±0.52	±0.22
		(n=7)	(n=3)	(n=6)	(n=5)	(n=4)	(n=5)	(n=5)	(n=8)
24		0.37	1.80	1.07	34.2	1.27	14.24	4.66	0.95
	t	±0.05	±0.07	±0.32	±5.4	±0.51	±3.05	±0.48	±0.56
		(n=13)	(n=3)	(n=14)	(n=6)	(n=4)	(n=5)	(n=8)	(n=17)
	sign.	n.s.	n.s.	n.s.	p<0.01	n.s.	n.s.	p<0.01	n.s.
		0.43	1.23	0.88	26.3	1.48	16.81	3.97	0.91
	C	±0.12	±0.02	±0.12	±4.1	±1.10	±1.31	±0.74	±0.11
		(n=8)	(n=3)	(n=7)	(n=5)	(n=4)	(n=4)	(n=6)	(n=14)
48		0.41	1.36	0.94	23.8	0.97	10.41	2.81	0.78
	t	±0.12	±0.05	±0.29	±13.7	±0.51	±3.15	±0.59	±0.18
		(n=13)	(n=4)	(n=16)	(n=8)	(n=5)	(n=10)	(n=8)	(n=14)
	sign.	n.s.	p<0.02	n.s.	n.s.	n.s.	n.s.	p<0.02	n.s.

C = control; t = treated; sign. = significance of differences (between controls and all acute L-Arg pancreatitis data); n.s. = non significant

Table II

Antioxidant system (AOS) and SODs activity data on liver homogenates of rat treated with a high amount of L-Arg. (see the abbreviations in Table I or in the text)

Hours after treatment	Groups	LP	GSH	GSSG	Activity of antioxidant enzymes				
		nmol MDA/ mg prot.	nmol/ mg prot.	nmol/ mg prot.	GP-ase U/mg prot.$\times 10^{-2}$	C-ase BU/mg prot.$\times 10^{-2}$	t-SOD U/mg prot	Mn-SOD U/mg prot.	Cu, Zn-SOD SOD U/mg prot.
24	C	0.30 ±0.06 (n=6)	6.59 ±0.55 (n=6)	0.63 ±0.12 (n=6)	13.23 ±1.20 (n=6)	0.79 ±0.03 (n=6)	53.92 ±8.63 (n=6)	3.17 ±0.77 (n=6)	50.74 ±9.0 (n=6)
	t	0.19 ±0.15 (n=6)	6.3 ±2.0 (n=7)	3.70 ±1.13 (n=4)	6.78 ±3.28 (n=6)	1.01 ±0.56 (n=6)	40.15 ±8.65 (n=9)	3.63 ±0.95 (n=9)	36.51 ±7.92 (n=9)
	sign.	p<0.02	n.s.	p<0.01	p<0.01	n.s.	p<0.02	n.s.	p<0.02
48	C	0.26 ±0.07 (n=6)	4.74 ±1.06 (n=6)	0.61 ±0.06 (n=6)	9.59 ±3.16 (n=6)	0.65 ±0.06 (n=6)	50.44 ±13.31 (n=6)	2.51 ±0.40 (n=6)	47.93 ±13.02 (n=6)
	t	0.18 ±0.01 (n=6)	6.3 ±1.6 (n=7)	1.66 ±0.36 (n=6)	8.07 ±2.34 (n=6)	1.23 ±0.63 (n=6)	31.09 ±13.09 (n=9)	3.66 ±1.13 (n=9)	27.44 ±14.02 (n=9)
	sign.	p<0.05	n.s.	p<0.01	n.s.	n.s.	p<0.05	p<0.05	p<0.02

C = control; t = treated; sign. = significance of differences (between controls and all acute L-Arg pancreatitis data); n.s. = non significant

Table III

Changes in AOS data in whole kidney homogenates (see the abbreviations in Table I)

Hours after treatment	Groups	LP	GSH	GSSG	Activity of antioxidant enzymes				
		nmol MDA/ mg prot.	nmol/ mg prot.	nmol/ mg prot.	GP-ase U/mg prot.$\times 10^{-2}$	C-ase BU/mg prot.$\times 10^{-2}$	t-SOD U/mg prot	Mn-SOD U/mg prot.	Cu, Zn-SOD SOD U/mg prot.
24	C	0.81 ±0.17 (n=6)	6.79 ±1.12 (n=6)	0.43 ±0.07 (n=6)	9.89 ±1.02 (n=6)	1.31 ±0.73 (n=6)	28.91 ±2.40 (n=6)	3.09 ±1.23 (n=6)	25.82 ±2.95 (n=6)
	t	1.05 ±0.37 (n=9)	7.68 ±1.75 (n=10)	0.50 ±0.31 (n=6)	3.99 ±0.69 (n=6)	1.03 ±0.49 (n=9)	25.10 ±5.27 (n=9)	1.80 ±1.08 (n=9)	23.30 ±4.73 (n=9)
	sign.	n.s.	n.s.	n.s.	p<0.01	n.s.	n.s.	n.s.	n.s.
48	C	0.75 ±0.27 (n=6)	6.25 ±0.94 (n=6)	0.40 ±0.04 (n=6)	7.36 ±1.64 (n=6)	1.55 ±1.06 (n=6)	30.21 ±2.26 (n=6)	3.50 ±1.45 (n=6)	26.71 ±3.05 (n=6)
	t	1.08 ±0.41 (n=9)	7.48 ±1.44 (n=10)	0.44 ±0.33 (n=6)	5.96 ±2.81 (n=6)	1.50 ±1.15 (n=9)	21.42 ±3.75 (n=9)	2.43 ±1.37 (n=9)	19.00 ±4.44 (n=9)
	sign.	n.s.	n.s.	n.s.	n.s.	n.s.	p<0.01	n.s.	p<0.01

C = control; t = treated; sign. = significance of differences (between controls and all acute L-Arg pancreatitis data); n.s. = non significant

Table IV

AOS data in pancreas homogenates in pancreatitis induced by administration of a high amount of L-Arg (see the abbreviations in Table I)

Hours after treatment	Groups	LP	GSH	GSSG	Activity of antioxidant enzymes				
		nmol MDA/	nmol/	nmol/	GP-ase	C-ase	t-SOD	Mn-SOD	Cu, Zn-SOD
		mg prot.	mg prot.	mg prot.	U/mg prot.$\times 10^{-2}$	BU/mg prot.$\times 10^{-3}$	U/mg prot	U/mg prot.	SOD U/mg prot.
24	C	0.27 ±0.04 (n=4)	9.76 ±1.7 (n=3)	1.96 ±0.47 (n=3)	1.24 ±0.23 (n=6)	0.65 ±0.03 (n=3)	7.38 ±2.02 (n=6)	1.08 ±0.27 (n=6)	6.30 ±2.24 (n=6)
	t	3.40 ±0.57 (n=3)	15.1 ±3.2 (n=6)	2.88 ±1.95 (n=6)	2.28 ±1.08 (n=3)	0.31 ±0.11 (n=6)	8.29 ±2.17 (n=6)	0.87 ±0.31 (n=6)	7.42 ±1.97 (n=6)
	sign.	p<0.01	n.s.	n.s.	p<0.05	p<0.01	n.s.	n.s.	n.s.
48	C	0.27 ±0.04 (n=4)	9.76 ±1.7 (n=3)	1.96 ±0.47 (n=3)	1.24 ±0.23 (n=6)	0.73 ±0.02 (n=3)	7.84 ±1.73 (n=6)	1.00 ±0.26 (n=6)	6.83 ±1.93 (n=6)
	t	5.00 ±2.75 (n=3)	11.4 ±1.8 (n=6)	2.16 ±1.74 (n=6)	3.15 ±0.75 (n=6)	0.33 ±0.13 (n=6)	5.71 ±1.57 (n=6)	0.36 ±0.28 (n=6)	5.35 ±1.35 (n=6)
	sign.	p<0.01	n.s.	n.s.	p<0.01	p<0.01	n.s.	p<0.01	n.s.

C = control; t = treated; sign. = significance of differences (between controls and all acute L-Arg pancreatitis data); n.s. = non significant

Table III presents data of the antioxidant systems in kidney homogenates.

L-Arg treatment induced an increase in the LP value at both times, similarly as for the increased GSH and GSSG values.

Of the peroxide-decomposing enzymes, GP-ase was significantly decreased after 24 hours, the C-ase activity was lower after 24 hours, but not significantly. The decreases in t-SOD, Cu, Zn-SOD, were significant 48 hours after treatment.

Table IV gives data of the antioxidant systems in the pancreas.

L-Arg administration induced significant increase in LP, similarly with the increased GSH and GSSG values. The GP-ase activity increase became significant at both times, while the C-ase activity decreased. Changes in the activities of t-SOD and Cu, Zn-SOD were decreased non-significantly after 48 hours, whereas the decrease in Mn-SOD at that time was significant.

Discussion

Sanfey et al. [4] proved indirectly that oxygen radicals play a significant role in pancreatitis models induced in different ways. The acinar enzyme secretion (the amylase and lipase in ex vivo perfused dog) was decreased by SOD+C-ase perfusion or allopurinol administration. It has been assumed that xanthine oxidase activation following the release of protein-decomposing enzymes, is responsible for the increase in superoxide radical (O_2^-) production. Other studies [5] in an acute reflux pancreatitis produced by occlusion of the common bile duct in rat have led to similar results.

Our studies concerned the changes in the antioxidant enzymes, and the antioxidant system in red blood cell hemolysates, and in homogenates of two major parenchymal organs (the liver and kidneys) and of the pancreas of rat 24 and 48 hours after L-Arg treatment.

It has been clearly established that nitric oxide and nitrogen oxide synthase are involved in the formation of the inflammatory symptoms of pancreatitis, i.e. inflammatory vasodilatation and the regulation of pancreatic acinar cell secretion [8, 9]. It is also well known that the decomposition of peroxynitrite or peroxynitric acid is the source of the most dangerous oxygen radical, the HO• hydroxyl radical.

$$\underset{\text{peroxynitrite}}{ONOO\bullet} \xrightarrow{H^+} \underset{\text{peroxynitric acid}}{ONOOH} \qquad \text{(a)}$$

$$ONOOH \rightarrow NO_2 + HO\bullet \qquad \text{(b)}$$

Peroxynitrite takes it origin from the reaction of superoxide and nitric oxide radicals [22]. Peroxynitrite and their mentioned decomposition products are very important inflammatory factors too (see reactions a, b).

Serum lipid peroxidation increases in acute pancreatitis and significant changes in antioxidant systems in the blood serum and hemolysate under similar conditions were reported earlier [23, 24].

The changes observed in the kidney in the present work indicate that because of the oxidative predominance ("oxidative stress") the antioxidant systems' have difficulty to compensate. (Lipid peroxidation, GSSG increase and antioxidant enzymes activity decrease.) The liver in our experiments, on the other hand, showed a better compensating capacity against oxidative overload.

The present antioxidant system studies confirms that GSH is the most active antioxidant in the pancreas [25]. The GSH–GSSG system together with the GP-ase activity responds to L-Arg induced oxidative stress with significant changes that can compensate the oxidative stress-induced increase in LP.

Further studies are needed to clarify the mechanism of changes in oxygen and nitric oxide radicals.

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Study of the bone marrow penetration of radioactivity after oral administration of radiolabelled girisopam (EGIS-5810) in mice

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Girisopam (EGIS-5810) is a potent anxiolytic compound [1]. Recent in vitro studies with the substance, in Chinese hamster ovary cells, indicated dose-dependent mutagenic activity [2, 3, 4, 5]. At the same time, in ex vivo bone marrow micronucleus tests performed after treating CFLP mice with extreme oral doses (875, 1300 and 1750 mg/kg) no mutagenic activity could be observed at any of the dose-levels [6]. On the basis of the above results, it seemed reasonable to study the absorption and distribution of radioactivity and particularly its bone marrow penetration after administering tritiated and ^{14}C-labelled girisopam at the same doses as those applied in the micronucleus test. The animals were sacrificed 30 minutes, 2 and 24 hours after treatment and the radioactivity content of blood, plasma and bone marrow was determined. For whole body autoradiography studies, the animals were sacrificed at the same time points, however they were treated with tritium-labelled girisopam. The results indicated that the absorption of radioactivity from the gastro-intestinal tract of the animals started immediately. The samples collected had well measurable radioactivity even 30 minutes after treatment. At the same time, it was also evident, that, in spite of the high doses, the absolute amount of radioactivity was rather low. At both dose-levels, the radioactivity concentration was the highest in samples collected 24 hours after treatment. This results indicated extremely delayed absorption. The radioactivity level of bone marrow was practically the same as that measured in blood. The samples of animals treated with the high-dose had higher radioactivity content, however the increase was not linearly proportional to the dose. Disproportionality can probably be explained by delayed absorption. The whole body autoradiography was in good agreement with the results of quantitative determinations.

This results confirmed the observations obtained by ex vivo micronucleus test. The radioactivity penetrated in the bone marrow resulting a long time exposure of the radioactivity without any mutagenic effect.

Keywords: girisopam, mutagenic activity, absorption, bone marrow, radioactivity

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Girisopam (EGIS-5810), chemical name: 1-(3-chlorophenyl)-7,8-dimethoxy-4-methyl-5*H*-2,3-benzodiazepine) is a potent anxiolytic compound [1].

Recent *in vitro* studies indicated that the compound and/or its metabolites exerted mutagenic activity. Chromosome aberration appeared in Chinese hamster ovary cells [2, 3, 4, 5] and the clastogenic effect showed dose-dependency. At the same time, examination of *ex vivo* bone marrow cells (micronucleus test) did not indicate mutagenic activity [6]. Considering these findings, it seemed necessary to investigate the presence of radioactivity in the bone marrow after administration of labelled girisopam at the same oral doses as those administered in the ex vivo micronucleus test. In the present study, male and female CFLP mice were treated with 875 and 1750 mg/kg oral doses of ^{14}C-labelled girisopam and blood, plasma and bone marrow were sampled at different time points to determine their radioactivity content.

In addition, the distribution of radioactivity in the organism was also investigated by the means of whole body autoradiography after the oral administration of tritiated girisopam. The recommendations of OECD Guidelines for Testing of Chemicals (No. 417) and the principles of Good Laboratory Practice issued by the National Institute of Pharmacy were observed all over the study [7, 8].

Materials and Methods

Experimental animals

For the purposes of the present study CFLP mice were selected since the above *ex vivo* micronucleus test [5] was carried out in this species. Both female and male animals were used. The animals were kept in an animal room having artificial light cycles and controlled air-change, temperature and humidity. The animals were offered standard pelleted feed and drinking water *ad libitum*, but they were fasted for 18 hours before the treatments.

Test substances

^{14}C-labelled girisopam (EGIS-5810): Manufacturer: Institute for Drug Research Ltd., IV Synthetic Laboratory, specific activity: 425.5 MBq/g, radiochemical purity: 98% checked by TLC. Total impurity detected by HPLC: 1.6%.

^{3}H-labelled girisopam (EGIS-5810): Manufacturer: "B"-level Isotope Laboratory of Biological Research Center of Hungarian Academy of Sciences, Szeged. Molar activity: 847.3 GBq/mmol, concentration of the radioactivity: 37 MBq/cm^3, volume:

2 cm^3 (in ethanol for spectroscopy), total radioactivity: 74 MBq. Radiochemical purity: 95%, checked by TLC.

Non-labelled test substance: girisopam: (EGIS-5810), chemical name: 1-(3-chlorophenyl)-7,8-dimethoxy-4-methyl-5*H*-2,3-benzodiazepine, molecular mass: 328.8, manufacturer: EGIS Pharmaceuticals Ltd. Chemical purity: 99.35% (on the basis of HPLC analysis) storage: at room temperature.

Other materials

Mucus of methylcellulose 1% in distilled water (Institute for Drug Research Formulation Department), tetraethylammonium hydroxide (TEH) 20% aqueous solution as tissue solubilizer (Merck, Darmstadt, Germany), hydrogenperoxide, 30% solution as bleaching agent (Reanal, Budapest, Hungary), carboxymethylcellulose sodium salt, purum (Fluka, Buchs, Switzerland) n-hexane, pure (Reanal, Budapest, Hungary), ethanol abs. p.a. (Reanal, Budapest, Hungary), PICO-FLUOR 40 liquid scintillation solution (Packard, Groningen, The Netherlands), Hyperfilm ^3H, (Amersham, Aylesbury, England), Heparin, 25000 IU/5 ml aqueous solution (Richter Gedeon Chemical Works, Budapest, Hungary).

Preparation of dosing formulation of ^{14}C-labelled girisopam

Labelled and non-labelled girisopam were weighed separately and mixed in a ratio of 1:5. The mixture was dissolved in ethanol and the solution was evaporated to dryness. Radiochemical purity of 98% was found by TLC analysis and the specific activity determined by LSC counting was 77.96 MBq/g.

Preparation of dosing formulation of ^3H-labelled girisopam

Tritiated girisopam was obtained as an ethanolic solution (2 cm^3). The amount of nonlabelled girisopam required for the treatment was also dissolved in ethanol. The two solutions were mixed then evaporated to dryness. Radiochemical purity of 98% was found and the specific activity amounted to 264.78 Mbq/g.

Dosage

The radioactivity levels of blood, plasma and bone marrow samples were examined after administration of 875 and 1750 mg/kg doses of ^{14}C-labelled girisopam.

The distribution of radioactivity in the animals' organism after the treatments was examined by whole body autoradiography at three time points. The animals were treated with ^{3}H-labelled girisopam, the administered dose was 1200 mg/kg.

The doses selected and the route of administration were the same as those applied in the toxicology studies and in the bone marrow micronucleus test. The doses were administered in the form of methylcellulose suspension by gavage.

Sampling and time points of sample collection

Thirty minutes, 2 and 24 hours after the treatments. The animals were exsanguinated by decapitation. Blood samples were collected into centrifuge tubes containing 250 IU of heparin. Fifty microliter aliquots of whole blood were measured into Packard liquid scintillation vials. The remaining blood samples were centrifuged and 100 µl plasma samples were measured into Packard liquid scintillation vials. Bone marrow samples were obtained from the femur, by blowing out the bone marrow into Packard liquid scintillation vials of known tare. The samples were weighed with 0.1 mg accuracy. The whole sample was subjected to radioactivity counting.

For the whole body autoradiography study the animals were over anaesthetized at the predetermined time points after treatment, then deep-frozen in hexane saturated with dry ice (–70 °C). The frozen bodies were embedded into aqueous carboxymethylcellulose gel. The blocks were frozen in hexane saturated with dry ice (–70 °C) and stored deep-frozen in a refrigerator (–20 °C) until processing. By the means of a PMW Cryomicrotome MP 450 (LKB, Stockholm, Sweden) frozen sections were made up. Autoradiograms of the sections were prepared using Hyperfilm-^{3}H film.

Sample preparation

The samples were solubilized and decolourized, then they were supplemented with 10 ml of PICO-FLUOR 40 liquid scintillation solution. (Representative background samples were obtained from untreated animals.)

Measurement of radioactivity

The radioactivity content of the samples was measured by liquid scintillation counting in a PACKARD TRI-CARB 2000 CA (Downers Grove, Illinois, U.S.A.) instrument. The radioactivity of each sample, including background samples was counted by an external ratio method using automatic quench and chemiluminescence correction. Net dpm (desintegration pro minute) values for the samples were obtained by subtracting the representative background activity from the activity measured in the

sample. Twofold value of background radioactivity was regarded as detection limit. The values of the radioactivity is expressed in μg-equivalent unit obtained by using the specific activity values (dpm/μg).

Results

Radioactivity concentration of whole blood, plasma and bone marrow samples is expressed as μg-equivalent/ml, or μg-equivalent/g units. The results are tabulated in Tables I and II (demonstrating the individual values, their means and the standard errors).

Table I

Radioactivity levels (means±s.e.m.) following administration of a single 875 mg/kg oral dose of ^{14}C-labelled girisopam (EGIS-5810)

Time (hour)	Blood (μg-equiv/ml)	Plasma (μg-equiv/ml)	Bone marrow (μg-equiv/g)
0.5	13.56 ± 1.55	22.23 ± 2.76	13.26 ± 2.04
2	23.55 ± 1.64	38.18 ± 2.45	20.71 ± 1.86
24	40.32 ± 8.60	53.73 ± 11.91	25.61 ± 6.31

Table II

Radioactivity levels (means±s.e.m.) following administration of a single 1750 mg/kg oral dose of ^{14}C-labelled girisopam (EGIS-5810)

Time (hour)	Blood (μg-equiv/ml)	Plasma (μg-equiv/ml)	Bone marrow (μg-equiv/g)
0.5	20.33 ± 1.43	35.17 ± 1.79	17.10 ± 1.00
2	29.91 ± 4.72	51.62 ± 8.36	30.21 ± 4.41
24	68.90 ± 6.40	107.84 ± 9.54	76.05 ± 8.41

The mean values and standard errors for each dose-group are also shown in bar diagrams (Figs 1 and 2).

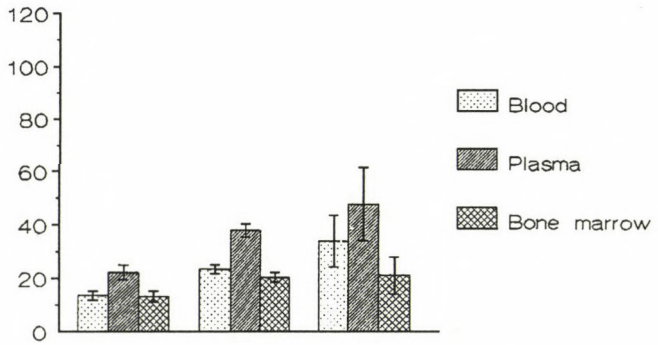

Fig. 1. Mean radioactivity levels (±S.E.M.) in blood, plasma and bone marrow after the oral administration of ^{14}C-labelled girisopam (EGIS-5810) to mice (dose: 875 mg/kg)

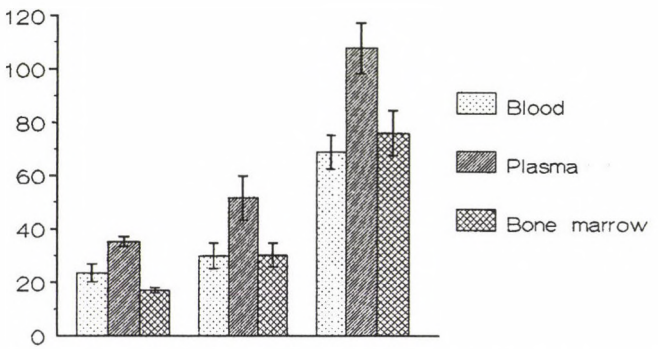

Fig. 2. Mean radioactivity levels (±S.E.M.) in blood, plasma and bone marrow after the oral administration of ^{14}C-labelled girisopam (EGIS-5810) to mice (dose:1750 mg/kg)

The relative density of the whole body autoradiograms in various organs and tissues was scored. The results are presented in Table III and Fig. 3.

Table III

Relative density of whole body autoradiograms after the oral administration of ^{3}H-labelled girisopam (EGIS-5810) (dose: 1170 mg/kg)

Animal code	1	2	3	4	5	6
time	male 30 min.	female 30 min.	male 2 hours	female 2 hours	male 1 day	female 1 day
Brain	2	2	2	2	2	2
Brown fat	2	2	2	2	2	2
Thymus	1	1	1	1	1	1
Bone	0	0	0	0	0	0
Bone marrow	1	1	1	1	2	2
Spinal cord	2	2	2	2	2	2
Stomach	5	5	5	5	5	5
Pancreas	–	–	–	–	–	–
Testes	1	–	1	–	1	–
Muscle	1	1	1	1	1	1
Cerebellum	1	1	1	1	1	1
Lacrimal gland	1	1	2	2	2	2
Spleen	1	1	1	1	2	2
Liver	3	3	3	3	3	3
Adrenals	1	1	1	1	1	2
Salivary gland	1	2	1	2	2	2
Oesophagus	4	3	1	1	1	0
Nose	1	1	2	2	2	2
Eyes	0	0	0	0	0	0
Heart	2	2	2	2	2	2
Lungs	1	1	2	2	2	2
Large intestines	0	0	0	0	5	5
Small intestines	0	1	5	5	5	5
Blood	1	1	1	1	1	1
Renal cortex	3	3	3	3	3	3
Renal pelvis	4	1	2	1	3	2
Fat	1	1	1	1	1	1

5: very strong, 4: strong, 3: moderate, 2: weak, 1: wery weak, 0: background, –: not examined

Discussion

As the figures and tables demonstrate, the absorption of radioactivity started very soon after the treatment, in the thirty minutes the blood levels of radioactivity were well detectable in the case of each animal. The radioactivity level of the bone marrow was practically the same as that of blood after the administration of 875 mg/kg dose. Thirty

minutes after treatment with the high-dose (1750 mg/kg), the radioactivity level of bone marrow was somewhat lower than that measured in blood. The radioactivity levels detected after the administration of the high-dose were significantly higher than those measured after treatment with the low-dose, nevertheless the increase was not linearly proportional to the dose most probably due to the delayed absorption of the high-dose. A similar picture could be seen 2 hours after treatment. The radioactivity of samples increased as the absorption progressed. Linear dose-proportionality could not be found at this sampling time either. The blood and bone marrow levels of radioactivity were practically the same at both dose-levels.

Maximum radioactivity levels of samples were measured 24 hours after treatment. At this time the blood levels of radioactivity measured in the two dose-group were nearly linear proportional to the dose. The radioactivity level of bone marrow also increased, indicating that the bone marrow penetration of radioactivity progressed parallel with the absorption. The high level of radioactivity measured in plasma samples from both dose-groups and at all sampling times indicated that the binding of radioactivity to the cellular fraction of blood is low.

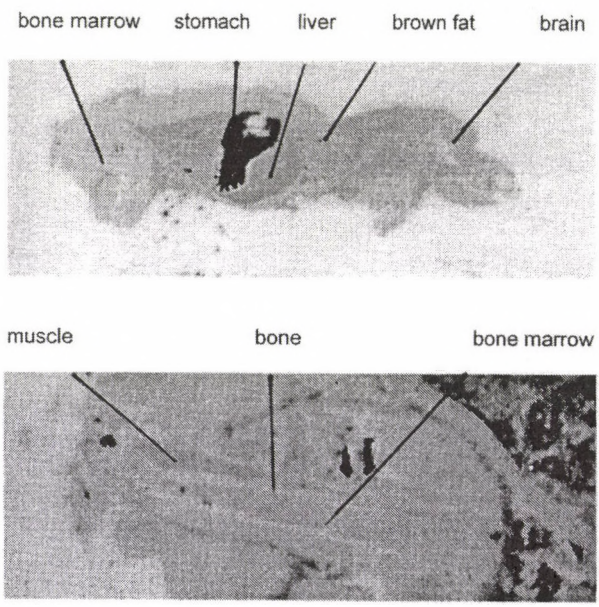

Fig. 3. Whole body autoradiogram of a male mouse in the 2nd hour following oral treatment with 1200 mg/kg dose of ^{3}H-labelled girisopam (EGIS-5810) (upper panel shows the whole autoradiogram and lower panel the region of the femur)

The results obtained by the whole body autoradiography examinations were in good agreement with those of quantitative determinations (Table III). The score of the autoradiograms clearly demonstrate the increase of radioactivity in various organs and tissues 30 minutes, 2 and 24 hours after treatment. The highest relative tissue density was observed 24 hours after dosing. Due to the oral administration, the density of gastrointestinal tract was extremely high at each sampling time. It could also be observed that the absorption of radioactivity was incomplete even at the last sampling time. As compared to that of the gastro-intestinal tract, the relative density of other tissues and organs was weak, or moderate. Moderate density was found at each sampling time in the liver and kidneys and lower than moderate was observed at the 30th minute and 2nd hour in the myocardium, salivary gland and brown fat. The density of the latter tissue increased by the 24th hour and could be regarded as moderate. In the renal pelvis of a female animal sectioned 30 minutes after treatment the relative density indicated the start of urinary excretion of radioactivity. The skin and bone had practically background radioactivity. The relative density of bone marrow was weak and it was approximately the same as that found in blood (vessels and the ventriculi of the heart). The relative density of brain tissue suggested that the radioactivity traversed the blood-brain barrier. Twenty-four hours after treatment, the radioactivity content of the gastro-intestinal tract was still extremely high probably due to the poor water solubility of the test substance and to the extreme doses and the consequent delayed absorption.

The small group-size (3–3 animals per sex) did not allow the statistical evaluation of sex differences. Nevertheless, the evaluation of the entire data set did not indicate any tendency for sex differences.

The results above detailed confirmed the observations obtained by *ex vivo* mutagenic (micronucleus test) study which did not indicate any mutagenic effect, although the bone marrow was exposed to the radioactivity representing parent compound and/or metabolites.

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Human liver cytochrome P-450 and conjugating enzymes. Hungarian data

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Cytochrome P-450 and the conjugating enzymes of the liver are the most important enzymes in the detoxification of xenobiotics. In order to get direct data in the Hungarian human population the activity of liver cytochrome P-450 enzymes (CYP1A2, 2A6, 2C19, 2D6, E1, 3A4) and of the conjugating enzymes has been determined in 11 Hungarian tissue donors died in accidents or in brain vascular catastrophe, by using specific enzymic reactions. In the activity of cytochrome P-450 enzymes a wide range has been found, a ten-fold difference being rather common. In the activities of conjugating enzymes the differences seem to be less.

Introduction

Cytochrome P-450 and conjugating enzymes are believed to be the most important enzymes responsible for the detoxification of xenobiotics. The relative activities of these enzymes, their variations in the individuals, the characteristics and amount of xenobiotics entering the organism create an equilibrium between the toxic effect of the drug (and its metabolites) and the detoxification process. On the other hand more and more data suggest that the above-mentioned equilibrium plays an important role in the formation of malignant, allergic and other long-term toxic processes. The results of the action of the drug metabolizing enzymes is not always detoxification. This transformation of xenobiotics is the result of the interaction between the enzymes and the xenobiotics, it is determined by the structure of the xenobiotics and in many cases the result is activation, i.e. toxic metabolite, and not

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inactivation. When activation prevails or detoxification becomes exhausted toxic processes are generated. Many of the enzymes responsible for activation and inactivation of xenobiotics show marked individual variation in their levels of expression. Much of this variation is due to the existence of genetic polymorphism in cytochrome P-450 and conjugating enzymes. Data are being accumulated that polymorphism in these enzymes may be associated with cancer susceptibility or resistance. Known polymorphism exists for CYP2D6, CYP2C19, N-acetyl-transferases, glutathion-S-transferase α, and in the inducibility for CYP1A. Moreover, CYP3A corresponds to about half of total liver cytochrome P-450 enzymes and is responsible for the elimination of most drugs. The individual variation of CYP3A enzyme in human results in differences in the elimination rate of many drugs. The inducibility of CYP1A, 2B, 2E1, 3A, glucuronyl- and glutathion transferases makes the question more complicated. Thus it is important for people dealing with drug development and toxicology to know the fate of a new drug in humans as early as possible. The use of human liver preparations makes possible to study: a) the in vitro metabolism of a drug under development, b) the role of the individual cytochrome P-450 enzymes in the transformation of the drug, c) the interaction of drugs, d) inductive potency of drugs, e) the importance of enzymes showing genetic polymorphism in the metabolism of the drug. For more details we refer to [13, 2, 6, 7].

Materials and Methods

Human livers were obtained from tissue donors died in accident or in brain vascular catastrophe, with the permission of the relevant Ethical Committee. The applied medication was recorded in every case. The livers were placed into ice cold physiological solution or (when not processed immediately) into fluid nitrogen. Microsomes and cytosol were prepared according to Hoeven and Coon [10]. The protein content, the amount of total cytochrome P-450 and the enzyme activities were determined according to internationally used methods, as follows: Protein content [12], total cytochrome P-450 [14], ethoxycoumarin O-dealkylase (ECOD, mixed: CYP1A and CYP2B) [1], phenacetin O-deethylase (CYP1A2) [5], coumarin 7-hydroxylase (CYP2A6) [16], (S)-mephenytoin 4'-hydroxylase (CYP2C19) [17], dextrometorphan O-demethylase (C4P2D2) [11], chlorzoxazone 6-hydroxylase (C4P2E1) [15], nifedipine oxydase (CYP3A4) [8], UDP-glucuronyltransferase (UDPGT) by using bilirubin [3] or 4-nitrophenol [4] as substrates, glutathion S-transferase (GST) by using chlorodinitrobenzene as the substrate [9].

Results and Discussion

The activity of cytochrome P-450 and conjugating enzymes have been determined in the liver of 11 donors (Table I). As it can be seen from the table that the range of the activity of the enzymes is rather wide, a tenfold difference is rather common, and in the case of CYP2A6 the activity ranged from an undetectable to a rather high value (2053 pmol/mg protein/min). If we take into consideration that the in vivo elimination of most of the drugs shows significant interindividual differences, it is not surprising that great differences can be detected in the activities of liver enzymes responsible for the elimination of drugs. It is interesting and seems to be worth noting that the differences in the activities of conjugating enzymes seem to be much less.

Table I

Activity of human liver cytochrome P-450, UDP-GT and GST enzymes (n= 11)

Enzymes	Mean±SD	Range
Total cytochrome P-450 (pmol/mg protein)	170 ± 79	83 – 365
ECOD (pmol/mg protein/min)	237 ± 175	28 – 528
CYP1A2 (pmol/mg protein/min)	265 ± 160	50 – 606
CYP2A6 (pmol/mg protein/min)	421 ± 576	0 – 2053
CYP2C19 (pmol/mg protein/min)	36 ± 26	6.0 – 91
CYP2D6 (pmol/mg protein/min)	327 ± 140	165 – 513
CYP2E1 (pmol/mg protein/min)	1022 ± 547	223 – 1799
CYP3A4 (pmol/mg protein/min)	417 ± 252	93 – 1048
UDP-GT substrate: bilirubin (pmol/mg protein/min)	827 ± 413	453 – 1727
UDP-GT substrate: 4-nitrophenol (nmol/mg protein/min)	21 ± 6.2	9.2 – 32
GST (nmol/mg protein/min)	713 ± 220	373 – 1096

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Short-term monitoring of the vascular resistance of the human skin microvasculature

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Objective and method: Laser Doppler flowmetry is a non-invasive tool in assessing the temporary changes of skin microcirculation. Another non-invasive equipment the Finapres 2300 finger blood pressure monitor provides a continuous blood pressure signal. The combination of the two devices allows short-term monitoring of the changes in the resistance of skin microvasculature. In order to assess the role of skin blood vessels in physiological responses to complex reflex tests Valsalva manoeuvre was performed by 12 healthy volunteers. For comparison a thermal stimulation (cold pressor) test was also done.

Results: The two tests resulted in skin blood flow responses of similar magnitude. The changes in calculated regional peripheral resistance (dRPR) indicated that both responses involved active vasoconstrictor mechanisms. It is of importance that the active vasoconstriction could be documented only at the late strain phase (V2) but not in the early strain phase (V1) of the Valsalva manoeuvre (%dRPR in V1=0.14 vs. V2=0.96, $p<0.05$).

Conclusions: In conclusion our findings support the theory that changes in the tone of the skin blood vessels parallel the changes in systemic vasculature in response to complex reflex tests. This is the first report which documents the feasibility of the continuous monitoring of the regional peripheral resistance.

Keywords: laser Doppler flowmetry, Valsalva manoeuvre, cold pressor test, regional vascular resistance

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Laser Doppler flowmetry (LDF) provides a continuous non-invasive quantitative measurement of tissue blood flow with high spatial and temporal resolution of the microcirculation [1, 2, 3, 4]. The laser Doppler method used to measure perfusion in discrete sections revealed several categories of rhythmic variations in the flow of human skin [5, 6, 7]. There are also indications that skin is involved in microcirculatory responses induced by cardiovascular reflexes in normal and pathological conditions [8, 9]. The technique, which primarily monitors flow in the arteriolar-capillary network quantifies the frequency shift of laser light caused by moving blood cells in the vessels. The LDF is highly sensitive to movement, positioning, and myriad other factors that can alter skin perfusion. The LDF technique has limitations and can be modified by several uncontrollable factors. The best application of LDF is therefore to determine the relative changes in microcirculatory blood flow to certain characterized stimuli instead of the determination of absolute blood flow [8, 9]. As skin blood flow can be measured simply, non-invasively, simultaneously and continuously it provides several advantages in autonomic reflex tests. The reduction in skin blood flow in response to the various stimuli can be used as an index of arteriolar tone, which in turn is determined by sympathetic nerve traffic.

The traditional laboratory reflex tests are accompanied by rapid blood pressure fluctuations. Formerly these blood pressure swings could be detected only by intra-arterial monitoring. The invasive technique however has several limitations. Technical, legal and ethical considerations have so far prevented its widespread use. More importantly the intravascular instrumentation itself may significantly alter the autonomic regulation [10]. Most recently however a continuous non-invasive blood pressure monitor, the Finapres has become commercially available. The reliability of this device has been already tested during laboratory conditions with satisfactory results [11]. The combination of the laser Doppler flowmetry and continuous blood pressure monitoring allows continuous determination of the local peripheral vascular resistance. The resistance changes in turn may help in differentiation of active and passive blood flow responses.

The aim of the present study therefore was to assess the feasibility of continuous monitoring of the cutaneous vascular resistance in healthy volunteers. Our further goal was to characterise passive and active local alterations of the cutaneous vascular resistance during autonomic reflex tests.

Methods

Healthy subjects were recruited to participate in this study. The study group consisted of 12 (6 male and 6 female) healthy subjects, who ranged from 18 to 28 years of age.

The subjects were studied in supine rest position, in the afternoon, 3 to 4 hours after meal. For our recordings, the study was not begun until stable baseline of blood pressure and skin blood flow signals for each subject was obtained. Two sets of measurements (Valsalva manoeuvre and cold pressor test) were taken on each subject. Each test was done in duplicate. The measured parameters returned to the baseline within 3 minutes following the tests. Another test was not performed until stability had been regained.

Regional peripheral resistance (RPR) was calculated in the strain (V1 point) and at the late strain phase (V2 point) of the Valsalva manoeuvre (Fig. 1) For characterisation of vasoconstrictor response to cold pressor test the lowest value of skin blood flow was used.

Measurement of skin blood flow

Relative blood cell perfusion was measured with a Periflux PF 3 LD monitor (Perimed, Stockholm, Sweden). This method uses the frequency shift of laser light (2 mW helium-neon laser source of 632.8-nm wavelength) induced by reflection on moving red blood cells to measure red blood cell flux. Skin laser Doppler flow values cannot be expressed in conventional physiological units unless certain specific conditions are fulfilled. In vivo, the readings are therefore expressed in perfusion units related to the Brownian motion in motility standard emulsion provided by the manufacturer. At standard temperature the emulsion produces a motility of 250 perfusion units. This corresponds to a 2.5 V at the analogue perfusion output. The measurement field of the laser Doppler skin probe is restricted to 1 mm^3 of the skin microvasculature [12].

The laser Doppler probe and probe holder were attached to the index finger pad of the left hand. In preliminary studies, we examined skin vasomotor reflexes at different sites including the forehead, forearm and index finger pad. Responses of these sites were fairly uniform, therefore we continued the measurements at the index finger pads, which contain only vasoconstrictor fibers. Skin temperature was found to modify the vasoconstrictor responses substantially. At a skin temperature of less than 28 °C, skin blood flow was very low and vasomotor responses were often unobtainable. At skin temperature of more than 40 °C blood flow was very high, and skin vasomotor reflexes were attenuated. Responses seemed to be optimal at 34 to 36 °C.

Continuous blood pressure monitoring

The blood pressure was measured continuously with Finapres 2300 non-invasive blood pressure monitor developed by Wesseling and coworkers. The measurement is based on the Penaz principle. The blood volume under an inflatable finger cuff is measured with an infrared photoplethysmograph, which is mounted inside the cuff. The blood volume as seen by the plethysmograph is clamped to setpoint value by appropriately adjusting cuff pressure in parallel with intraarterial pressure by means of an electropneumatic servosystem. The volume clamp setpoint is regularly adjusted to keep the pressure difference across the arterial wall, the transmural pressure, at zero. At zero transmural pressure, cuff pressure equals intraarterial pressure, which is then determined indirectly by measuring cuff pressure [13, 14].

Valsalva manoeuvre

Each subject was asked to maintain a column of mercury at 40 to 50 mm for 15 seconds with forced expiration, and then to resume normal expiration. A small air leakage was allowed to prevent closure of the glottis.

The Valsalva manoeuvre is commonly used for assessing autonomic function in humans. The effects of the Valsalva manoeuvre on arterial blood pressure is well documented. [15] Initially, arterial pressure increases steeply for a few seconds and this is attributed mainly to the effects of the imposed pressure on the intrathoracic and intraabdominal arteries. The pressure then falls due to decreased venous return. Toward the end of the strain phase blood pressure recovers as heart rate and vascular resistance increase to compensate for the lower cardiac output. On release of the Valsalva manoeuvre, pressure transiently falls, then increases to above control. The overshoot phase is accompanied by rebound bradycardia. In our experiments initial blood pressure increase set the V 1 point, and the deepest point of the second phase on blood pressure recording set the V2 point (Fig. 1) [15].

Cold pressor test

The subjects were asked to immerse their right hand (the hand contralateral to the laser Doppler velocimeter) into a basin of ice cold water (0 Celsius degree) to the level of the right wrist for 20 seconds. Immersion of part of the body in ice cold water causes an increase in arterial blood pressure and efferent sympathetic activity (Fig. 2)[16].

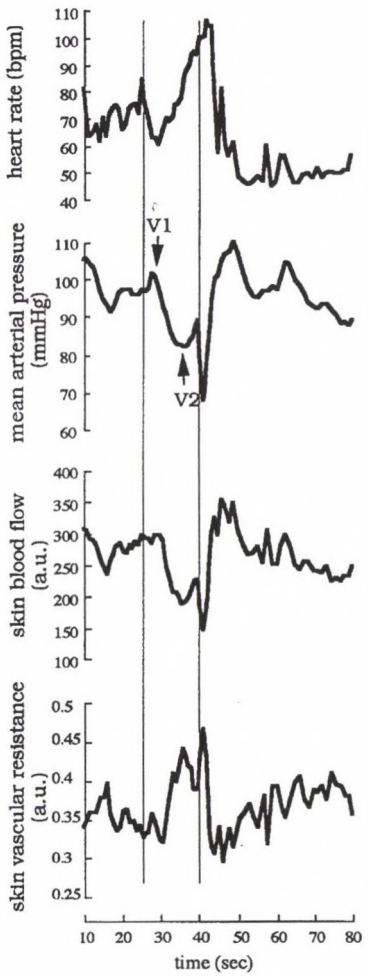

Fig. 1. The heart rate, mean arterial blood pressure, skin blood flow, and skin vascular resistance changes are presented during Valsalva manoeuvre. The lines indicate the onset and the release of the manoeuvre. The initial blood pressure increase (V1) and the deepest point of the strain phase (V2) on the blood pressure trend curve are set

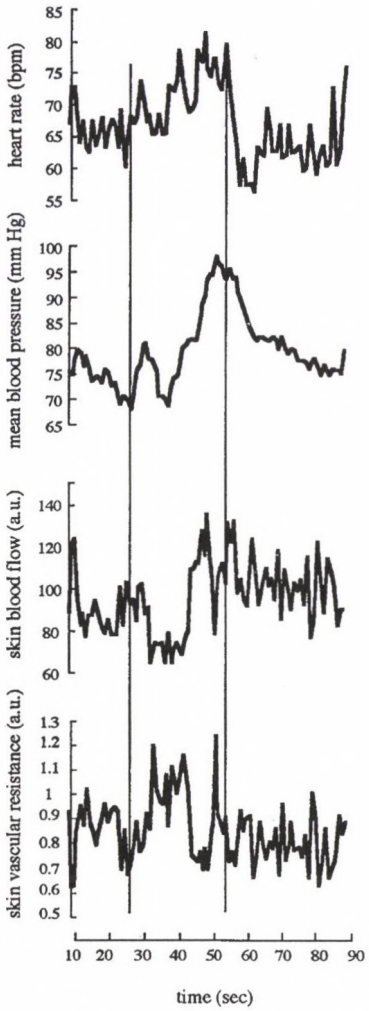

Fig. 2. The heart rate, blood pressure, skin blood flow and regional peripheral resistance during the cold pressor test are presented. The lines indicates the onset and the end of the ice water immersion

Calculation of the regional peripheral resistance

The skin blood flow (SBF) and mean arterial pressure (MAP) were used to calculate the regional peripheral resistance

$$RPR = MAP/SBF$$

The skin blood flow (SBF) and mean arterial pressure (MAP) were also used to express the relative change in regional peripheral resistance (dRPR %) during the examined reflexes according to the following formula:

$$dRPR = (RPRx - RPRbase)/RPRbase$$

where x means any points of the reflex tests.

For characterization the vasomotor responses in our study we used the previously defined V1 and V2 points during the Valsalva manoeuvre. For characterisation of vasoconstrictor response to cold pressor test the maximal change in skin blood flow was used.

Statistical analysis

Normally distributed data were compared by using the one-way repeated measurement ANOVA. To isolate which group is differ from the others we used a multiple comparison procedure (Bonferroni's *t*-test). Non-Gaussian data were analyzed by using the Friedman repeated measurement ANOVA test. Student-Newman-Keuls method was performed as all pairwise multiple comparison method.

Results

Valsalva manoeuvre (Tables I and III)

The typical skin blood flow and blood pressure responses to Valsalva manoeuvre is shown in Fig. 1. Skin blood flow responses followed a pattern of four phases that corresponded to the four blood pressure phases of the Valsalva manoeuvre. Comparison of skin blood flow, mean arterial pressure and calculated regional peripheral resistance at the baseline, V1 point and at V2 point levels are summarized in Table I. The initial phase of the Valsalva manoeuvre resulted in a significant increase in mean arterial pressure and skin blood flow, however the calculated regional peripheral resistance remained unchanged. Toward the end of the strain phase there was a drop in mean arterial pressure below baseline. At this point there was also a marked decrease in skin blood flow. The calculated regional peripheral resistance showed significant elevation.

Table I

The mean arterial pressure, the skin blood flow and the regional peripheral resistance at the three characteristic points of the Valsalva manoeuvre

	Baseline	V1	V2
MAP (mmHg)	77.595 ± 8.3	104.79 ± 9.2*	70.96 ± 9.29*
SBF (PU)	546.55 ± 275.22	655.195 ± 222.62*	329.27 ± 294.42*
RPR	0.18 ± 0.096	0.178 ± 0.062	0.37 ± 0.23*

MAP = mean arterial pressure; SBF = skin blood flow; RPR = regional peripheral resistance * = $p<0.05$ vs baseline

Table II

The mean arterial pressure, the skin blood flow and the regional peripheral resistance responses to cold pressor test

	Baseline	Cold pressor test
MAP (mmHg)	70.33 ± 7.17	96.396 ± 10.48*
SBF (PU)	524.25 ± 252.92	390.709 ± 246.53*
RPR	0.168 ± 0.083	0.36 ± 0.228*

MAP = mean arterial pressure; SBF = skin blood flow; RPR = regional peripheral resistance * = $p<0.05$ vs baseline

Table III

The extent of the mean percentage changes in regional peripheral resistance (dRPR%) at different points of the cardiovascular reflex tests

	dRPR%
V1	0.139
V2	0.956
Cold pressor test	0.962

dRPR% = (RPRx – RPRbase)/RPRbase

Cold pressor test (Tables II and III)

Cold immersion of the contralateral hand resulted in significant increase in mean arterial pressure. The skin blood flow at the same time exhibited a significant reduction. As a consequence the calculated regional peripheral resistance showed a marked – more than double-fold – elevation. In Table III we summarized the extent of the percentage change in regional peripheral resistance at different points in our reflex tests.

Discussion

According to the traditional view changes in the resistance of skin vessels are governed mainly by thermoregulatory reflexes [17, 18]. Thus the relative changes in skin blood flow during complex reflex tests such as Valsalva manoeuvre could be regarded as reflections of the changes in systemic blood flow. Recently however Low et al. have documented that these changes in skin blood flow are results of active local regulating mechanisms [19]. Our goal was to assess the responses of the skin vasculature to thermal and baroreflex changes. Changes were characterized by calculating relative resistance from the continuously recorded blood flow signals. Our findings are in concordance with that of Low et al. The thermal and the baroreflex responses involved comparable temporary fluctuations in skin blood flow. Physiological responses to cold exposure are well documented [16, 17, 18]. It is of importance that the initial strain phase of the Valsalva manoeuvre was parallelled by proportional changes in the skin blood flow and finger blood pressure. Thus the calculated skin resistance remained unchanged. Toward the end of the strain phase however the decrease in blood pressure was accompanied by an inproportionally marked decrease in blood flow, signalling increasing skin resistance. The same tendency has been already documented in the changes in systemic vascular resistance during the Valsalva manoeuvre [20, 21]. Cutaneous blood flow shows a parallel behaviour with blood pressure modification under Valsalva manoeuvre while it clearly decreases when blood pressure rises during cold pressore test. The opposite relationship between cutaneous blood flow and blood pressure behaviour during the two stimuli is probably determined by different reflexogenic modifications triggered by the Valsalva manoeuvre and cold pressor test. The response to the cold exposure certainly involves similar active vasoconstriction. The extent of the active vasoconstriction characterised by the percentage changes of regional peripheral resistance was very similar during the two tests.

Low et al. were the first to suggest a possibility to calculate skin vascular resistance [19]. In their study they utilized traditional blood pressure measurements with mercury sphygmomanometer, which allows only infrequent sampling. Owing to the continuous blood pressure measurements our findings are more accurate then the previous data. Indeed the continuous monitoring of the skin vascular resistance with our system seems to be realistic. The feasibility of this monitoring however is not yet evaluated, and merits further studies.

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The role of nitric oxide in the regulation of coronary flow and mechanical function of isolated, perfused rat hearts

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The role of nitric oxide (NO) in the regulation of coronary flow and mechanical function under basal conditions and when exposed to nifedipine was studied in perfused rat hearts.

Inhibition of basal release of NO by bolus injections of N^G-nitro-L-arginine (L-NNA) (90 mM) and N^G-nitro-L-arginine methyl ester (L-NAME) (185, 370 and 740 mM) induced significant decrease in coronary flow, contractile force and heart rate.

L-NAME in the doses of 185, 370 and 740 mM also decreased significantly contractile force and heart rate during treatment with 170 μM nifedipine. However, the same doses of L-NAME caused an insignificant reduction in coronary flow in the presence of nifedipine.

These findings suggest that NO has an important role in the regulation of coronary flow and mechanical performance under basal conditions and during the hearts exposed to nifedipine. Nifedipine may attenuate the myocardial ischaemia induced by the loss of NO production.

Keywords: nitric oxide, isolated perfused rat heart, N^G-nitro-L-arginine methyl ester (L-NAME), nifedipine, coronary flow

The endothelium plays a major role in regulating vascular tone by releasing relaxing and contracting factors and by modulating vascular responses to various stimuli [9]. It has been known that the vascular endothelium releases a potent vasorelaxant, endothelium derived relaxing factor (EDRF), in response to a number of agonists [8]. Recent studies showed that EDRF is either nitric oxide [22] or a NO containing moiety [21]. NO is formed from the terminal guanidino nitrogen of the L-arginine [23] and involves in the regulation of vascular tone [32], blood pressure [25],

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platelet function [24], neurotransmission [12] and host defence [19]. Endothelial NO biosynthesis from its precursor L-arginine can be inhibited by several L-arginine analogues such as L-NNA and its methyl ester, L-NAME [16, 20, 26].

Basal release of NO has been reported in isolated coronary vessels and in the isolated hearts, suggesting a role for NO in the control of coronary vascular tone [1, 2, 5, 6, 18]. This endogenous secretion is quantitatively sufficient to influence the coronary tone and its inhibition by L-N^G-monomethylarginine (L-NMMA), a specific inhibitor of NO formation, increases coronary perfusion pressure in a dose-dependent manner [2, 18]. A reduced release of NO under basal conditions and in response to pharmacological stimulus has been reported in atherosclerotic arteries. This reduced ability would also predispose the arterial wall to the constrictor effects of substances released as a result of platelet disruption and increases the risk of trombosis [5]. On the other hand, recently experimental and clinical evidence has accumulated suggesting that calcium antagonists may exert antihypertensive and antiatherogenic effects [14].

No data are met about the role of NO in the control of coronary flow and mechanical function during coronary vasodilation induced by nifedipine, an L-type calcium channel antagonist. We preferred nifedipine because it has the most powerful vasodilating properties of the clinically available first-generation Ca^{2+} antagonists [31]. Moreover, it has been reported that nifedipine has an early onset and long-lasting coronary vasodilatory effect [13]. In the present study, we have investigated the role of NO in the regulation of contractile force, heart rate and coronary flow under basal conditions and when exposed to nifedipine in isolated, perfused rat hearts in which endogenous NO production was inhibited by L-NNA or L-NAME.

Methods

Wistar rats of either sex weighing between 200–300 g were used in all the experiments. One hour after the administration of 1000 IU heparin i.p., the chest was opened under light ether anesthesia, and the heart was excised rapidly and then placed into the ice-cold (0–4 °C) modified Krebs-Henseleit solution (mKHs) until contractions ceased. After the heart was cleaned off surrounding fat and other tissues, aorta was immediately tied to a stainless steel perfusion cannula and heart was perfused retrogradely by the nonrecirculating Langendorff technique. The pulmonary artery was incised to facilitate complete coronary drainage in the ventricles. The perfusion solution was mKHs with following ingredients (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. mKHs was continuously oxygenated with 95% O_2 and 5% CO_2; temperature was maintained at 37 °C and pH at 7.4. The hydrostatic perfusion pressure was set at 70 mmHg. Before starting the

experiments a resting tension of 1 g was applied to each heart to yield an optimal contractile force. To measure the contractile force, a thread was attached to the apex of the heart by a metal hook and connected to a force displacement transducer (Nihon Kohden TB 611T). Changes in tension were amplified by an amplifier (Nihon Kohden AP 620G) and recorded on a recorder (Nihon Kohden WI 641G) of a polygraph (Nihon Kohden RM 6000). Tension was calculated by using the following formula taken from the operator's manual of Nihon Kohden TB 611T force displacement transducer

$$A = (A_0 \times B \times C)/(B_0 \times C_0)$$

where A is tension to be measured (g), A_0 is calibration value (input equivalent, g), B is sensitivity control setting position during measurement, B_0 is sensitivity control setting position during calibration, C is amplitude of the recorded contraction curve during measurement (mm) and C_0 is pen amplitude reading during calibration (mm). Heart rate was determined from the polygraph tracings of the contractile force at a paper speed of 2.5 mm/s. Coronary flow was measured by collecting the amount of fluid leaving the heart in one minute and expressed as ml/min/g wet heart weight. The hearts were equilibrated for 30 min to establish a stable baseline.

In the first group of experiments, L-NNA (90 mM) and three doses of L-NAME (185, 370 and 740 mM) were injected in a bolus fashion (100 µl) into the perfusate, 2 cm proximal to the aortic cannula. A recovery period of at least 15 min was allowed between each dose of L-NAME. In the second group of experiments, the responses to L-NAME injections were assessed in the presence of nifedipine. Nifedipine (170 µM) was infused into the side arm of the perfusion cannula at a constant rate of 0.1 ml/min by means of an infusion pump (B. Braun-Melsungen AG). After 2 min infusion of nifedipine L-NAME in the same dosage was injected in a bolus fashion (100 µl) into the perfusate and thereafter nifedipine infusion continued for 5 min.

L-NAME (Sigma) was dissolved in isotonic saline. L-NNA (Sigma) was dissolved in mKHs acidified by bubbling CO_2 for 15–20 min and applied to the hearts after pH of solution rose to 7.4. For preparing nifedipine solution, a 10 mg nifedipine (Nidilat®) capsule containing dissolved substance was cut open with a razor blade and squeezed out completely into the mKHS. The solution of nifedipine was protected from the light. All solutions were prepared daily.

Values obtained before L-NNA or L-NAME administration were taken as controls. Statistical evaluation was performed by two-way analysis of variance. Values are presented as mean ± SE and a P value less than 0.05 was considered to be significant.

Results

Bolus injections of L-NAME in doses of 185, 370 and 740 mM significantly decreased cardiac contractile force, heart rate and coronary flow (Table I). L-NAME resulted in a rapid but transient decline in contractility which peaked at about 10 s from administration (Fig. 1). Thereafter contractile force increased gradually toward the control values within 5 min but remained depressed below the control levels (Fig. 1). Both chronotropic and coronary responses to L-NAME had a time course similar to that of contractile force, although the decrease in heart rate and coronary flow peaked about 1–2 min after the L-NAME injections (Fig. 1). During the 15 min recovery period between different doses of L-NAME contractility (except 370 mM L-NAME), heart rate and coronary flow did not reach the control level, because there was a spontaneous decrease in these parameters in the course of experiment.

The bolus injections of 90 mM L-NNA into the perfusion medium also caused a significant decrease in contractile force, heart rate and coronary flow (Table II). As shown in Fig. 2 the time course of the changes in these parameters induced by L-NNA was similar to that of L-NAME and L-NNA-induced decrease in contractile force was maximal about 30 s after the injections (Fig. 2).

Table I

The baseline values of contractile force, heart rate and coronary flow and maximal decreases of these parameters after administration of L-NAME in perfused rat hearts

	Control	L-NAME (185 mM)	Control	L-NAME (370 mM)	Control	L-NAME (740 mM)
Contractile force(mN)	77.5 ± 7.54 (8)	41.25 ± 4.98* (8)	73.5 ± 8.27 (8)	29 ± 3.38* (8)	75 ± 4.69 (10)	226 ± 3.39* (10)
Heart rate (beat/min)	227.63 ± 13.18 (8)	195.88 ± 14.1* (8)	218.29 ± 6.87 (7)	182 ± 12.77* (7)	197.57 ± 8.73 (7)	156.57 ± 13.92* (7)
Coronary flow (ml/min/g)	12.51 ± 1.61 (9)	7.36 ± 0.96* (9)	10.2 ± 1.39 (9)	6.11 ± 1.02* (9)	8.34 ± 0.98 (10)	3.69 ± 0.53* (10)

Values represent the mean±SE of the number of experiments shown in parentheses. *Significantly different from the corresponding control ($p<0.001$).

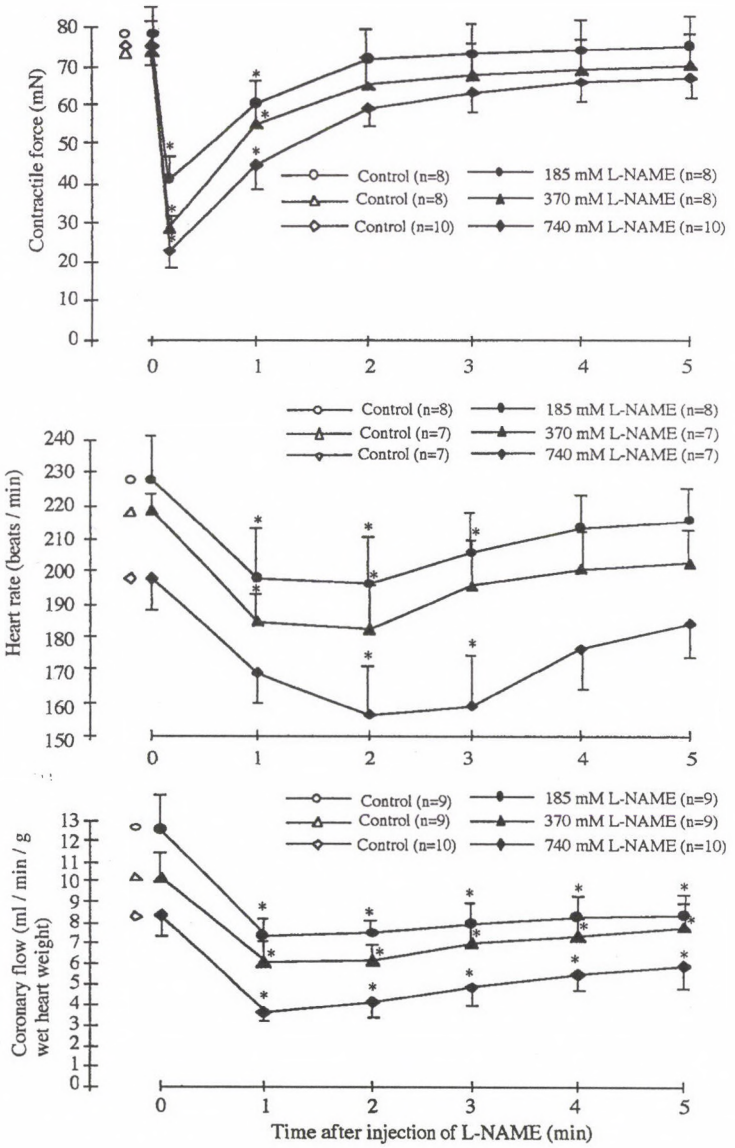

Fig. 1. Time course of the effects of L-NAME on contractile force, heart rate and coronary flow in isolated perfused rat hearts. Time 0 corresponds to the beginning of L-NAME administration. Vertical bars show standard errors. *Significantly different from the corresponding control ($p<0.001$)

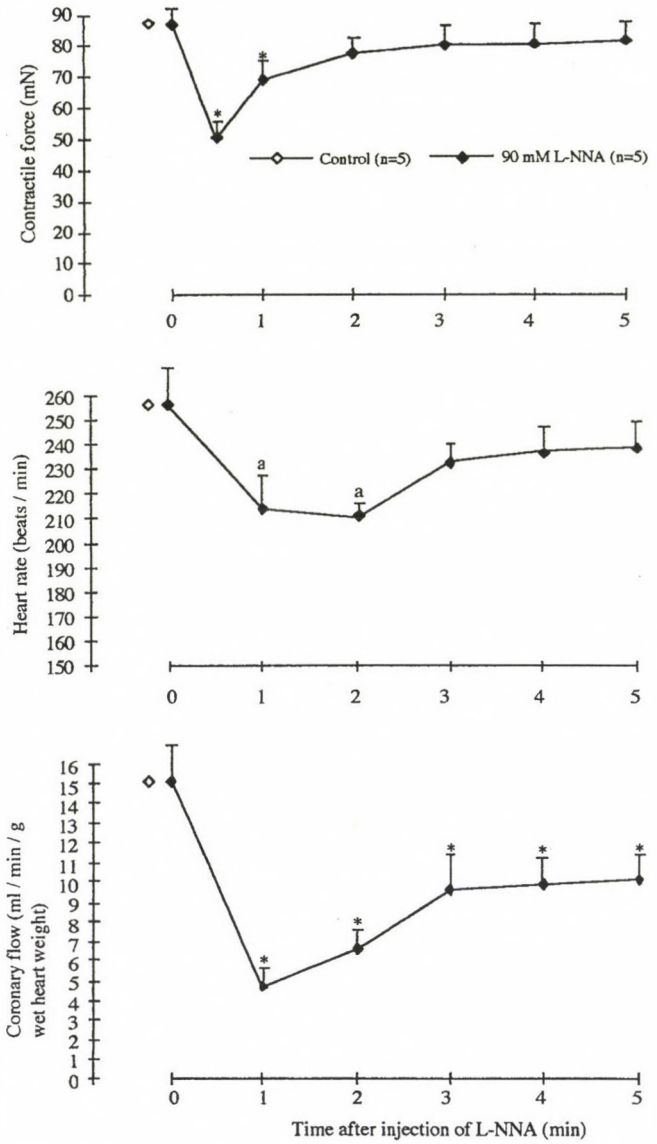

Fig. 2. Time-dependency of the effects of L-NNA on contractile force, heart rate and coronary flow. *Significantly different from the control ($p<0.001$), a. Significantly different from the control ($p<0.01$)

Table II

The baseline values and maximal decreases in contractile force, heart rate and coronary flow in response to L-NNA

	Contractile force (mM)	Heart rate (beat/min)	Coronary flow (ml/min/g)
Control	86.4 ± 4.62	254.6 ± 16.95	15.06 ± 1.78
L-NNA	50.4 ± 4.31*	210 ± 4.73[a]	4.7 ± 0.7*

Each value represents the mean±SE of 5 experiments. *Significantly different from the control ($p<0.001$), a. significantly different from the control ($p<0.01$).

In hearts exposed to 170 mM nifedipine, L-NAME in same doses produced a decrease in contractility, heart rate and coronary flow. Tension and heart rate were significantly lower than control values. However, the reduction in coronary flow was not statistically significant (Table III). As shown in Fig. 3, during nifedipine infusion L-NAME produced a rapid decrease in contractile force within 10 s and this change was similar to that seen in hearts administered L-NAME alone. The initial decline in contractile force and heart rate was followed by a recovery, after which these parameters declined slowly (Fig. 3). In contrast to the initial rapid decline and recovery of contractile tension and heart rate, only small decrease in coronary flow was observed.

Table III

The values of contractile force, heart rate and coronary flow induced by nifedipine and the maximal decreases in these parameters after administration of L-NAME in the presence of nifedipine

	Nifedipine (170 μM)	L-NAME (185 mM)	Nifedipine (170 μM)	L-NAME (370 mM)	Nifedipine (170 μM)	L-NAME (740 mM)
Contractile force (mN)	55.75 ± 7.71	30.75 ± 6.18*	43.25 ± 5.11	17.25 ± 3.93*	34.13 ± 5.32	9.13 ± 2.46*
Heart rate (beat/min)	245.25 ± 18.16	232.63 ± 17.4[b]	219.88 ± 18.69	200.88 ± 18.53*	191.5 ± 20	169.63 ± 18.28*
Coronary flow (ml/min/g)	18.07 ± 1.02	16.06 ± 1.12	13.72 ± 1.43	12.4 ± 1.29	10.05 ± 1.38	9.2 ± 1.17

Each value represents the mean±SE of 8 experiments. *Significantly different from the corresponding nifedipine ($p<0.001$), b. significantly different from the corresponding nifedipine ($p<0.05$).

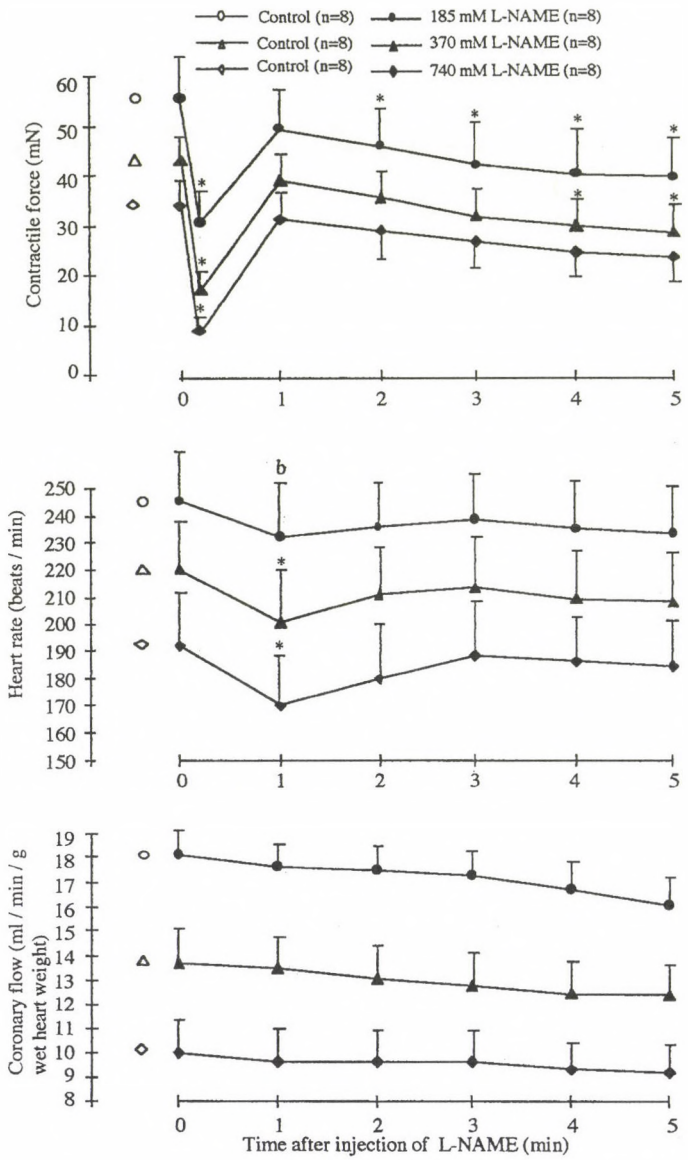

Fig. 3. Time-dependency of the effects of L-NAME in the presence of nifedipine on contractile tension, heart rate and coronary flow. *Significantly differenct from the control ($p<0.001$), b Significantly different from the control ($p<0.05$)

Discussion

In the present study, we have observed that bolus injections of L-NAME or L-NNA induces a significant decrease in myocardial contractility. We also observed that L-NAME significantly reduces contractility in the presence of nifedipine. Nifedipine inhibits transmembrane Ca^{2+} influx in the myocardium, restricts atrial and ventricular contractility and causes vascular smooth muscle relaxation [13]. Similar to our findings a negative inotropic effect has been reported following administration of L-NAME to conscious rat and it has been suggested that this effect may have been due to a direct action of this compound on the myocardium or to a reduction in coronary flow resulting from its potent vasoconstrictor actions [11]. However, since in our study the decrease in myocardial contractility was associated with the decrease in coronary flow, the diminution in cardiac contractility after L-NAME application cannot be attributed to a direct action on the myocardium. It has been demonstrated that the inhibition of basal release of NO by L-NMMA did not produce a significant effect on the contractile activity of isolated cardiac myocytes, thereby excluding a direct toxic or negative inotropic effect. Furthermore, the concentration of L-NAME providing maximal diminution in coronary flow produced a significant decrease in mechanical parameters in perfused rat hearts and no drop in mechanical function was observed in working rat hearts perfused with a combination of L-NMMA and glyceryl trinitrate to avoid diminution in coronary flow [3]. Therefore we suggest that the depressed contractility observed in the present study may possible relate a decline in coronary flow. Further studies on the cardiac actions of inhibitors of NO synthesis are required. On the other hand, NO released from endocardial cells by the Ca^{2+}-dependent NO synthase [27] plays a role in the physiological modulation of myocardial contractility by increasing the level of cyclic GMP in cardiac muscle and thus exerting a negative inotropic effect [4, 7, 29, 30]. Recently a Ca^{2+}-dependent NO synthase activity in the myocardium and freshly isolated cardiac myocytes was observed [28]. Indeed, a continuous basal release of NO by this enzyme in the myocyte may regulate myocardial contractility in an autocrine fashion.

The present results show that the inhibition of NO synthesis in the absence and in the presence of nifedipine produces a reduction in heart rate. Similarly, previous studies in rats have shown that administration of L-NMMA or other inhibitors of NO synthase leads to bradycardia [11, 17, 26, 34]. It has been concluded that an increase in vagal efferent activity was primarily responsible for the bradycardia [34]. In contrast, in other studies heart rate decreases were not observed following administration of inhibitors of NO synthesis [10, 35] suggesting that anaesthesia may block the baroreflex response [10]. It is possible that in our present study the heart rate decreased due to coronary vasoconstriction induced by the inhibitors of NO synthase, as has been reported to

occur after administration of L-NNA to conscious rabbits [15]. It has been known that nifedipine has a coronary vasodilator effect [13]. Our results suggest that this effect may be attenuated by a stronger vasoconstrictor effect of L-NAME, leading a coronary vasoconstriction.

In this study, we found that the inhibitors of NO synthesis both L-NNA and L-NAME significantly decreased the coronary flow in the absence of nifedipine. The results confirm the findings of previous studies which showed that inhibition of NO synthesis produces coronary vasoconstriction in the isolated guinea-pig and rat hearts [3, 18]. Similarly, it has been observed that the inhibition of NO synthase decreases resting coronary blood flow by increasing coronary vascular resistance in vivo [10]. Moreover, L-NAME was found to increase tension in preconstricted human coronary arteries [5]. Our results suggest that the decreased coronary flow induced by the two inhibitors of NO synthesis is related to reduction in basal NO production, and consequently with inhibition of NO-mediated basal vasodilator tone in coronary vasculature. Furthermore, we observed that L-NAME produced an insignificant decrease in coronary flow in the presence of nifedipine indicating that the vasoconstrictor effect of L-NAME normally apposes the vasodilatatory action of nifedipine. It has been reported that nifedipine injected into the coronary artery has a direct vasodilatory action which is related to its calcium antagonistic effect on vascular smooth muscle [13]. We suggest that the decrease in coronary flow after L-NAME injections in nifedipine-treated hearts was probably a consequence of inhibition of NO production. Our findings indicate that nifedipine may protect against vasoconstriction induced by NO synthase inhibition with L-NAME.

In conclusion, the present study demonstrates that inhibition of basal production of NO by L-NAME or L-NNA induces a state of ischaemia due to reduced coronary flow and a concomitant drop in cardiac mechanical function. NO is involved in the regulation of contractile force, heart rate and coronary flow under basal conditions and during the heart is exposed to nifedipine. Our finding that inhibition of NO synthesis by L-NAME or L-NNA results in coronary vasoconstriction in vitro, support the proposal [33] that pathophysiological loss of normal endothelial function may predispose to coronary vasospasm and ischaemia. Nifedipine may attenuate the myocardial ischaemia induced by the loss of NO production.

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Biochemical variations of male scent markers alter the attractiveness in the female rats, *Rattus norvegicus*

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The present investigations were carried out to evaluate whether the variations in the biochemical composition of male scent markers may cause differences in the attractiveness to female rats, *Rattus norvegicus*. The scents originated from the preputial glands, cheek glands, skin, urine, faeces, and ventral body rubbing. The females responded to all these scent sources of male but they spent more time to ventral body rubbing, preputial glands and urine. However, the duration of response was differ from one another, which may be due to the quantitative and qualitative variations of biochemical composition of male scent sources. The females responded to male odours in the following order; ventral body rubbing, preputial gland, urine, cheek gland, skin extract and finally to the faeces as observed in the present investigation. Thus, The present work concluded that the lipids play a major role in the sexual attraction through the sex pheromones released by the male to the females of the same species.

Keywords: preputial gland, cheek gland, urine, faeces, biochemical variation, rat

Communication signals play a conspicuous role in the sociobiology of diverse groups of animals. Mammals deploy many kinds of delightful signalling systems such as visual, accustic, tactile, olfactory and gustatory communications. Among these the olfactory communication has special advantages than the other signalling systems [3, 22]. The vital role of scent sources are: sexual attraction [11], territorial marking [21], individual identification [9], alarm signalling and convey messages related to sexual status and reproductive ability. The major sources of behaviourally attracted odours in mammals are the liberated substances of specialized integumentary glands [1, 14, 27,

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35], urine [6], faeces [10, 16, 29], preputial glands [5, 28, 38], cheek glands and ventral marking [32, 36].

The previous reports provide us with a lucid picture of the behavioural role of various olfactory cues of mammals. The histochemical estimation and behavioural studies were individually carried out in urine and in a few scent markers. Nevertheless, there is no report available on the biochemical composition of scent sources and their elegance in attractiveness of male to female rats. Therefore, an attempt has been made to evaluate the influences of biochemical composition of male scent sources on the attractiveness of the virgin females.

Materials and Methods

The females and males used in the present study belonging to the albino rats, *Rattus norvegicus*. The animals were individually housed in polypropylene cages (40×25×15 cm), with 2.5 cm of rice husk lining the bottom as bedding material. They were fed with pelleted food (Lipton India Ltd) and water *ad libitum*. The females (wt. 145±3.60 g) were 12–16 weeks old regularly cycling virgins. The males (wt. 173± 4.67 g) were 16 weeks old and of proven fertility.

The females were tested for their preferences to male odours of different sources such as, preputial glands, cheek glands, extraction of skin, urine, faeces and ventral side of the adult male conspecifics.

Scents were smeared on the microscope slide (2.5×7.6 cm) and placed on one end of the cage (40×25×15 cm) and the empty slide was placed on the other end of the experimental cage. The experimental animal released at the middle of the cage. The time of experimental rat spent to investigate the scented slide was recorded continuously for each 5-minute test. In the present study the responder was obviously licking and sniffing the smeared and control slides. Each slide was used in one trial and then discarded. Responses to male odours were tested for 30 times (5×6 males). The bedding material was changed during each test.

In this experiment, a mirror and stop watch were used to record the frequency. The slides were visited on both end of the cage. The main purpose of using the mirror was to avoid unnecessary disturbances caused by experimenters. Unless otherwise noted, all scent fluids were collected freshly for the present investigation at each trial. The preputial glands, cheek glands, and skin scents were collected by homogenizing the respective organs (1% level) in water. A microscope slide was rubbed on the ventral side of the scent donor for collecting the scent from the ventral surface. The urine was collected just before use by holding the scent donor over a Petri dish by gently squeezing the flank region. Faecal scent was collected by picking up one or two freshly

dropped faecal boli with clean forceps and rubbing onto a microscope slide with a few drops of water.

After collecting the scent sources such as ventral body rubbing, urine and faeces, males were sacrificed by cervical dislocation and the respective scent sources such as preputial gland, cheek gland, skin were removed surgically. These scent sources have been subjected partially to behavioural study and rest of the major portion has been served for biochemical estimations such as carbohydrates [15] lipids [12] and proteins [25]. However, in this investigation the experimenters were unable to find suitable method to estimate the biochemical nature of the ventral body rubbed scent sources of the male rat. All the data were subjected to statistical analysis: standard deviation, Student's *t*-test and one way ANOVA [41].

Results

Behavioural response

Regularly cycling females showed conspecific response to the male odours rescued from the ventral body surface, skin, urine, faeces, preputial glands, and cheek glands. The sensitiveness of females to male odours was showed in the following order: ventral body rubbing, preputial gland, urine, cheek gland, skin extract and finally to the faeces. The aim of this experiment has been to demonstrate that the females showed affluent attraction towards ventral body rubbing preputial glands, and urine. The females visited more frequently the male odouriferous slides and spent there a long time as compared to the blank slide (Table I).

Biochemical analysis

A drastic variation in the biochemical composition of the scent sources was observed in this experiment (see Table II). The lipid content was higher in the preputial glands (688 mg/g of the sample) and cheek glands and skin extract have moderate level of lipid content, by contrast, urine and faeces have a trace amount of lipids.

There was a conflicting report obtained in the protein estimation. Thus, the protein concentration was higher in skin extract (296 mg/g of the sample) when compared to other scent sources which have been used in the present investigation. Nonetheless, the urine and faeces have the lowest level of proteins.

In general, the amount of total carbohydrates were very meagre in most of the scent sources when compared to lipids and proteins. However, the level of carbohydrates was elevated in faeces (144 mg/g of the sample) and very low in urine.

Table I

Olfactory responses of female rat to male odour of own species

Stimulus	No. of visits per 5 minutes	Duration of response (in sec)
Ventral rubbing	4.33±1.20**	17.82±0.72**
Blank slide	1.70±0.27	7.83±1.30
Preputial gland	3.90±0.50**	15.16±1.04**
Blank slide	1.50±0.50	4.62±1.19
Urine	3.66±0.47**	12.44±2.00**
Blank slide	1.69±0.56	6.00±2.85
Cheek gland	3.37±0.84**	10.42±1.78*
Blank slide	1.70±0.47	4.32±2.90
Skin extract	2.85±0.48**	9.33±1.69**
Blank slide	1.60±0.45	3.83±0.84
Faeces	2.66±0.47	7.24±5.10**
Blank slide	2.00±0.82	5.80±1.08

* Represents significant change from the blank slide at $p<0.05$ level (t-test).

± Represents the standard deviations of five observations.

** Represents the significant changes observed at 0.01% level (One Way ANOVA)

Table II

The biochemical nature of the scent sources of male rat (Rattus norvegicus)

Particulars	Carbohydrates	Proteins	Lipids
Preputial gland	0.102±0.154	0.204±0.0013	0.688±0.01
Cheek gland	0.127±0.0023	0.206±0.004	0.561±0.02
Skin extract	0.038±0.005	0.296±0.007	0.504±0.01
Urine	0.015±0.0001	0.054±0.0001	0.195±0.02
Faeces	0.144±0.003	0.082±0.011	0.098±0.1

± Represents the standard deviations of five observations.

Unit of biochemical composition is g/g of the sample.

Discussion

It is known that mammals are equipped with integumentary glands on the head regions [26] and ventral side of the animal [18, 19, 24]. These glands were more efficient in transferring diverse communication signals during social interaction [40].

In the present work the male ventral body rubbing and preputial glands showed more attractive power to female rats as compared to other scent sources such as, cheek gland, skin, urine, faeces. The reason may be that all the scent sources expelled their adductive substances through dermal layers [1, 21] and the end products of scent markers have biochemically been processed and liberated as a pure attractant into the external environment [4, 26]. It has also been reported in other rodents [11, 32, 36].

The females were less responsive to the skin extract because the skin may have impurities and they were not biochemically processed. The sacrifice of the animal also leads to the reduction in attractiveness by way of suppressing the release of scents. In conclusion, this study indicates that the skin extract does not have an appreciable level of scents because the skin is not a reservoir of scents.

This report coherent with the previous findings suggest that cheek glands act as social communicators [3].

The preputial gland is the site of sex odour production which is dependent on the presence of male sex hormone [13, 23, 30]. It is one of the specialized sebacious glands of myomorphous rodents [5]. In the present study all the scent sources were taken from the male since the mammalian scent marking is predominantly a male behaviour [37].

There are several reports available to indicate that urine acts as a main source of social communicator in rodents [7, 8, 17, 20, 34]. In the present investigation females were more attracted by the male urine than the faecal extract. The excretory substances such as urine and faeces were highly involved in sexual behaviour. Thus, the subcutaneous gland secretions; as perhaps that of some ungulate tarsal, preputial and cheek glands is the potent sources of pheromones. A part of the preputial gland secretion is directed through urine [33]. The faeces may serve as a substrate for scent marking with anal gland secretions, which were deposited more frequently on the faeces [2].

The scent sources originated from adipose tissues and were involved in the sexual attraction [1, 33]. The present investigation proved that the scent glands have vast amount of lipids so they have significant level of attraction to the opposite sex. This was supported by several documentations [31, 39]. Furthermore the finding that sebaceous glands are under the control of the steroid hormonal regulation has also been well proved earlier. The primary source of the scents were found in the ventral body rubbing, preputial glands, urine, and cheek glands as biochemically finalized form of lipoid substances which are expelled to the environment to attract the opposite sex.

Hence, the present investigaton comes to a conslusion that the quantity of the biomolecules especially the lipids may have a remarkable role in the sexual attraction, and the quality of the biological substance execute the notable function in sexual behavior among the sexual partners of rats.

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Further prove on oxidative stress in alloxan diabetic rat tissues

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After intravenous administration of alloxan monohydrate (AL) diabetes developed in rats. Forty-eight hours after the injection the animals were sacrificed, their blood was collected in heparin containing tubes and the tissues were dissected and frozen (–70 °C) until their homogenization for pro- and antioxidant testing.

Our results can be summarised as follows:

(i) In the blood hemolysate the lipid peroxidation slightly elevated and the activity of antioxidant enzymes and reduced glutathione decreased.

(ii) Similar phenomena could be observed in the different examined organ homogenates. The organs tested for pro- and antioxidant system were as follows: the liver, heart, skeletal muscle, kidney and pancreas.

In our present work we attempt to confirm the data in support of the oxidative predominance over antioxidants in oxidative stress of AL diabetic rats.

Keywords: oxidative stress, diabetes, alloxan, antioxidant system

Alloxan (AL) was used a long time ago to induce experimental diabetes in variety of experimental animal species [13, 22]. It is also known that the pancreatic islet β-cells are very vulnerable to the damage caused by oxygen derived free radicals [8], because their protective (antioxidant) systems are rather weak [9]. The diabetogenic action of the AL owing to a high rate of selective uptake and intracellular

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generation of free oxygen radicals were observed mainly superoxide (O_2^-) of islet β-cells [1, 12, 15].

Following i.v. injection of alloxan monohydrate to mice three step of response in plasma glucose has been observed:

(i) Hyperglycemic response lasting for 4 hours.

(ii) The second hypoglycemic action of AL lasts several hours owing to the leakage of insulin and other proteins, hormones from β-cells.

(iii) In the third period, after 24 hours massive diabetes develops [2].

In our earlier work we proved the oxidative predominance in experimental diabetes in rats. In this work we did not select the alloxan and streptozotocin treated rats, the only point was that the rats were glucosuric [17].

In our present work, therefore, we examined the short-term effect of diabetogenic AL on the pro- and antioxidant systems of rat blood and organ homogenates.

We studied the influence of AL treatment on the quantity of reduced and oxidized glutathione (GSH and GSSG), lipid peroxidation (LP) and antioxidant enzymes, namely superoxide dismutases, catalase, glutathione peroxidase and glutathione reductase.

Materials and Methods

All chemicals, enzymes and other reagents were of analytical grade or purest quality purchased from Sigma, Aldrich, Merck and Reanal (Budapest, Hungary).

Experiments were carried out with male Wistar rats [270–310 g body weight (b.w.)] housed individually at room temperature 23 °C, given food and water *ad libitum* and maintained on 12/12-hour light cycle. Diabetes was induced in rats after a 24-hour fasting, with a single i.v. injection of AL-monohydrate 100 mg/kg b.w. dissolved in physiological saline (ph.s.).

Control rats were treated with equal volume of ph.s. solution. Forty-eight hours after the AL injection, when all of the treated animals become hyperglycemic, they were anesthetized with i.p. injections of sodium hexobarbital (50 mg/kg b.w.) and sacrificed.

The mentioned experiments were repeated three times with five, altogether with 15 rats.

Blood glucose levels of normal and diabetic rats are as follows:

(i) Non-diabetic control blood glucose levels (n=10) were 6.32±0.65 mM/L

(ii) and 16.51±6.75 mM/L (n=10).

Blood glucose levels were determined using o-toluidine method [10].

After decapitation blood samples were taken from the heart in heparin-containing tubes. Centrifugation at 3,000 g for 10 min. plasma was separated as supernatants. The packed red blood cells (RBC) were collected and washed three times with ph.s. and separated by centrifugation. It was hemolysed by cold distilled water in a ratio of 1:10.

Heart and skeletal muscles, liver, kidney, lung, spleen and brain were rapidly dissected, washed with ph.s. and frozen at –70 °C until analysis. Samples [about 1 g wet weight (w.w.)] of different tissues were weighed and homogenized with 1:5 ratio cold ph.s. solution. The homogenates and the whole hemolysate were suitable for LP measurement [21].

For further analysis, the homogenates were centrifuged at 15,000 g for 10 min. and the supernatant aliquots were used for the quantitative determination of various parameters.

Enzyme activity measurements

Superoxide dismutase (SOD; EC 1.15.1.1) activities were determined by the epinephrine-adrenochrome method [16, 18].

In the case of hemolysate 0.5 ml packed RBCs were hemolysed with 4.5 ml cold distilled water. After centrifugation, the supernatant aliquots were used further. Before the enzyme activity assay, 0.75 ml chloroform:ethanol (1:2) was added to 1.0 ml hemolysate [19]. After repeated centrifugation, the supernatant aliquots were used for SOD (Cu, Zn-SOD) activity measurements.

The meaning of epinephrine-adrenochrome method is that epinephrine spontaneously transforms to adrenochrome in the presence of air at pH 10.2. During the autocatalytic transformation superoxide anions (O_2^-) are formed. The autoxidation is inhibited by SOD, depending on the activity of SOD in the sample. The unit of SOD is regarded the amount of enzyme which causes a 50% inhibition in extinction changes within 1 min. compared to the control. Samples activity measurement were made at 25 °C and 480 nm.

In general, in the case of tissue homogenates the supernatant aliquots were used for the total SOD activity measurements without any pretreatment.

In the presence of CN^- (5×10^{-3} M KCN) the Mn-SOD (or mitochondrial SOD) could be determined from the same aliquots with the same method [3]. The differences between t-SOD – Mn-SOD=Cu, Zn-SOD.

Glutathione peroxidase (GPx-ase; EC 1.11.1.9) activity was measured by the so-called "chemical" method [20] in the presence of cumene hydroperoxide and reduced glutathione (GSH) as substrates. In this case the GSH was measured before and 10 min. after the addition of the supernatant aliquots to the solution. Ellman's reagent

[5,5'-dithiobis-(2-nitrobenzoic acid DTNB)] was used for the GSH measurement at 412 nm [23]. One unit of GPx-ase converts 1 micromole GSH to GSSG (oxidized glutathione)/min. at 30 °C and pH 7.4.

Catalase (C-ase; EC 1.11.1.6) activity was determined by H_2O_2 extinction decrease at 240 nm. It was measured for 10 min at 30 °C [4]. The activities were given in Bergmeyer units (BU), it means that 1 BU is the amount of C-ase which can decompose 1000 mg H_2O_2 during 1 min.

Glutathione reductase (GR-ase; EC 1.6.4.2) was measured by NADPH extinction decrease at 340 nm, 30 °C and pH 7.4 [20]. One unit of GR-ase was defined as 1 micromole of GSSG reduced/min.

The measurement of **GSH** quantity was carried out spectrophotometrically with Ellman's reagent [23] and **GSSG** by the method of Tietze [25].

Protein content was measured by Folin reagent using plasma albumin as standard [14].

Statistical evaluation: the reported data are means of measurements and their SEM values. For statistical evaluation Student's *t*-test was used.

Differences were considered significant if $p<0.05$.

Results

Table I presents the amounts of total tiobarbituric acid reactive substances (TTBARS or LP values) found in the plasma and in the hemolysate, GSH values of diabetic and control rats. Forty-eight hours after AL treatment the measured plasma values did not change. The measured elevated LP in the hemolysates were typical, but the changes were not significant. As it has been expected the LP values were mildly higher and the SOD, C-ase and GR-ase activities decreased, the GPx-ase activity increased.

Further in the Table, the control and AL treated (diabetic) rat tissue pro- and antioxidant changes were compared in the following organs: spleen, heart and keletal muscle.

Table I

Pro- and antioxidant measurements from the aliquots of plasma, hemolysate, spleen, heart and skeletal muscle homogenates

Organs	Plasma		Hemolysate		Spleen		Heart muscle		Skeletal muscle	
Parameters	Control	AL	Control	AL	Control	AL	Control	AL	Control	AL
GSH	0.17×10^{-2}	0.19×10^{-2}	9.81×10^{-2}	7.38×10^{-2}	11.03	6.01	11.53	10.50	13.77	14.50
nM/mg	±0.06	±0.01	±2.01	±1.03	±0.36	±0.34	±0.30	±0.60	±0.25	±0.40
	(n=4)	(n=3)	(n=5)	(n=8)	(n=4)	(n=5)	(n=5)	(n=5)	(n=5)	(n=5)
GSSG					0.04	0.16	0.11	0.10	0.12	0.08
nM/mg	–	–	–	–	±0.008	±0.12	±0.09	±0.01	±0.063	±0.001
					(n=4)	(n=5)	(n=5)	(n=5)	(n=3)	(n=5)
LP	0.37	0.34	1.08	1.46	1.21	0.76	0.45	0.89	1.06	1.49
mM	±0.04	±0.03	±0.33	±0.16	±0.1	±0.6	±0.01	±0.1	±0.06	±0.26
MDA/mg	(n=16)	(n=4)	(n=18)	(n=7)	(n=5)	(n=5)	(n=5)	(n=5)	(n=5)	(n=5)
t-SOD			37.32	24.18	6.47	5.52	16.83	18.67	11.34	9.77
U/mg	–	–	±2.96	±4.50	±0.51	±0.60	±1.41	±2.71	±1.06	±0.92
			(n=5)	(n=8)	(n=4)	(n=5)	(n=5)	(n=5)	(n=5)	(n=5)
Mn-SOD					0.15	0.23	2.65	2.13	1.73	1.68
U/mg	–	–	–	–	±0.07	±0.03	±0.26	±0.19	±0.50	±0.12
					(n=4)	(n=5)	(n=5)	(n=5)	(n=5)	(n=5)
Cu, Zn-SOD					6.33	5.29	14.18	16.53	9.60	8.10
U/mg	–	–	–	–	±0.46	±0.58	±1.24	±2.62	±0.66	±0.84
					(n=4)	(n=5)	(n=5)	(n=5)	(n=5)	(n=5)
GPx-ase			3.60	8.82	2.63	2.17	5.55	4.71	1.39	0.94
$U/mg\times10^{-2}$	–	–	±0.37	±1.41	±0.01	±0.15	±0.12	±0.28	±0.24	±0.07
			(n=11)	(n=9)	(n=2)	(n=5)	(n=3)	(n=5)	(n=3)	(n=5)
C-ase			218	120	1.20	7.78	1.17	4.55	0.28	0.83
BU/mg	–	–	±11	±12	±0.15	±0.47	±0.05	±0.97	±0.02	±0.16
$\times10^{-4}$			(n=5)	(n=8)	(n=4)	(n=5)	(n=5)	(n=5)	(n=5)	(n=5)
GR-ase	0.18	0.16	1.78	1.20						
$U/mg\times10^{-2}$	±0.02	±0.02	±0.16	±0.26	–	–	–	–	–	–
	(n=4)	(n=6)	(n=4)	(n=8)						

Values are means ±SE.

Table II

Summary of the measured pro- and antioxidant values from the liver, brain kidney, lung and pancreas

Organs	Liver		Brain		Kidney		Lung		Pancreas	
Parameters	Control	AL	Control	AL	Control	AL	Control	AL	Control	AL
GSH	5.78	5.17	24.18	16.14	10.04	11.9	11.17	6.57	9.76	71.8
nM/mg	±0.1	±0.32	±0.82	±2.09	±0.09	±0.6	±0.17	±0.57	±1.7	±23.1
	(n=3)	(n=5)	(n=4)	(n=5)**	(n=3)	(n=5)**	(n=3)	(n=5)**	(n=3)	(n=5)
GSSG	0.43	0.19	3.34	4.79	0.13	0.55	0.09	0.25	0.21	0.0
nM/mg	±2.9	±0.05	±0.56	±1.56	±0.05	±0.13	±0.05	±0.07	±0.11	±0
	(n=3)	(n=5)	(n=4)	(n=5)	(n=3)	(n=5)**	(n=3)	(n=5)*	(n=3)	(n=5)**
LP	0.42	0.25	2.16	2.97	1.28	0.88	0.83	0.67	0.39	0.19
nM MDA/mg	±0.01	±0.02	±0.06	±0.92	±0.01	±0.08	±0.01	±0.05	±0.03	±0.04
	(n=3)	(n=5)**	(n=4)	(n=5)	(n=3)	(n=5)**	(n=3)	(n=5)**	(n=3)	(n=5)**
t-SOD	40.53	77.5	13.29	24.23	36.16	32.96	4.16	13.62	9.37	13.33
U/mg	±1.45	±8.76	±1.19	±3.8	±3.84	±6.35	±0.58	±1.43	±0.57	±2.07
	(n=3)	(n=5)**	(n=3)	(n=5)**	(n=3)	(n=5)	(n=5)	(n=5)**	(n=4)	(n=5)*
Mn-SOD	1.76	1.95	2.64	1.90	1.69	2.93	1.06	0.50	0.95	0.40
U/mg	±0.11	±0.42	±0.37	±0.25	±0.27	±0.53	±0.18	±0.18	±0.06	±0.15
	(n=3)	(n=5)	(n=3)	(n=5)*	(n=3)	(n=5)**	(n=5)	(n=5)**	(n=4)	(n=5)**
Cu, Zn-SOD	38.77	75.55	10.65	22.33	34.47	30.02	3.10	13.12	8.43	12.93
U/mg	±1.36	±8.76	±0.82	±3.88	±3.66	±6.34	±0.41	±1.60	±0.51	±2.81
	(n=3)	(n=5)**	(n=3)	(n=5)**	(n=3)	(n=5)	(n=5)	(n=5)**	(n=4)	(n=5)*
GPx-ase	5.08	4.53	2.57	3.31	1.61	2.03	5.85	5.54	1.59	1.19
$U/mg \times 10^{-2}$	±0.08	±0.44	±0.07	±0.52	±0.06	±0.16	±0.18	±0.25	±0.05	±0.19
	(n=3)	(n=5)	(n=3)	(n=5)	(n=3)	(n=5)*	(n=3)	(n=5)	(n=3)	(n=5)
C-ase	35.0	112/0	0.46	1.01	22.7	53.1	1.98	5.60	3.85	0.36
$BU/mg \times 10^{-4}$	±3.0	±11.0	±0.08	±0.15	±0.80	±3.0	±0.32	±0.26	±0.21	±0.55
	(n=5)	(n=5)**	(n=4)	(n=5)**	(n=4)	(n=5)**	(n=5)	(n=5)**	(n=5)	(n=5)**

(Mean±SE). A value of $p<0.05$* or $p<0.01$** were considered to be significant.

The following comparisons were made between the control and AL-treated animals spleen homogenates:

(i) The LP decreased significantly.

(ii) The amount of oxidized glutathione (GSSG) increased, but the amount of GSH significantly decreased in the organ. GPx-ase activity decreased and the C-ase activity increased markedly.

(iii) t-SOD decrease and the Mn-SOD mildly increased, while the decrease of Cu, Zn-SOD was significant.

In the case of heart and skeletal muscle (sample taken from the right posterior tight muscle) homogenates the following changes were observed:

(i) In the heart and skeletal muscle the LP were increased, the increase in the heart muscle homogenates was significant.

(ii) The GSSG and GSH mildly the GPx-ase significantly decreased.

(iii) SODs mildly elevated in the heart muscle, except for Mn-SOD which was reduced. In the skeletal muscle homogenates SODs decreased after the AL treatment and Cu, Zn-SOD activity significantly decreased.

Table II shows the detected pro- and antioxidant changes in the rat tissues treated with AL. The following changes are summarized in the Table II.

In the liver:

(i) The LP, GSH and GSSG quantities in the diabetics decreased.

(ii) GSH decreased significantly, but GPx-ase, SODs and C-ase activities were increased. The activity enhancement of the antioxidants were significant in the latter two enzymes.

In the brain tissue the following changes were observed after AL treatments:

(i) The LP and GSH quantity increased in diabetic rats.

(ii) GSH decreased significantly but the GPx-ase, SODs and C-ase activities are increased. The activity enhancement of the antioxidants were significant in the latter two enzymes.

In the kidney homogenates the following quantitative changes appeared after diabetes:

(i) The LP values decreased significantly. On the other hand, GSH and GSSG increased significantly.

(ii) From antioxidant enzymes the H_2O_2 decomposing C-ase and GPx-ase activities increased significantly.

The decrease of t- and Cu, Zn-SOD was not significant, Mn-SOD activity, however, elevated significantly in diabetic kidney.

In the lung, the following pro- and antioxidant changes occurred after AL treatment:

(i) LP, GSSG increase was significant in the diabetic homogenates. GSH decreased significantly.

(ii) GPx-ase mildly decreased, but C-ase, t-SOD and Cu, Zn-SOD increase were significant. Mn-SOD activity decreased significantly.

In the pancreas homogenates:

(i) LP, GSH quantity decreased significantly. On the other hand, GSH increase was markedly high.

(ii) Activities of GPx-ase and C-ase were decreased. C-ase activity was significantly decreased.

(iii) Increase in activity of SOD was significant, with the exception of MnSOD which significantly decreased.

Discussion

Numerous earlier as well as the latest reviews summarise the experiences with AL, as the "longer time" used experimental diabetogenic agent [12, 15, 22].

It appears, that the main reasons of diabetogenic action of AL are that AL rapidly accumulates in pancreatic β-cells and their exquisite sensitivity to superoxide (O_2^-) and peroxides. On the other hand, the alloxan interacts with the sugar binding site of the glucokinase, oxidizing two SH groups of enzyme, inactivate it [12]. The alloxan therefore, inhibits the glucose induced insulin secretion and ultimately leads to the necrosis of the pancreatic β-cells.

Reviewing our pro- and antioxidant studies in blood and various tissues, and considering that C-ase and GPx-ase participate in approx. 50% in H_2O_2 decomposition [7], the following conclusions can be drawn:

If the decrease of GSH, increase of GSSG and enhancement of LP and C-ase activity and decrease of SOD activity are considered as a sign of oxidative stress, it can be definitely stated that some sign of oxidative stress (that is prooxidant predominance) can be observed in blood and in significant parts of organs in acute AL diabetes [24].

The phenomena observed are as follows:

(i) GSH concentration of plasma and hemolysate decreases. Similar trend can be observed in spleen, heart muscle, liver, brain and lung tissues. Enhanced amount of GSH can be measured in skeletal muscle, kidney and pancreas.

Amount of GSSG increases in blood, brain, kidneys and lungs after AL treatment. One of the signs of oxidative stress influencing the redox state of glutathione – generally appears either in the RBC hemolysate or in some of the organs.

(ii) Let's study further the signs of oxidative stress, e.g. the change of LP. (a) LP does not change in the plasma, (b) it decreases in the spleen, liver, kidney, lung and in

pancreas. (c) It increases in hemolysates, heart muscle, skeletal muscle. brain (apparently, it is the most active in tissues involved in glucose metabolism and in blood carrying glucose). Spleen, liver, kidneys, lungs and pancreas react with LP decrease to AL diabetes.

Upon acute AL treatment t-SOD activity decreases in hemolysate, spleen, skeletal muscle and kidney, while it increases in heart muscle, liver, brain, lung, pancreas.

(iii) Significant C-ase activity can be observed in every organ homogenate with exception of the kidney and RBC homogenate. Slight or marked enhancement in Mn-SOD indicates the effect of toxic substance, currently that of AL [5].

Our present studies confirmed our earlier results pertaining to prooxidant predominance observable both in human and experimental diabetes [17].

From our results we concluded, that in human diabetes – first of all – secondary complications (neuritis, vasculitis etc.) can be prevented with antioxidants, that is with ingredients inducing the balance of prooxidant redox state [6, 11].

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Isometric handgrip exercise-induced muscarinic vasodilation in the human skin microvasculature

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The existence of an active vasodilator system in the human skin microvasculature is well documented, but its physiological role and the underlying mechanisms are not completely understood. Cutaneous blood flow increases during isometric handgrip exercise. To examine whether this response is mediated by active vasodilation, isometric handgrip exercise testing was performed in nine healthy subjects. Local iontophoresis of atropine was applied to the forearm skin. Skin blood flow (SBF) monitoring by means of laser Doppler flowmetry was combined with continuous noninvasive blood pressure monitoring. SBF monitoring was performed at a site pretreated with atropine and an adjacent control area. Mean arterial pressure (MAP) was recorded noninvasively. Cutaneous vascular resistance (CVR) was calculated as MAP/SBF for the atropine treated and the control areas. Changes in CVR were expressed as percent deviation from the baseline (dCVR). Isometric handgrip exercise resulted in a marked reduction in CVR at the control site (dCVR: –66±4%). In contrast, the CVR was not significantly altered at the atropine-treated site (2.4±7%). It is concluded that isometric exercise induces an atropine-sensitive vasodilation which is mediated by muscarinic receptors in the human skin.

Keywords: cholinergic, vasodilation, cutaneous vascular resistance, static exercise

Cutaneous vasodilation may result from a withdrawal of the sympathetic adrenergic activity and/or activation of an independent vasodilator system. Active vasodilation plays an important role in thermoregulatory responses [5]. In contrast, the mechanism of cutaneous vasodilation during static exercise is controversial [3, 4, 9]. The onset of the static exercise is accompanied by vasodilation, mediated by

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sympathetic cholinergic nerve fibers in the muscles [8]. Cholinergic vasodilation may also be operative in the skin during isometric handgrip exercise.

Methods

Nine healthy male volunteers, ranging in age from 19 to 32 years, were recruited to participate in the study. All subjects provided written informed consent. Blood pressure (MAP), heart rate, skin blood flow (SBF), skin temperature were continuously monitored. Since the responsiveness of the skin microvasculature may vary with the ambient temperature [5], we studied the reflex responses at constant skin temperature (see below). To block muscarinic receptors, atropine was applied to a 0.6 cm^2 circular area of the non-exercising forearm skin by means of iontophoresis 30 min before the onset of recording [5]. The subjects were studied in a supine resting position, in the afternoon, 3 to 4 h after food intake. The subjects were instrumented for measurements of heart rate, finger blood pressure, skin temperature and SBF. The recording was started when stable baselines for blood pressure, heart rate and SBF were observed. Subsequent to a 5-min period of baseline data collection, isometric handgrip exercise was performed by each individual. Post-exercise data acquisition was continued until all parameters had returned to baseline values.

Relative blood cell perfusion was measured by means of a double-channel skin perfusion monitor (Perimed). The laser Doppler probes and the probe holders were attached to the ventral side of the treated (non-exercising) forearm. SBF was determined in the atropine-treated and in control areas. The control area was approximately 5 cm proximal to the treated region. The temperature probe was placed in between the two laser Doppler probe holders. Blood pressure was measured continuously on the non-exercising side by means of a Finapres 2300 (Ohmeda) noninvasive blood pressure monitor. The ECG was monitored by a Siemens Sirecust 730 ECG monitor. The R wave of the electrocardiogram and the plethysmographic signals were fed via an amplifier, a filter and analog to digital converter into an IBM–AT compatible computer.

All subjects performed isometric handgrip exercise (IHG) at 30% maximal voluntary contraction for 2 min with an exercise dynamometer. Maximum voluntary contraction was determined for each subject just before the beginning of the experimental session. Subjects were instructed to avoid a Valsalva manoeuvre during IHG.

Cutaneous vascular resistance (CVR) was determined as the ratio of MAP and SBF.

The percentage change in CVR (dCVR) was expressed as follows:

$$dCVR = (CVRx - CVRbaseline)/CVRbaseline$$

where x denotes the maximum change during the test.

Statistical analysis

Data with normal distribution were compared by using the one-way ANOVA for repeated measures. Non-Gaussian data were analyzed using the Friedman' test. An alfa level of $p<0.05$ was considered to be significant.

Results

Skin temperature (mean±S.E.M) of the volunteers was 33.9±1.2 °C; no significant fluctuations were detected during static exercising.

The SBF and MAP responses to IHG are shown in Figures 1 and 2. MAP increased continuously during the manoeuvre (baseline: 91±4 mm Hg, maximum: 122± 4 mm Hg, $p<0.05$). On termination of isometric exercise, MAP returned to baseline level. SBF increased significantly in the control area (baseline: 335±88 a.u., maximum: 1396±286 a.u., $p<0.05$) and a rapid post-exercise recovery was observed. SBF did not change significantly in the atropine-treated region throughout the test (baseline: 509±38 a.u maximum: 531±132 a.u., p=NS). CVR decreased significantly at the control site (–0.66±0.1 a.u., $p<0.05$ vs. baseline). In contrast, no significant CVR changes were seen in the region treated with atropine (0.02±0.06 a.u., p=NS). The maximum changes in CVR differed significantly between the atropine-treated and control areas.

Discussion

The objective of the present study was to determine microvascular responses of the non-exercising forearm skin during contralateral isometric exercise and to characterize the underlying mechanisms. A cholinergic cutaneous vasodilation response was hypothesized to accompany static exercise. We therefore utilized a self-controlled study design, comparing atropine-treated and control skin areas. The major finding of our study is that isometric exercise-induced human skin vasodilation is cholinergically mediated. This result is at variance with previous reports [7]. Roddie et al. reported vasodilation in the forearm tissues during body heating. This response was not prevented by intraarterial administration of atropine, but was abolished the sweating reaction effectively. A non-cholinergic, non-adrenergic vasodilator nerve activity was postulated as the probable mechanism [7].

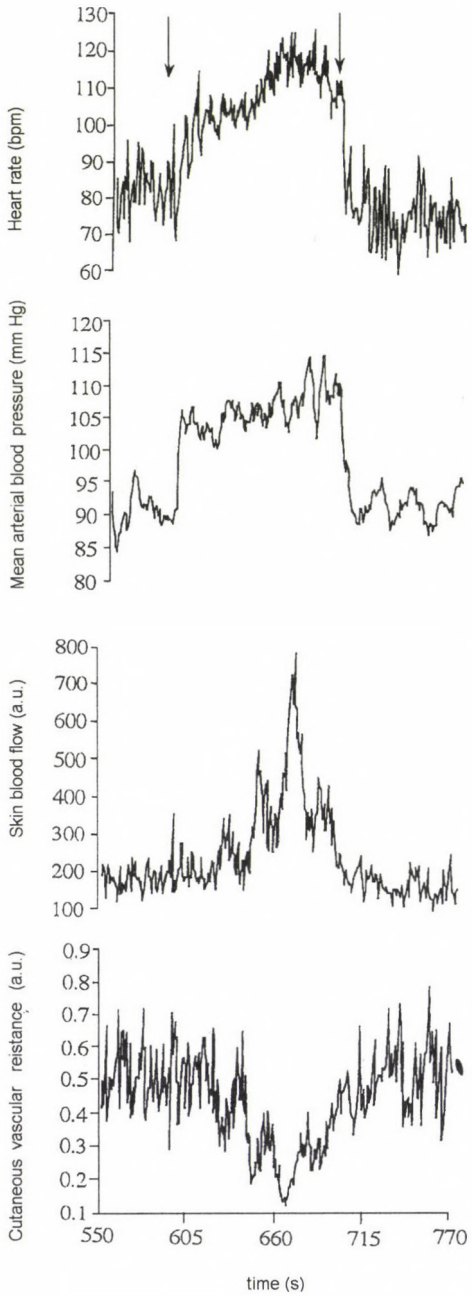

Fig. 1. Typical heart rate, MAP, and SBF response to 2-min IHG at the control site. MAP increases continuously during the maneuver. On termination of IHG MAP returns to the baseline level. SBF increases, and a rapid post-exercise recovery period can be observed. The change in CVR shows active vasodilation

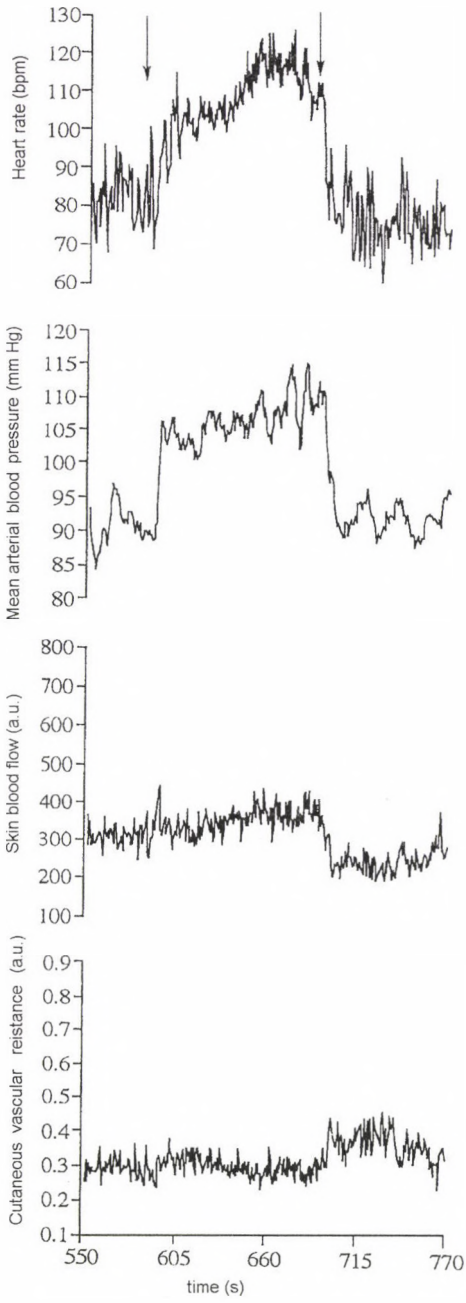

Fig. 2. Typical heart rate, MAP, and SBF response to 2-min IHG at the atropine-treated site. No significant SBF and CVR changes can be seen in that region

Sanders et al. found an active vasodilation in the non-exercising forearm muscles at the beginning of isometric handgrip exercise, despite the well-known activation of sympathetic vasoconstrictor fibers. They have documented that this response is primarily mediated by cholinergic mechanisms. Withdrawal of the adrenergic vasoconstrictor tone and the activation of β2 receptors could both be excluded [8]. It has been reported that an acethylcholine-mediated active vasodilation is operational in the skin microvasculature [2, 6]. Our present findings further extend the observation of these studies. The cutaneous cholinergic vasodilation system could be triggered by isometric exercise.

Acethylcholine induced vasodilation is currently attributed to the release of endothelium derived relaxing factors including nitric oxide [1]. By analogies drawn from this study, there is a possibility of nitric oxide-mediated dilation of the human skin microvasculature as well.

In conclusion, our results provide further evidence in favour of the presence of a cutaneous cholinergic vasodilator system which can be activated by isometric exercise.

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Familial adult onset X-linked hypophosphataemic osteomalacia

(Report of a family; clinical and experimental studies)

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From four patients (a great-grandmother, grandmother, her daughter and her grandson) suffering from a very severe form of familial X-linked hypophosphataemic osteomalacia (XLH), belonging to a 23-number-kindred of five generations, the youngest patient a 24-year-old man with an adult onset XLH was treated with phosphate and calcitriol for two years. Phosphate was given in increasing doses (500–6000 mg elemental phosphate) by mouth for a relatively short-term period and calcitriol in high doses per os combined with intermittent intravenous administration. Long-term treatment consisted of daily three grams of phosphate and 1,25 μg calcitriol by mouth combined with daily 2 μg calcitriol intravenously for one week every month. Dramatic clinical improvement occurred accompanied with definite radiological and scintigraphical changes. Serum phosphate increased from 0.525±0.478 mmol/l to 1.054± 0.041 mmol/l ($p<0.001$) in response to 3000 mg phosphate. A close correlation ($r=0.69$) was found between serum phosphate and urinary phosphate excretions ($p<0.001$) and an inverse correlation ($r=-0.31$) was found between serum phosphate and tubular reabsorption of phosphate ($p<0.01$). Serum and urinary calcium values, parathormone as well as renal functions did not change. Administration of high doses of phosphate seemed to be an effective and probably safe form of treatment in XLH provided that development of hyperparathyroidism is prevented by the coadministration of high doses of calcitriol.

Keywords: hypophosphatemic osteomalacia, calcitriol, plasma phosphate, tubular realasorption of phosphate, hypoparathyroidism

Familial X-linked hypophosphatemic osteomalacia (XLH) is a debilitating disorder which may affect several generations. Its pathophysiology comprises of decreased phosphate reabsorption and insufficient synthesis of vitamin D in the

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proximal tubules, both of which lead to the development of metabolic bone disease [4]. The presumed pathogenetic factors include 1. genetic defects of osteoblasts [14]; 2. increased fractional excretion of filtered phosphate (role of "phosphatonin"? [8]); 3. disorders of vitamin D synthesis [13], 4. disturbances in calcium and phosphate metabolism [2, 4] and, 5. secondary hyperparathyroidism [3, 5, 7, 9, 11, 12]. The purpose of this study is 1. to present the data pertaining to 4 patients belonging to a 23-kindred number of a five generation family; 2. to investigate the phosphate metabolism in a 24-year-old male member of the family; 3. and also to study the therapeutic effects of the combination of large doses of phosphate with peroral calcitriol and intermittent intravenous calcitriol pulses.

Family history

First generation

According to the family members' anecdotal reports, the great-grandmother (patient HB's mother and the representative of the first generation) suffered from a "rheumatic disease". Intense pain persisted from the age of 40. Many bone fractures occurred and she became disabled. She died at age of 51 (Fig. 1).

Second generation

The second generation patient (HB) developed bone disease at about the age 35. However it was only diagnosed 25 years later. She died when she was 64. In her final 2 years she was bedridden. Her first symptoms consisted of muscle weakness and pains in the chest and pelvis. Serial bone fractures occurred in her humerus and wrist and in the legs. Her walking ability gradually worsened, she started to hobble, and developed the characteristic waddling gait due to the gluteus medius muscle weakness. Laboratory tests showed the following: Serum phosphate 0.55 mmol/l, serum alkaline phosphatase 344 U/l and tubular reabsorption of phosphate (TRP) 69%. 25-OH vitamin D level 97 nmol/l. Urinary calcium excretion 1.26 mmol/day, phosphate excretion 7.62 mmol/day and serum calcium level 2.5 mmol/l. The X-ray pictures showed signs of serious osteomalacia, many bone fractures and Milkman-Looser pseudofractures of the ribs, both hands and femur, and tibia (Fig. 2). Low dose phosphate and standard ("classical") vitamin D treatment did not cure her bone disease.

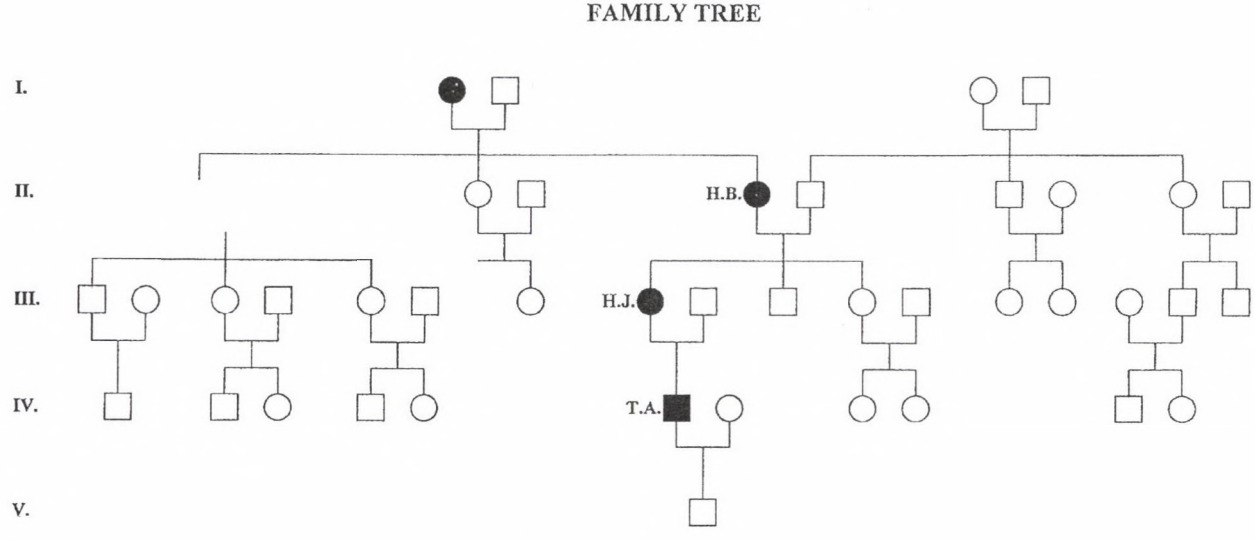

Fig. 1. The family tree

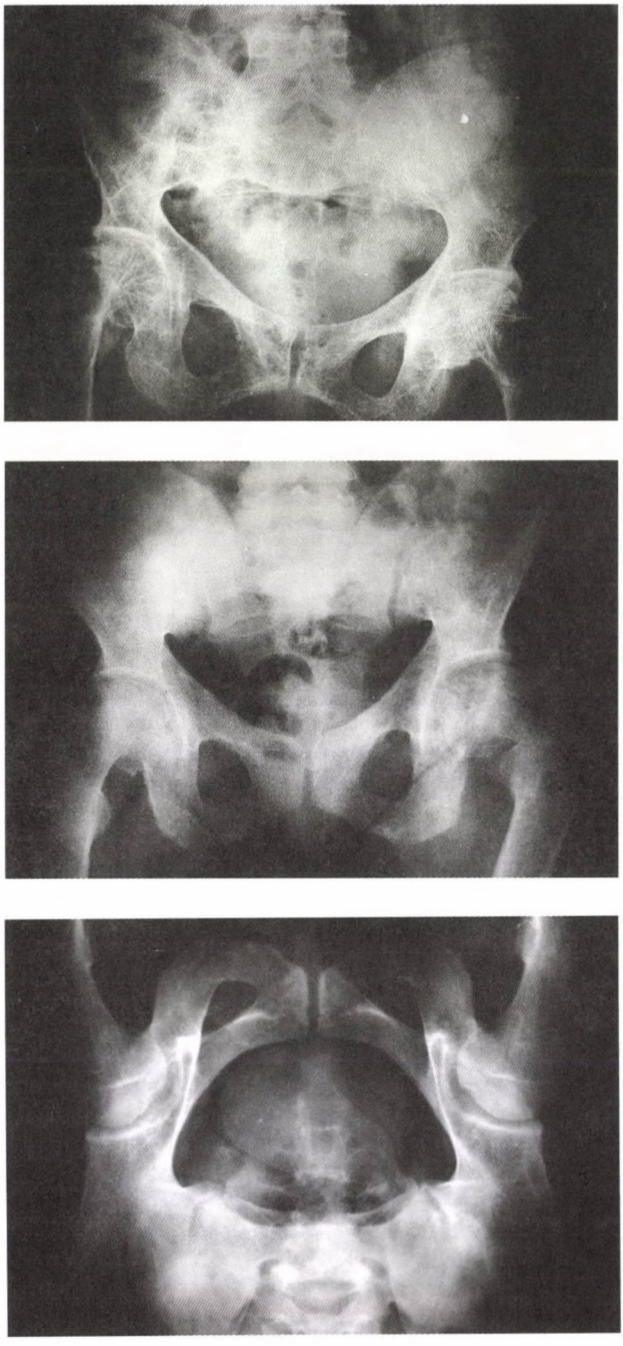

Fig. 2. Radiographical pictures (A-B-C) of osteomalacic pelvic abnormalities in patients HB (A), HJ (B), and TA (C)

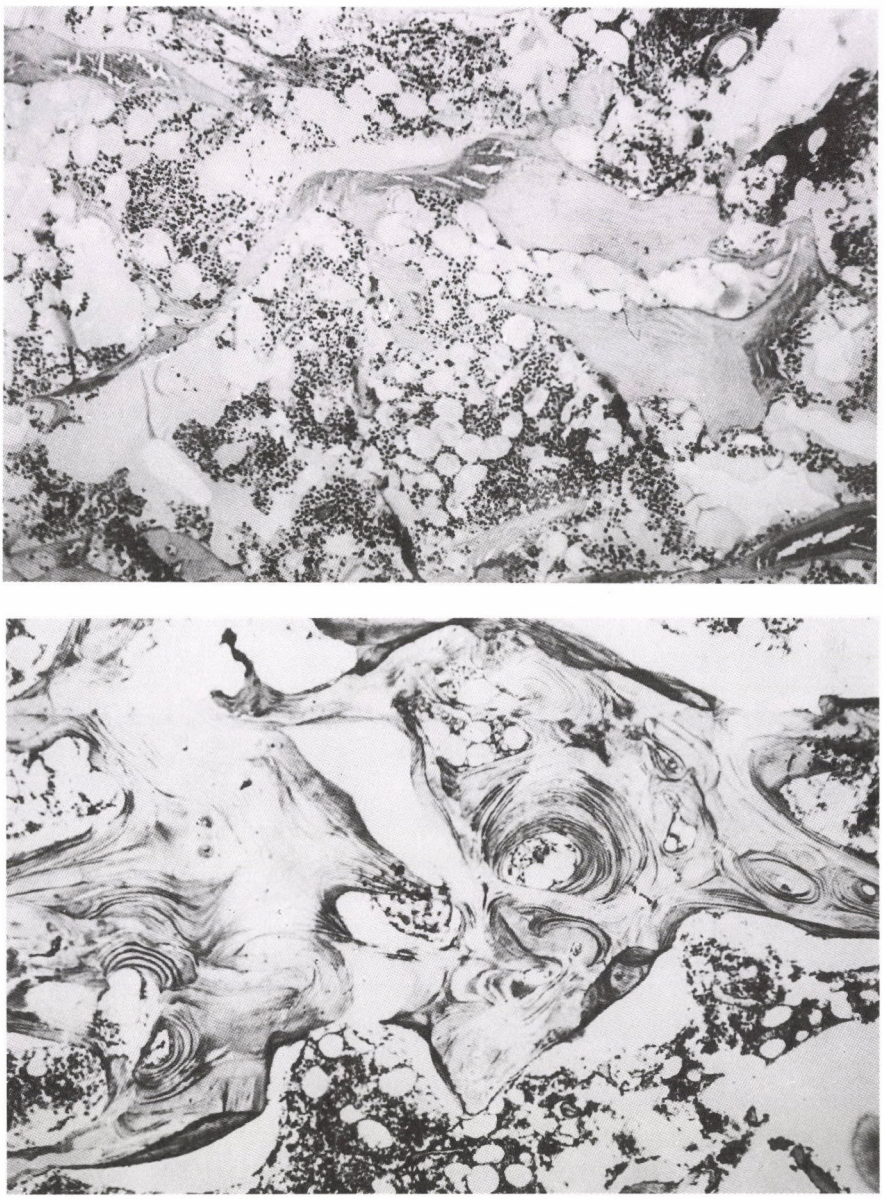

Fig. 3. Histological pictures. Post mortem histological examination of patient HJ's ribs: A. Undecalcined material with special staining by Giemsa and Goldner: the bone struts have irregular shape and widened markedly. The calcified and uncalcified areas can be differentiated well. B. Undecalcined material with special staining by Gallyas: the bone struts are irregularly widened. The collagen fibres show a laminar structures, the calcified regions are dark while the osteomalacic areas are pale

Third generation

HB's daughter (HJ), one of the four members of the third generation, was also healthy in her childhood. At the age of 30, however, she developed the same complaints as her mother's: a waddling gait and pains in her chest and extremities. Even after a hip fracture she could make some temporary recovery. At 37, her osteomalacia was diagnosed by X-ray examinations (Fig. 2). The result of the laboratory tests were as follows: serum phosphate 0.4 mmol/l, alkaline phosphatase 432 U/l, TRP 75%, blood level of vitamin 25-OH-D_3 100 nmol/l, PTH level 4.8 pmol/l, urinary calcium excretion 1.6 mmol/day, phosphate excretion 14 mmol/day, serum calcium level 2.4 mmol/l. Administration of the standard dose of vitamin D did not cure the osteomalacia. She died at 39, due to alcoholic hepatic failure accompanied by cerebral edema. The post-mortem histological findings of ribs, pelvis and femur proved the diagnosis of osteomalacia: undecalcined material with special staining by Giemsa and Goldner and by Gallyas showed that the bone struts were massive and bulky, were widened through irregular shape and also the osteoid parts were significantly broadened (Fig. 3).

Fourth generation

TA, a 24-year-old male (the proband patient, son of HJ), was admitted to our department for the first time in July, 1994. He represents the fourth generation. He is the sole member of the family whose bone disease has seemingly improved through treatment. His complaints had started six months previously. He experienced tenderness along the dorsal and lumbar spine, a stinging pain in the lower ribs which prevented him from lying on his stomach. He developed shuffling, waddling gait which caused difficulty in going up and down stairs. Following a sprain in 1991, his ankle remained permanently painful. Physical examination revealed no cardiac, pulmonary and abdominal abnormality. His blood pressure was 165/100 mm of Hg. Height was 188 cm. Body weight was 75 kg. The dorsal kyphosis was mildly increased and the lumbar lordosis became smooth; there were tenderness along the lumbar spine, restricted movement in both ankles and significant tenderness in the lower ribs and ankles.

Laboratory data on admission was as follows: serum sodium 142 mmol/l, potassium 4.2 mmol/l, total calcium 2.11 mmol/l, ionized calcium 1.124 mmol/l, phosphate 0.6 mmol/l, creatinine 86 mmol/l, total bilirubin normal, total protein 73 g/l, alkaline phosphatase 615 U/l, blood glucose: 4.7 mmol/l, blood urea nitrogen: 7.6 mmol/l, blood cell count normal, urine analysis normal, parathormone 3.2 pmol/l, 25-OH-vitamin D 186 nmol/l (normal range: 60–200 nmol/l), urinary phosphate

excretion 13.16 mmol/day, calcium excretion 0.584 mmol/day, TRP 86.6%. Blood gas analysis was as follows: pH 7.34, HCO_3 21.8 mmol/l, pCO_2 41.3 mm of Hg, pO_2 72.5 mm of Hg. Renal tubular acidosis was excluded by blood- and urine gas analysis combined with bicarbonate and phosphate loading. The X-ray pictures showed signs of osteomalacia (Fig. 2). Bone scintigraphy with 600 mBq ^{99m}Technetium-methylene-diphosphonate (July, 94; Fig. 4) showed severe extensive radiopharmacon uptake in the sacro-iliac region, in the right femoral head and acetabulum, in ramus superior of os pubis, and both tarsus and metatarsus. Increased activity was seen over the skull (os frontale, right os temporale), sternum (costo-chondral junctions), ribs (paravertebrally left Th1-6, and right Th1-8 as well as in the medial axillary line of ribs 7-11, on the right side and 9-12 on the left). No significant change in the accumulation of isotope could be detected on whole body scintiscans in November, 94 and January, 95. A definite decrease of abnormal radiopharmacon uptake was found, however, at June, 96.

The patient was readmitted on August 22, 1994. The treatment was started under close clinical observation by administering calcitriol intravenously every second day, gradually increasing the dose up to 2 mg. It was then supplemented by oral phosphate.

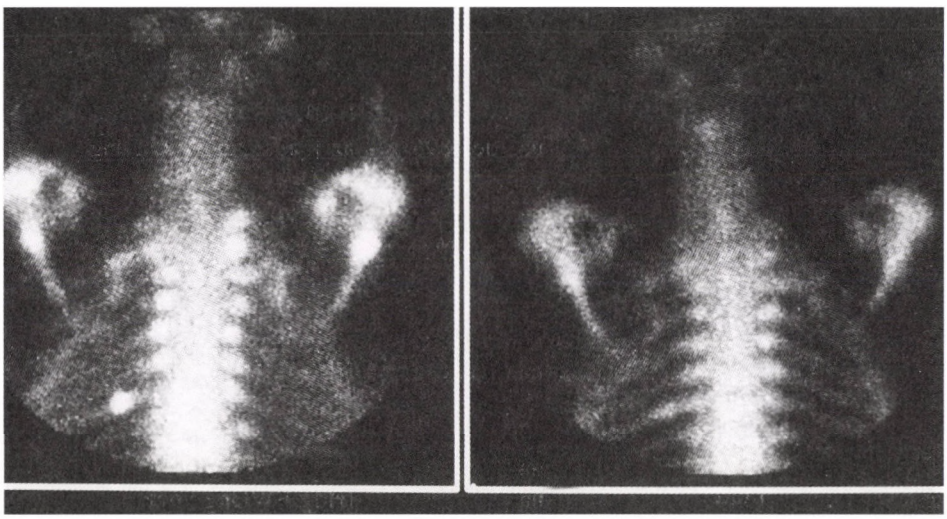

Fig. 4. Bone isotopic studies in patient TA before and after treatment. The decrease in the accumulation of the isotope in the bones after phosphatecalcitriol therapy is conspicuous

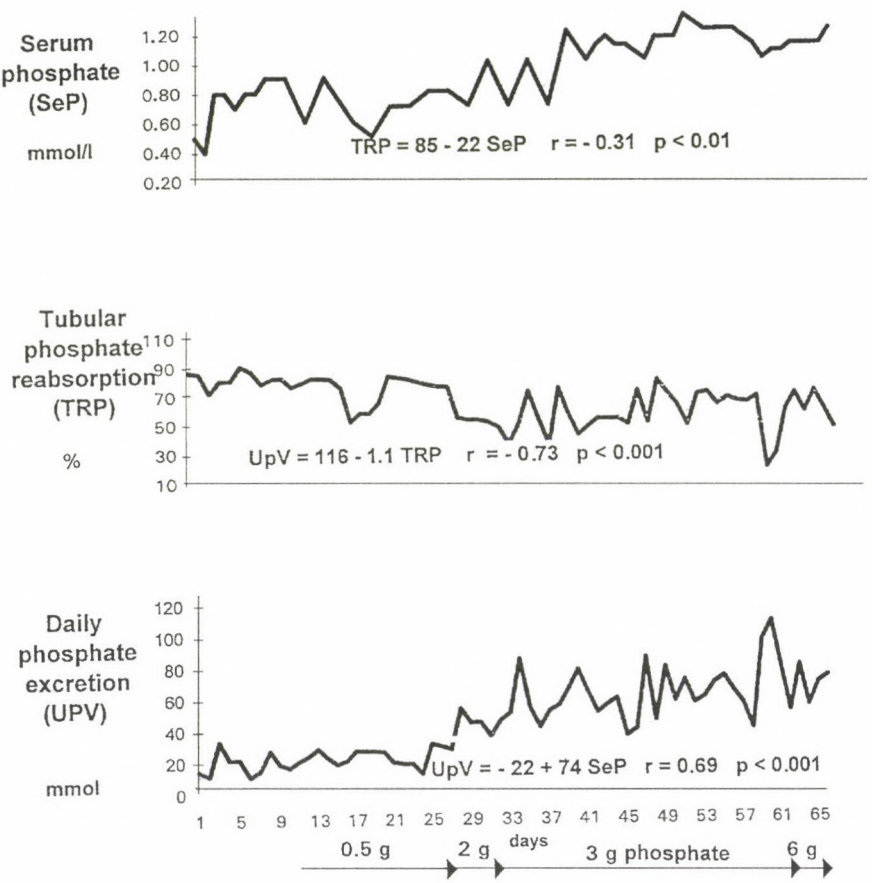

Fig. 5. Interrelationships between phosphate intake and serum and urine phosphate values

At the beginning 0.5 g/day, subsequently 2 g/day and later 3 g/day elemental phosphate (Fig. 5). When 6 g/day was temporarily given it had to be decreased to 3 g/day because of diarrhoea. Potassium chloride 2 g/day was given, too. Later calcitriol 1.25 μg was also taken orally which was continued on a daily basis. He took also daily 3×20 mg

oxprenolol and 10 mg enalapril because of hypertension. The patient was discharged in the middle of November. In order to administer intravenous calcitriol under close observation, he was readmitted one week in four in December, 1994, January, March, May, June and August 1995. During these periods of hospitalisation *per os* calcitriol (1,25 μg/day) treatment was supplemented by 4 μg, – then 2 μg per day *intravenously administered* calcitriol.

The patients condition started to improve six weeks after the initiation of therapy, in September 1994. First the stiffness in his femoral muscles decreased, followed by the pain in his ribs and spine. In the middle of October he was able to lie on his stomach again, and his walking ability significantly improved. By November his ankle pain also decreased. In December he was able to climb stairs without any difficulty, even taking them in two at a time. In the spring of 1995, within six months of starting treatment, the patient declared himself free of complaints. Only by very careful observation, could a slight shuffling gait be detected. In summer of 1995 he was able to work as a shop assistant, play football, and walk unlimited distances. In the spring of 1996, he was able to walk a distance of 10 kilometres without any difficulty. (The case of this patient has been presented previously [10].)

Fifth generation

The fifth generation, TA's one-year-old daughter (four-year-old at the time of the writing) is healthy, and the blood tests and bone scintigraphy have not revealed any trace of rickets.

Methods

Serum and urine creatinine, sodium, potassium, calcium, and phosphate determinations were carried out by standard laboratory methods in the central laboratory of the hospital. Serum ionized calcium measurements and blood and urine gas analysis were performed by an AVL analysator. Serum and urine osmolality were measured by an osmometer. Intact PTH levels were determined by Biorad RIA kits (normal range 1–8 pmol/l). Calculations for daily excretions of glomerular ions, sodium (UNaV), potassium (UKV), calcium (UCaV), phosphate (UPV), and renal functional parameters, glomerular filtration rate (GFR), urine flow (V), osmolal clearance (Cosm), free water clearance (CH_2O), osmolal and free water clearances expressed in the percentage of glomerular filtration rate (Cosm×100/GFR, CH_2O× 100/GFR) were based on 24-hour urine collection. The amount of phosphate excretion related to glomerular filtration per day expressed in mmol/24 h (UPV/GFR/24 h), as

well as the percentage of tubular phosphate excretion and reabsorption per filtrated phosphate were also calculated. Percentage of excreted phosphate per filtrated phosphate = Urine phosphate (mmol/l) × serum creatinine (mmol/l) × 100/Serum phosphate (mmol/l) × urine creatinine (mmol/l) = %. Tubular reabsorption of phosphate (TRP) = percentage of reabsorbed phosphate per filtrated phosphate = 100 – percentage of excreted phosphate per filtrated phosphate = %. Data are expressed as mean and standard error of mean (mean±SEM). For statistical analysis standard biometric methods were used (paired *t*-test and calculation of correlation coefficients).

Results

Before and under treatment 17 parameters were examined and calculated during the first-two months of hospitalisation (Tables I–III). In Table I changes in parameters of phosphate metabolism and PTH levels are summarised; Table II presents renal functional data and Table III shows serum levels and urinary excretion of sodium, potassium and calcium before treatment and during administration of 500 mg and 3000 mg of elemental phosphate. In addition to this the patient also obtained phosphate through the regular hospital diet.

Table I

Changes in some parameters of phosphate metabolism and parathormone (PTH) levels in response to administration of phosphate

	SeP, mmol/l	UPV, mmol/24 h	UPV/GFR, l/24 h	TRP, %	PTH, pmol/l
Basal	0.525±0.478	13.17	0.0889	86.609	3.2 4.7
N	4	2	2	2	2
500 mg P	0.745±0.039	21.801±1.671	0.164±0.012	77.967±2.245	
N	11	16	16	15	–
3000 mg P	1.054±0.041	61.741±3.086	0.448±0.023	56.982±2.499	5.377±0.879
N	16	32	32	32	9
Signif.	p<0.001	p<0.001	p<0.001	p<0.001	

N = number of days (determinations)

SeP = serum phosphate levels

UPV = daily urinary phosphate excretion

UPV/GFR/24 h = daily phosphate excretion related to glomerular filtration per day

TRP % (tubular reabsorption of phosphate) = percentage of phosphate reabsorption per filtrated phosphate

PTH = parathormone

Table II

Changes in specific renal functions in response to administration of phosphate

	Diuresis, ml/24 h	GFR, ml/min	Uosm, mOsm/kg	Cosm×100/GFR, %	T^cH_2O×100/GFR, %
Basal	383.3±16.67	89.42±14.01	945±56	1.067±0.254	0.751±0.192
N	3	3	3	3	3
500 mg P	884±45.27	98.21±5.82	641.4±33.22	1.471±0.125	0.808±0.093
N	22	22	22	22	22
3000 mg P	1060.6±37.55	97.53±4.58	696±31.44	1.911±0.115	1.103±0.098
N	33	33	33	33	33
Signif.	$p<0.01$	NS	NS	$p<0.05$	$p<0.05$

N = number of days (determinations)
GFR = glomerular filtration rate
Uosm = urine osmolality
Cosm = osmolar clearance
T^cH_2O = free water reabsorption

Before treatment phosphate excretion was almost normal in spite of the very low serum phosphate concentrations. The phosphate excretion related to glomerular filtration rate (UPV/GFR/24 h) was 0.0889, definitely higher than the "usual" values in simple phosphate wastage (0.01–0.03). This indicated a marked tubular insufficiency of phosphate reabsorption. It was also supported by the borderline pretreatment value (86%) of TRP as well as the significant decrease in TRP (56%) after "normalization" of serum phosphate concentration (Fig. 6). Increased phosphate intake resulted in significant elevation of serum phosphate levels, urinary phosphate excretions and drop in tubular phosphate reabsorption. The different correlations between these values are seen in Fig. 5.

Before starting treatment the patient's diuresis was remarkably low (Table II) and the urine was very concentrated. During increased phosphate intake the diuresis rose and the urine became more diluted. Renal functions, however, did not deteriorate during high phosphate intake, which were marked by unchanged glomerular filtration rate and preserved concentrating capacity (proportion of T^cH_2O to Cosm). During treatment sodium excretion significantly increased (Table III). Serum potassium decreased, probably because phosphate was administered in sodium salts and sodium diuresis was accompanied by increased excretion of potassium. The patient had to be given regularly potassium supplementation.

Table III

Changes in blood and urine values in response to administration of phosphate

	SeNa, mmol/l	SeK, mmol/l	UNaV, mmol/24 h	UKV, mmol/24 h	SeCa, mmol/l	ionCa^{++}, mmol/l	UCaV, mmol/24 h
Basal	141.5	4.3	56.73±9.53	34.67±6.49	2.208±0.036	1.257±0.0128	0.584
N	2	2	3	3	4	4	2
500 mg P	141.8±0.536	3.66±0.106	141.89±11.74	30.99±3.11	2.190±0.0168	1.203±0.011	2.264±0.237
N	11	11	22	22	11	7	19
3000 mg P	142±0.408	3.57±0.04	178.74±11.95	40.32±3.08	2.254±0.022	1.210±0.013	1.957±0.102
N	16	16	33	33	16	9	33
Signif.	NS	NS	$p<0.05$	$p=0.05$	NS	NS	NS

N = number of days (determinations)

SeNa = serum sodium levels

SeK = serum potassium levels

UNaV = daily urinary sodium excretion

UKV = daily urinary potassium excretion

SeCa = serum calcium levels

ionCa^{++} = serum ionised calcium levels

UCaV = daily urinary calcium excretion

NS = non significant

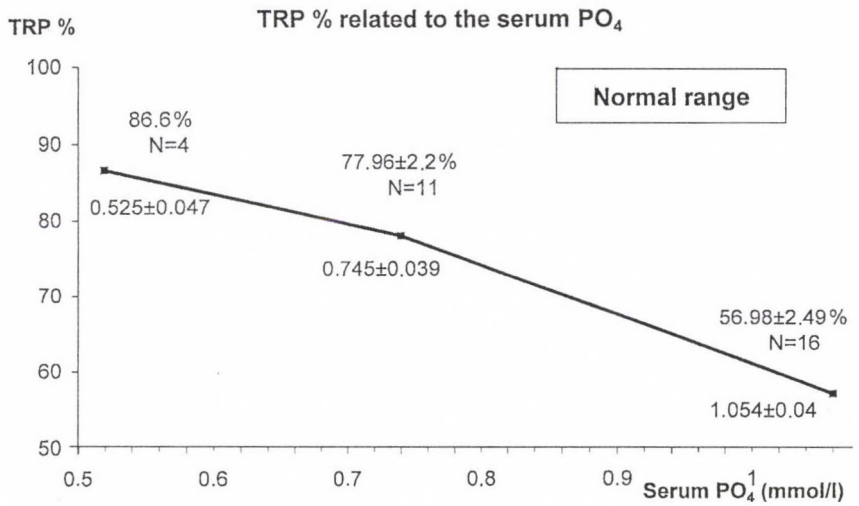

Fig. 6. Interrelationship between tubular reabsorption of phosphate (TRP) and serum phosphate levels. Change in TRP when plasma phosphate levels were increased into the normal range by high phosphate intake

Discussion

Although XLH was already described by Albright et al. [1] in 1937, and the existence of its animal model the "HYP mouse" has been known since 1976 [13, 14], the causes and the mechanisms of the disease are not completely clear as yet [8].

Hypophosphataemic osteomalacia occurred in 4 members of 5 generations of the described family, where the inheritance showed an X-linked dominant pattern. In all affected members – except TA – XLH seemed to shorten the lifetime.

The proband patient's phosphate excretion was not abnormally low even before treatment, in spite of the decreased (0.4–0.6 mmol/l) serum phosphate concentrations [6] (Table I). The quotient of daily phosphate excretion and GFR/24 h in our case was 0.09 ("normal value" is 0.01–0.02) suggesting that the kidney could not retain phosphate even in the phosphate depleted condition [9]. TRP, which hardly differed from the normal in basal situation (86.6% vs 88%) became abnormal (57%) when examined during normalization of serum phosphate to 1 mmol/l by administering high doses of phosphate. There was an inverse correlation between tubular reabsorption and excretion of phosphate (Fig. 5) as well as a close correlation between serum phosphate levels and phosphate intake (excretions of phosphate) (Fig. 5).

The ultimate defect responsible for the hypophosphataemia may be *1. a primary osteoblastic defect* leading to a vitamin D resistant condition. In addition, both the lack of the elevation of 1-25-$(OH)_2$-D_3 vitamin (calcitriol) levels in response to hypophosphataemia and the disturbance in the metabolism of vitamin D (enhanced metabolic clearance and mitochondrial transformation) may have contributed to the insufficient effect of vitamin D in the prevention of bone disease and possibly the suppression of the parathormone release by blocking the receptors of the parathyroid gland [4, 6, 13]; *2. decreased reabsorption of phosphate in the proximal tubules* as a result of an intrinsic tubular functional defect and/or the effect of a "phosphaturic hormone" [8]; *3. abnormal nocturnal elevation of the parathormone levels ("nocturnal hyperparathyroidism")* as a consequence of inability to mobilize calcium night-time from the unresponsive osteomalatic bone [2, 3]; *4. therapy itself*, hypocalcaemia due to starting phosphate administration, provoking increased parathormone levels [3–5, 7, 8]. Parathyroid gland hyperplasia may develop, the fast cell proliferation may result in mutations, and the monoclonal cell multiplication can cause parathyroid adenoma [5]. In XLH disease the development of not only secondary but tertiary hyperparathyroidism has been described as well proving for gland hyperplasia and adenoma formation [7].

In our patient, during combined phosphate-calcitriol treatment, the glomerular filtration rate did not deteriorate and both the serum total and ionized calcium levels remained stable (Tables II, III). The parathormone levels did not elevate (Table I). There was no increase in PTH levels at midnight, which was investigated twice (7.4 pmol/l and 1 pmol/l versus values in the morning: 6 pmol/l and 3.8 pmol/l).The only exception was (during a less than one month period), when the patient took phosphate but neglected calcitriol: PTH increased from 3–5 pmol/l (normal) to 14.8 pmol/l.

Administration of high doses of phosphate seemed to be an effective and safe treatment of our patient with XLH [provided [11] that the development of hyperparathyroidism had been prevented by the coadministration of high doses of calcitriol and careful observation excluded any sign of vitamin D intoxication (hypercalcaemia, hypercalcuria, nephrocalcinosis etc.)]. Regular daily peroral doses of calcitriol were combined with intermittent daily intravenous bolus administration of calcitriol (one week every month). It was established by Reichel and his co-workers [12] that the blood level of calcitriol was more important than the amount of the drug administered in suppression of parathormone release. The same amount of calcitriol administered in continuous infusion was less effective than that administered in bolus injection [12]. Therefore we combined the regular peroral calcitriol treatment of our patient with intermittent intravenous administration. Our patient's osteomalacia was

practically healed and his condition remained stable under maintenance treatment. It is fair to assume, therefore, that the affected members of the patient's family may have benefited from the same treatment.

Acknowledgements

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Addendum

Disorders of renal phosphate wasting are the most common hereditary forms of rickets and osteomalacia in western countries. Isolated renal phosphate wasting may result from a number of genetic disorders including X-linked hypophosphatemic rickets (XLH); hereditary hypophosphatemic rickets with hypercalcuria (HHRH); hypophosphatemic bone disease (HBD); and autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR) (Econs MJ and McEnery PT; Autosomal dominant hypophosphatemic rickets/osteomalacia: clinical characterization of a novel renal phosphate wasting disorder. J. Clin. Endocrinol. Metab. 82, 674–681 (1997).

In X-linked hypophosphatemic rickets (HYP) positional cloning efforts led to the identification and cloning of the responsible gene, now referred to as PEX, a member of the M 13 metalloprotease family. Its likely functions to degrade a phosphate wasting hormone (phosphatonin) or to activate a phosphate conserving hormone. "The HYP consortium: A gene (PEX) with homologies to endopeptidases is mutated in patients with X-linked hypophosphatemic rickets. Nat. Genet. 11, 130–136 (1995)."

After the completion of our studies and writing new data emerged. In the "rapid publication" of Econs MJ, McEnery PT, Lennon F. and Speer MC (Autosomal dominant hypophosphatemic rickets is linked to chromosome 12p13. J. Clinical Investigation 100, 2653–2657 (1997)) the authors determined the chromosome location of the ADHR gene. They localized the disease gene to an 18-cM interval on the short arm of chromosome 12.

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Sensitization of rat gastrointestinal tract to acetylcholine and histamine produced by X-radiation

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Abdominal x-radiation produces both acute and chronic disturbances of gastrointestinal motility. Anaesthetized Albino-Oxford rats received one-session x-radiation (absorbed dose 10 Gy) of whole abdomen. Two hours after irradiation the rats were sacrificed and segments of their gastrointestinal tract (gastric fundus, jejunum, ileum and ascending colon, were mounted in isolated organ bath. Acetylcholine and 5-hydroxytryptamine produced tonic contractions of all gut segments, while histamine did so only with gastric fundus. While contractile effect of 5-hydroxytryptamine was not affected by x-radiation, the responses of all gut segments on acetylcholine were potentiated and shifted towards lower concentrations. After x-radiation histamine produced concentration-dependent tonic contraction of previously unresponsive jejunum and ascending colon. The results of our study suggest that x-radiation produces acute sensitization of rat gastrointestinal tract to acetylcholine and histamine.

Keywords: x-radiation, gastrointestinal tract, acetylcholine, histamine, smooth muscles

Nearly 50% of all cancer patients eventually receive therapeutic radiation [11], a lot of them having abdominal region included in the radiation field. The adverse effects of radiation on gastrointestinal tract could be acute and chronic. Chronic radiation

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enteropathy manifests itself years after the treatment with obstruction, diarrhea or formation of fistulas. The acute radiation enteropathy is followed by vomiting, diarrhea and convulsive pain in the patients, usually leading to interruption of the treatment [10]. The first appearance of diarrhea is usually in second week from the beginning of the radiotherapy, but minor gastrointestinal disturbances could be recorded from the beginning of the treatment. The symptoms of vomiting and diarrhea within the first week are directly related to changes in bowel motility caused by radiation [6]. In fasted dogs, as early as few hours after first radiation fraction, increased frequency of abnormal motility patterns (giant migrating contractions and retrograde giant contractions) was noticed [6]. The dogs developed nausea, vomiting and diarrhea during the first day of irradiation.

It is well known that radiation with doses higher than 7.5 Gy damages intestinal mucosa, interfering with mucosal transport of water and solutes, and releasing a lot of neurotransmitters and local hormones with profound effect on motility [9]. However, ionizing radiation in usual therapeutic doses directly affects neurons and smooth muscle cells, too, primarily by changing the number and/or functional capacity of membrane receptors [7]. Direct deleterious effect on the contractile mechanism itself (myofibrils, e.g.) was observed only after exposure to enormous absorbed dose of X-rays (about 20,000 Gy) [1]. Reports on the irradiation effects on particular types of neural and smooth muscle receptors in the gut are still missing. The aim of our study was to investigate acute influence of rat gut irradiation by X-rays on contractile effects of three gut neurotransmitters and/or local hormones:acetylcholine, histamine and 5-hydroxytryptamine.

Materials and Methods

The animals and the irradiation

Twenty white, Albino-Oxford rats of both sexes, weighing between 250 and 400 grams, were used in the study after a 12-hour fast. Six rats served as controls, another six rats were anaesthetized with thiopentone-sodium (60 mg/kg of body weight, subcutaneous injection in the back) and yet another eight rats were anaesthetized in the same manner and their abdomen was immediately irradiated by X-rays during about 10 minutes (total absorbed dose 10 Gy, energy 10 MeV, distance 1 m, velocity of the dose 300 Rad/minute, field 8×7 cm, SIEMENS MEVATRON 12). Both the only anaesthetized and anaesthetized and irradiated rats were killed by blow on a head and exsanguinated after 2 hours from the moment anaesthesia ensued. The control rats were

also killed by blow on a head and exsanguinated. The gastric fundus, segments of jejunum and ileum and ascending colon were taken off the rats and mounted in isolated organ baths.

The isolated preparations

Gastric fundus. The fundus of the stomach (differing from other parts of stomach by its whitish-gray color) was excised by scissors and put in dissecting pan filled with Krebs solution (NaCl 5.54, KCl 0.35, $MgSO_4 \times 7H_2O$ 0.29, $CaCl_2$ 0.28, KH_2PO_4 0.16, $NaHCO_3$ 2.1 and glucose 2.1 g/l). The fundus at first was made flat by two opposite cuts along the great curvature. The isolated preparation was constructed according to Vane [18]: a few opposite cuts parallel with the great curvature were made on the fundus (a distance between two next cuts was 1.5 mm) producing a very long and narrow isolated preparation (4 cm in length and 1.5 mm in width), containing full thickness of the gastric wall.

Jejunal segment. A four centimeter segment of the jejunum, 30 cm proximal from the ileo-cecal junction was excised and put in the dissecting pan. Its mesenterium was stripped off and it was mounted in isolated organ bath according to Magnus [12]: only one wall of opposite ends of the intestine was tied to the bottom of the bath and the lever, respectively.

Ileal segment. A four-centimeter segment of the ileum, 3 cm proximal from the ileocecal junction was excised and put in the dissecting pan. Its mesenterium was stripped off and it was mounted in isolated organ bath in the same way as the jejunal segment.

Ascending colon. A four-centimeter proximal segment of the ascending colon was excised and put in the dissecting pan. Its mesenterium was trimmed off and it was mounted in isolated organ bath in the same way as the jejunal segment.

The bath and the lever

Each isolated preparation was mounted in the 15 ml isolated organ bath. One end of the preparation was fixed to the bath bottom and the other for the short arm of isotonic, frontal writing lever. The bath was filled with Krebs solution, aerated continuously with 95% O_2 and 5% CO_2, and maintained at 37 °C. All strips were loaded with 1.0 g weight. For isolated preparations of rat fundus additional stretching weight of 1.0 g was used. The magnification was 8:1. Recordings were made on a smoked drum. A vibrator was attached to the lever holder to reduce friction of the writing point.

Agonists

All isolated preparations were allowed to equilibrate for about 45 minutes before drugs were added. The effects of each concentration of each agonist were tried on isolated preparations from at least 6 different animals. Each agonist was added to the bath cumulatively. After recording the effect of its last dose the bath was drained, washed three times and refilled with fresh Krebs solution. Next agonist cumulating on the same isolated preparation was tried after 20 minutes pause.

Substances

In our study following substances were used: acetylcholine chloride, histamine dihydrochloride and 5-hydroxytryptamine hydrochloride (Sygma Chemical Co, St Louis, USA).

Statistics

Concentration-response curves for the agonists were constructed using linear regression according to the least-squares analysis. Effective concentration of an agonist that produced 50% of maximal response (EC_{50}) was calculated for each agonist together with its confidence limits (1.96 × standard error). The results were considered statistically significant when $p \leq 0.05$. The difference between effects of an agonist on isolated preparations from irradiated and anaesthetized, only anaesthetized and control rats was tested by two-way analysis of variance [17]. The difference was considered significant if $p \leq 0.05$.

Results

Spontaneous activity

The isolated preparations from rat fundus did not show spontaneous activity. About half of isolated preparations of jejunum and ileum showed spontaneous phasic contractions with low amplitude (below 5 mm on recordings) and frequency of 3–6 cycles per minute. All preparations of ascending colon showed spontaneous phasic contractions with moderate amplitude (below 10 mm on recordings) and frequency of 3–6 cycles per minute.

Neithes anasthesia only nor anasthesia together with irradiation did produce significant effect on spontaneous contractions.

Effects of acetylcholine

The effects of acetylcholine on rat gastric fundus. Acetylcholine (1.83×10^{-8} M to 1.63×10^{-5} M) produced concentration-dependent tonic contraction of the isolated gastric fundus from control rats ($r=0.99$; $p\leq0.01$; $EC_{50}=4.24\pm0.03\times10^{-7}$ M; maximal contraction on the recorder was 30.0±7.6 mm), anaesthetized rats ($r=0.99$; $p\leq0.01$; $EC_{50}=2.27\pm0.01\times10^{-7}$ M; maximal concentration on the recorder was 46.2±10.2 mm) and anaesthetized and irradiated rats ($r=0.98$; $p\leq0.01$; $EC_{50}=5.73\pm0.24\times10^{-8}$ M; maximal contraction on the recorder was 48.5±11.6 mm) (Fig. 1). The anaesthesia only did not affect significantly only the effect of acetylcholine ($F=2.75$, $p>0.05$), but anaesthesia and irradiation together shifted the concentration-response curve of acetylcholine to left and upwards ($F=39.74$, $p\leq0.001$).

Effects of acetylcholine on rat jejunum. Acetylcholine (1.83×10^{-8} M to 5.30×10^{-5} M) produced concentration-dependent tonic contraction of the isolated jejunum from control rats ($r=0.98$; $p\leq0.01$; $EC_{50}=9.27\pm0.02\times10^{-7}$ M; maximal contraction on the recorder was 37.2±4.6 mm), anaesthetized rats ($r=0.95$; $p\leq0.05$; $EC_{50}=1.05\pm0.004\times10^{-5}$ M; maximal contraction on the recorder was 21.8±5.8 mm) and anaesthetized and irradiated rats ($r=0.98$; $p\leq0.01$; $EC_{50}=0.02\times10^{-7}$ M; maximal contraction on the recorder was 54.4±10.0 mm) (Fig. 2). The spontaneous activity of jejunum was not affected. The anaesthesia only produced significant shift of acetylcholine concentration-response curve only to the right with a decrease of the maximal effect ($F=41.91$, $p\leq0.01$), but anaesthesia and irradiation together shifted the concentration-response curve of acetylcholine to the left and upwards ($F=20.03$, $p\leq 0.001$).

Effects of acetylcholine on rat ileum. Acetylcholine (1.83×10^{-8} M to 5.30×10^{-5} M) produced concentration-dependent tonic contraction of the isolated ileum from control rats ($r=0.96$; $p\leq0.01$; $EC_{50}=9.23\pm0.02\times10^{-7}$ M; maximal contraction on the recorder was 23.0±5.7 mm), anaesthetized rats ($r=0.97$; $p\leq0.01$; $EC_{50}=4.59\pm0.02\times 10^{-7}$ M; maximal contraction on the recorder was 45.0±07.2 mm) and anaesthetized and irradiated rats ($r=0.99$; $p\leq0.01$; $EC_{50}=1.18\pm0.02\times10^{-7}$ M; maximal contraction on the recorder was 54.8±15.9 mm) (Fig. 3). The spontaneous activity of ileum was not affected. The anaesthesia only did not influence only the effect of acetylcholine ($F=2.75$, $p>0.05$), but anaesthesia and irradiation together shifted the concentration-response curve of acetylcholine to the left and upwards ($F=38.46$, $p\leq0.001$).

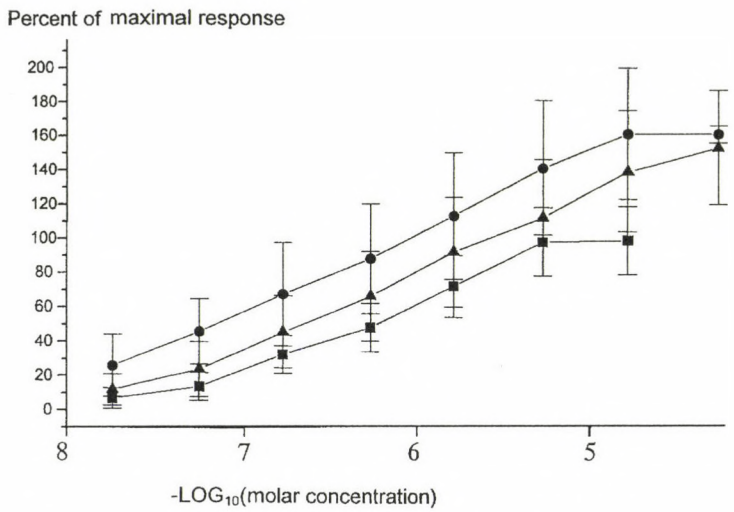

Fig. 1. Effects of acetylcholine on isolated gastric fundus from control, anaesthetized and both anaesthetized and irradiated rats. Each point represents a mean response from 6 different animals. Error bars = standard deviations.

–■– Acetylcholine-control; –●– Acetylcholine+radiation+anaesthesia; –▲– Acetylcholine+anaesthesia

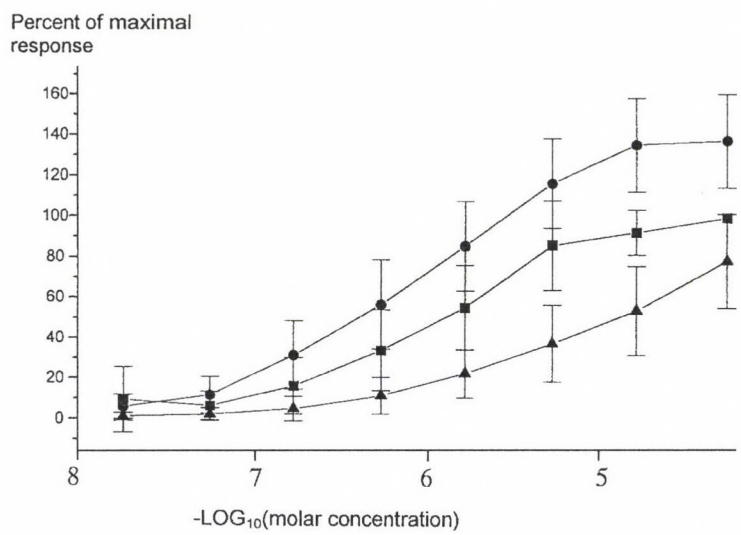

Fig. 2. Effects of acetylcholine on isolated jejunum from control, anaesthetized and both anaesthetized and irradiated rats. Each point represents a mean response from 6 different animals. Error bars = standard deviations.

–■– Acetylcholine-control; –●– Acetylcholine+radiation+anaesthesia; –▲– Acetylcholine+anaesthesia

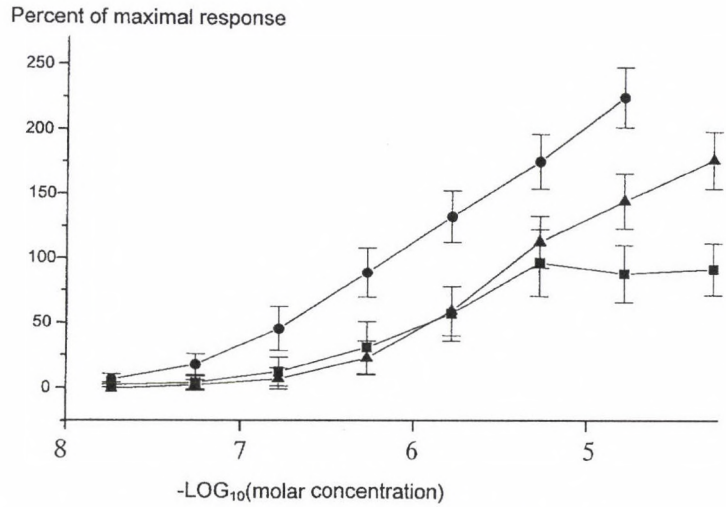

Fig. 3. Effects of acetylcholine on isolated ileum from control, anaesthetized and both anaesthetized and irradiated rats. Each point represents a mean response from 6 different animals. Error bars = standard deviations.

–■– Acetylcholine-control; –●– Acetylcholine+radiation+anaesthesia; –▲– Acetylcholine+anaesthesia

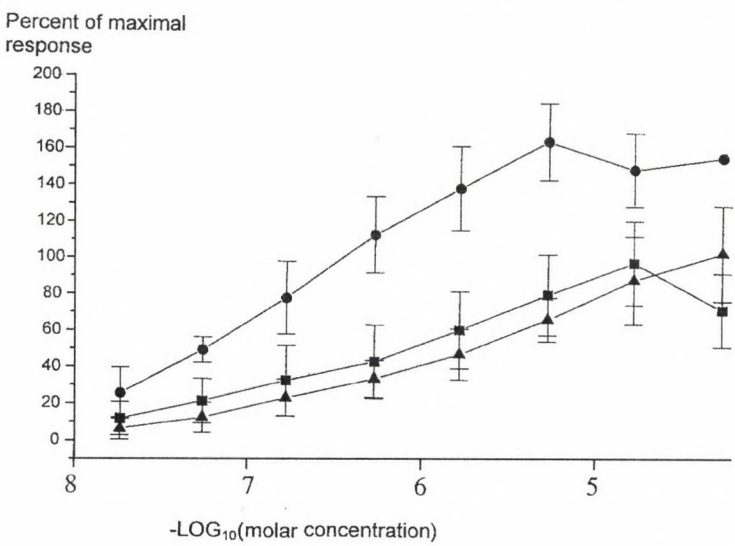

Fig. 4. Effects of acetylcholine on isolated ascending colon from control, anaesthetized and both anaesthetized and irradiated rats. Each point represents a mean response from 6 different animals. Error bars = standard deviations.

–■– Acetylcholine-control; –●– Acetylcholine+radiation+anaesthesia; –▲– Acetylcholine+anaesthesia

Effects of acetylcholine on rat ascending colon. Acetylcholine (1.83×10^{-8} M to 5.30×10^{-5} M) produced concentration-dependent tonic contraction of the isolated ascending colon from control rats ($r=0.94$; $p\leq0.05$; $EC_{50}=6.45\pm0.03\times10^{-7}$ M; maximal contraction on the recorder was 36.1±14.7 mm), anaesthetized rats ($r=0.99$; $p\leq0.01$; $EC_{50}=1.01\pm0.002\times10^{-6}$ M; maximal contraction on the recorder was 38.2±12.1 mm) and anaesthetized and irradiated rats ($r=0.94$; $p\leq0.01$; $EC_{50}=3.22\pm0.49\times10^{-8}$ M; maximal contraction on the recorder was 59.8±16.6 mm) (Fig. 4). The spontaneous activity of ascending colon was not affected. The anaesthesia only slightly decreased the effect of acetylcholine ($F=9.26$, $p<0.05$), but anaesthesia and irradiation together shifted the concentration-response curve of acetylcholine far to the left and upwards ($F=45.54$ $p\leq0.001$).

Effects of histamine

The effects of histamine on rat gastric fundus. Histamine (1.81×10^{-8} M to 1.61×10^{-4} M) produced concentration-dependent tonic contraction of isolated gastric fundus from control rats ($r=0.96$; $p\leq0.05$; $EC_{50}=1.66\pm0.05\times10^{-6}$ M; maximal contraction on the recorder was 20.0±6.8 mm), anaesthetized rats ($r=0.99$; $p\leq0.01$; $EC_{50}=2.44\pm0.02\times10^{-7}$ M; maximal contraction on recorder was 23.5±10.0 mm) and anaesthetized and irradiated rats ($r=0.99$; $p\leq0.01$; $EC_{50}=2.60\pm0.02\times10^{-7}$ M; maximal contraction on the recorder was 32.3±7.4 mm) (Fig. 5). Both anaesthesia only ($F=10.26$, $p\leq0.01$) and anaesthesia + irradiation ($F=33.44$, $p\leq0.01$) produced significant shift of histamine concentration-response curve to the left and upwards (Fig. 5). However, the effect of irradiation could not be distinguished from the effect of anaesthesia ($F=0.04$, $p>0.05$).

Effects of histamine on rat jejunum. Histamine (1.81×10^{-8} M to 1.61×10^{-4} M) did not affect either the tone or the spontaneous activity of isolated jejunum from the control or anaesthetized rats, but it produced a concentration-dependent tonic contraction of the isolated jejunum from anaesthetized and irradiated rats ($r=0.93$; $p\leq0.05$; $EC_{50}=5.02\pm0.003\times10^{-6}$ M) (Fig. 6).

Effect of histamine on rat ileum. Histamine (1.81×10^{-8} M to 1.61×10^{-4} M) did not affect either the tone or the spontaneous activity of the isolated ileum from control, anaesthetized and both anaesthetized and irradiated rats.

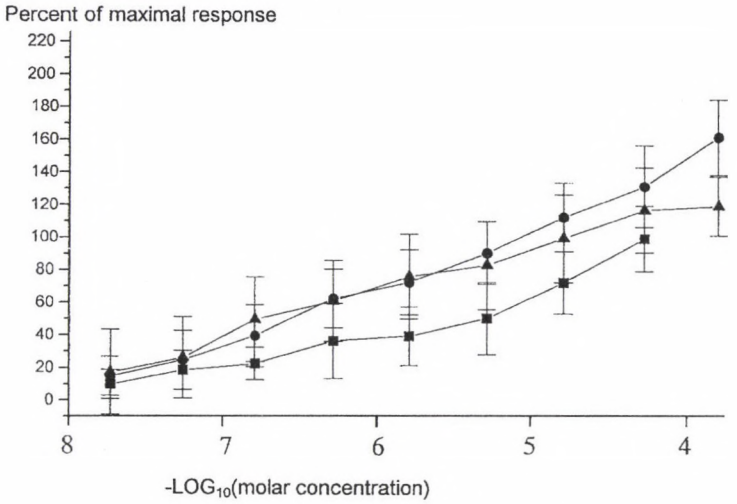

Fig. 5. Effects of histamine on isolated gastric fundus from control, anaesthetized and both anaesthetized and irradiated rats. Each point represents a mean response from 6 different animals. Error bars = standard deviations.

–■– Histamine-control; –●– Histamine+radiation+anaesthesia; –▲– Histamine+anaesthesia

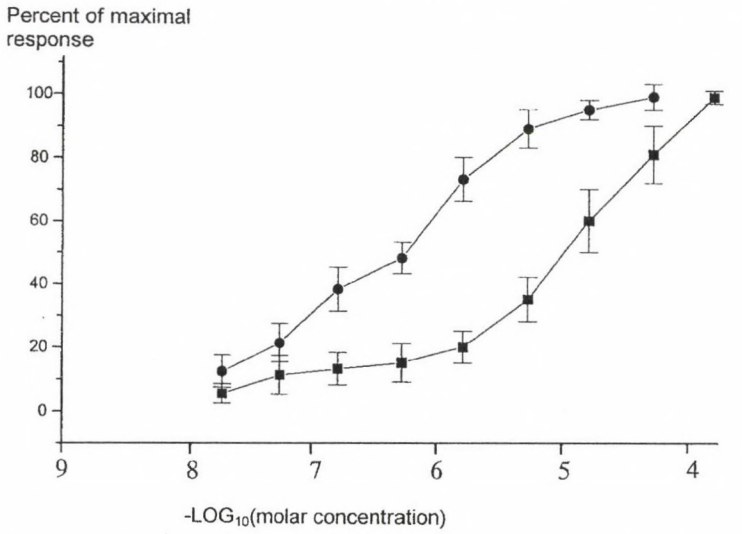

Fig. 6. Effects of histamine on isolated jejunum and ascending colon of both anaesthetized and irradiated rats. Each point represents a mean response from 6 different animals. Error bars = standard deviations.

–■– Histamine-jejunum; –●– Histamine-ascending colon

Effect of histamine on rat ascending colon. Histamine (1.81×10^{-8} M to 1.61×10^{-4} M) did not affect either the tone or the spontaneous activity of the isolated ascending colon from control or anaesthetized rats, but it produced concentration-dependent tonic contraction of the isolated ascending colon from both anaesthetized and irradiated rats ($r=0.90$; $p\leq0.05$; $EC_{50}=1.16\pm0.005\times10^{-5}$ M) (Fig. 6).

Effects of 5-hydroxytryptamine

The effect of 5-hydroxytryptamine on rat gastric fundus. The 5-hydroxytryptamine (1.11×10^{-9} M to 1.68×10^{-5} M) produced concentration-dependent tonic contraction of the isolated gastric fundus from control rats ($r=0.96$; $p\leq0.01$; $EC_{50}=2.13\pm0.02\times10^{-7}$ M; maximal contraction on the recorder was 45.5±12.0 mm), anaesthetized rats ($r=0.99$; $p\leq0.01$; $EC_{50}=8.26\pm0.01\times10^{-8}$ M; maximal contraction on the recorder was 44.2±8.6 mm) and both anaesthetized and irradiated rats ($r=0.99$; $p\leq0.01$; $EC_{50}=2.27\pm0.04\times10^{-7}$ M; maximal contraction on the recorder was 42.0±14.6 mm) (Fig. 7). The anaesthesia alone ($F=3.98$, $p>0.05$) and anaesthesia and irradiation together ($F=0.05$, $p>0.05$) did not affect significantly the effect of 5-hydroxytryptamine.

Effect of 5-hydroxytryptamine on rat jejunum. The 5-hydroxytryptamine (1.89×10^{-8} M to 1.68×10^{-5} M) produced concentration-dependent tonic contraction of the isolated jejunum from control rats ($r=0.89$; $p\leq0.05$; $EC_{50}=2.58\pm0.05\times10^{-7}$ M; maximal contraction on the recorder was 27.0±3.0 mm), anaesthetized rats ($r=0.98$; $p\leq0.01$; $EC_{50}=3.18\pm0.002\times10^{-6}$ M; maximal contraction of the recorder was 19.8±13.0 mm) and both anaesthetized and irradiated rats ($r=0.99$; $p\leq0.01$; $EC_{50}=2.40\pm0.02\times10^{-6}$ M; maximal contraction on the recorder was 28.5±2.1 mm) (Fig. 8). The anaesthesia alone ($F=5.57$, $p\leq0.05$) and anaesthesia and irradiation together ($F=16.99$, $p\leq0.05$) produced significant decrease of contractile response of rat jejunum on 5-hydroxytryptamine. However, the effect of x-radiation could not be differentiated from the effect of anaesthesia ($F=0.09$, $p>0.05$).

Effect of 5-hydroxytryptamine on rat ileum. The 5-hydroxytryptamine (1.89×10^{-8} M to 1.68×10^{-5} M) produced concentration-dependent tonic contraction of the isolated ileum from control rats ($r=0.93$; $p\leq0.05$; $EC_{50}=3.30\pm0.03\times10^{-7}$ M; maximal contraction on the recorder was 10.5±3.0 mm), anaesthetized rats ($r=0.97$; $p\leq0.01$; $EC_{50}=4.64\pm0.002\times10^{-7}$ M; maximal contraction on the recorder was 14.7±5.7 mm) and both anaesthetized and irradiated rats ($r=1.98$; $p\leq0.01$; $EC_{50}=2.42\pm0.02\times10^{-7}$ M;

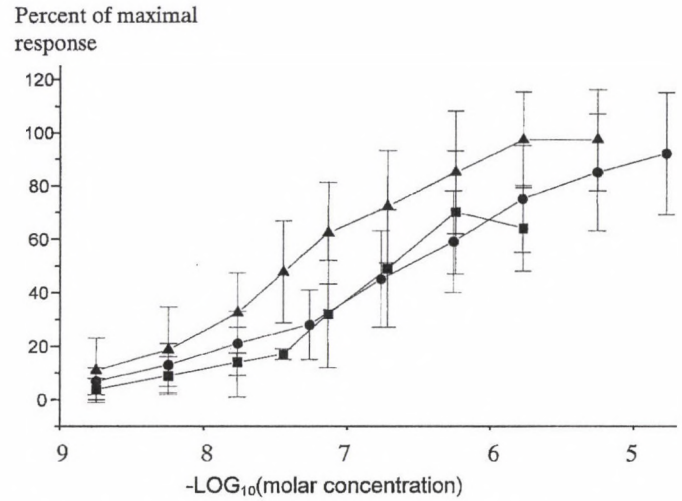

Fig. 7. Effects of 5-HT on isolated gastric fundus from control, anaesthetized and both anaesthetized and irradiated rats. Each point represents a mean response from 6 different animals. Error bars = standard deviations.
–■– 5-Hydroxytryptamine-control; –●– 5-Hydroxytryptamine+radiation+anaesthesia;
–▲– 5-Hydroxytryptamine+anaesthesia

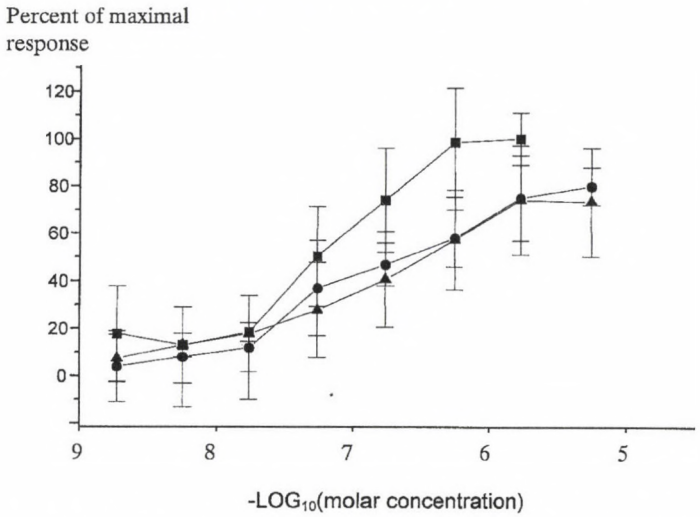

Fig. 8. Effects of 5-HT on isolated jejunum from control, anaesthetized and both anaesthetized and irradiated
rats. Each point represents a mean response from 6 different animals. Error bars = standard deviations.
–■– 5-Hydroxytryptamine-control; –●– 5-Hydroxytryptamine+radiation+anaesthesia;
–Δ– 5-Hydroxytryptamine+anaesthesia

maximal contraction on the recorder was 24.0±6.1 mm) (Fig. 9). The anaesthesia alone ($F=2.49$, $p>0.05$) and anaesthesia and irradiation together ($F=0.48$, $p>0.05$) did not affect the concentration-response curve for 5-hydroxytryptamine.

Effect of 5-hydroxytryptamine on rat ascending colon. The 5-hydroxytryptamine (1.89×10^{-8} M to 1.68×10^{-5} M) produced concentration-dependent tonic contraction of the isolated ascending colon from control rats ($r=0.95$; $p\leq0.01$; $EC_{50}=1.94\pm0.03\times10^{-7}$ M; maximal contraction of the recorder was 15.3±6.2 mm), anaesthetized rats ($r=0.99$; $p\leq0.01$; $EC_{50}=7.90\pm0.20\times10^{-8}$ M; maximal contraction on the recorder was 24.3±8.0 mm) and both anaesthetized and irradiated rats ($r=0.89$; $p\leq0.05$; $EC_{50}=3.16\pm1.12\times10^{-8}$ M; maximal contraction on recorder was 22.6±6.3 mm) (Fig. 10). The anaesthesia alone ($F=4.72$, $p\leq0.05$) and anaesthesia and irradiation together ($F=10.34$, $p<0.01$) produced significant increase of contractile response of rat jejunum on 5-hydroxytryptamine. However, the effect of x-radiation could not be differentiated from the effect of anaesthesia ($F=2.20$, $p>0.05$).

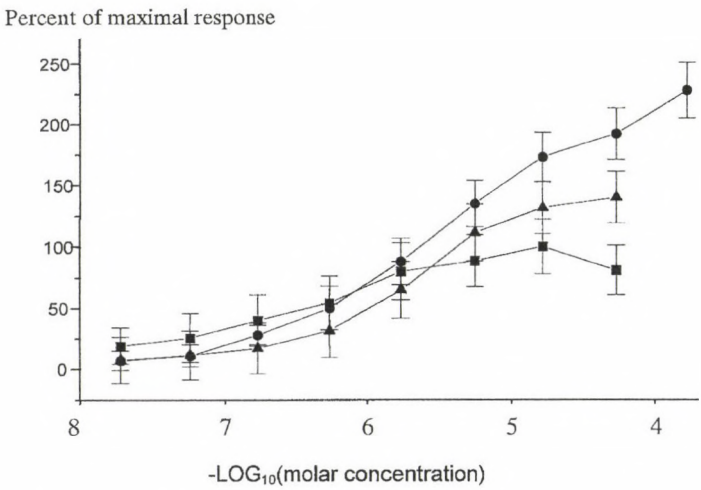

Fig. 9. Effects of 5-HT on isolated ileum from control, anaesthetized and both anaesthetized and irradiated rats. Each point represents a mean response from 6 different animals. Error bars = standard deviations. –■– 5-Hydroxytryptamine-control; –●– 5-Hydroxytryptamine+radiation+anaesthesia; –▲– 5-Hydroxytryptamine+anaesthesia

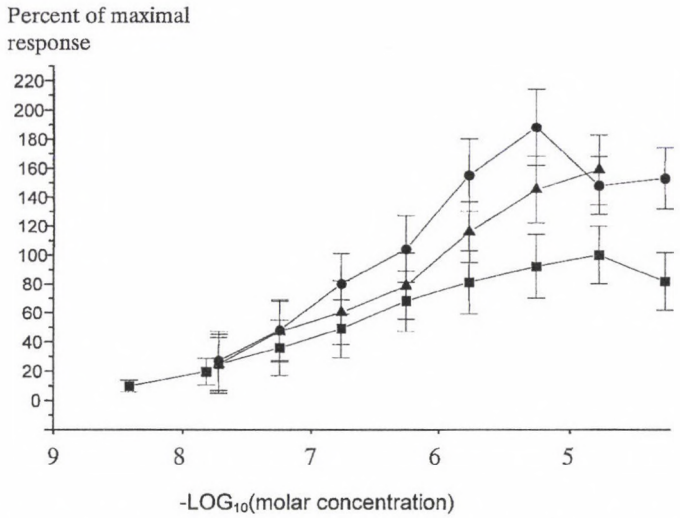

Fig. 10. Effects of 5-HT on isolated ascending colon from control, anaesthetized and both anaesthetized and irradiated rats. Each point represents a mean response from 6 different animals. Error bars = standard deviations.
–■– 5-Hydroxytryptamine-control; –●– 5-Hydroxytryptamine+radiation+anaesthesia; –▲– 5-Hydroxytryptamine+anaesthesia

Discussion

It is well known that ionizing radiation either directly or by formation of free radicals produces damage on macromolecules in living cells leading to loss of function and sometimes, to activation. However, not all macromolecules are affected in the same extent after certain absorbed dose [14]. Some of them are less vulnerable owing to smaller degree of tertiary structure entropy, and some are so outnumbered that inactivation (or activation) of one part will not affect total cell function (e.g. spare receptors). Acute gastrointestinal side effects of abdominal irradiation (vomiting, colicky pain and diarrhea) represent final outcome of changes in function of many enzyme and receptor systems. In our study we have examined acute influence of x-radiation of abdomen on contractile effects of acetylcholine, histamine and 5-hydroxytryptamine.

The abdominal irradiation in our study rendered all examined segments of gastrointestinal tract more sensitive and responsive to acetylcholine. This effect was independent of barbiturate anaesthesia. Gastrointestinal smooth muscle cells express muscarinic receptors, while intrinsic neurons express both muscarinic and nicotinic receptors [3, 8]. Both excitatory and inhibitory intrinsic neurons possess muscarinic and

nicotinic receptors on their membranes [19]. Contractile effect of acetylcholine observed in our study represents sum of its direct action on muscarinic receptors on smooth muscle cells and the effects of both excitatory and inhibitory neurotransmitters released from intrinsic neurons after binding of acetylcholine for receptors on their membrane. Potentiating effect of x-radiation could be consequence of two events: (1) direct sensitization of muscarinic receptors on smooth muscle cells resulting in both increased affinity and increased intrinsic efficacy, or (2) inactivation of inhibitory intrinsic neurons which are also targets for acetylcholine action. Which of these two events occurred in reality remains to be established by further experimentation. In any case, potentiating effect of radiation could be neutralized by means of muscarinic antagonists.

On the other hand, contractile effect of 5-hydroxytryptamine was not affected by x-radiation in neither of the examined segments. The 5-hydroxytryptamine is one of the most abundant neurotransmitters in the gut [13]. It has a direct contractile action on gut smooth muscle cells, but it also stimulates both excitatory cholinergic neurons and inhibitory neurons within the gut wall [2, 16].

Furthermore, it reduces the output of acetylcholine from cholinergic neurons with connections in myenteric plexus [15]. However, x-radiation did not affect any of these sites of 5-hydroxytryptamine action, questioning significant role of this neurotransmitter in radiation-induced gastrointestinal adverse effects. Of course, 5-hydroxytryptamine could be released from neurons or mucosal endocrine cells by radiation and increase gut motility, but this potential effect is out of scope of current experimental model. The lack of modulating effect of x-radiation on 5-hydroxytryptamine effects favors the first explanation of potentiation of acetylcholine-induced contractions by x-radiation; inhibitory intrinsic neurons are physiologically stimulated by both cholinergic and tryptaminergic nerve fibers and express both muscarinic and 5-HT receptors on their membranes. If inhibitory intrinsic neurons had been damaged by x-radiation, the contractile effect of 5-hydroxytryptamine should have been affected, too. It seems that muscarinic receptors on smooth muscle cells were principal target for X-rays.

Although histamine occurs in the gastrointestinal tract in substantial quantities (mostly concentrated in mast cells) its physiological role in regulation of gut motility has never been proved. More likely, histamine affects gut motility during various inflammatory conditions. Rat gastrointestinal smooth muscles are relatively insensitive to histamine; in our study, only gastric fundus was contracted by it in control rats. However, x-radiation sensitized both rat jejunum and ascending colon to histamine in our study: concentration-dependent contractions by histamine were observed after irradiation. Histamine receptors in the gut wall were found on both smooth muscle cells and cholinergic excitatory neurons [4, 5]. Sensitization of the isolated preparations by

X-rays either could be caused by direct activation of histamine receptors, or it only reflected potentiation of the effect of acetylcholine released from intrinsic cholinergic neurons. In either case, histamine released during x-radiation will significantly affect gut motility and could participate among causes of radiation-induced diarrhea.

The results of our study suggest that x-radiation sensitizes rat gastrointestinal tract to acetylcholine and histamine. Both substances occur physiologically in the gastrointestinal tract, playing roles of neurotransmitter and paracrine factor, respectively. It seems that symptomatic treatment of radiation-induced changes in gut motility should enroll blockers of both muscarinic and histamine receptors. Muscarinic receptor blocker have already been used in the therapy, but with varying success. Employing histamine H1 blockers instead (or combined with) should bring additional benefits. First, they have both antimuscarinic and antihistaminic actions; second, they have significant antiemetic and sedative properties.

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The effect of xylene inhalation on the rat liver

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In this study, 11284 mg/m^3 (2600 ppm) of xylene was administered for 8 hours a day to pregnant rats by means of inhalation, starting from the sixth day of their pregnancies. Furthermore, while a group of non-pregnant rats inhaled the same amount of xylene during the same period, the control group inhaled clean air. Consequently, in addition to the embryotoxic effects of xylene, the effects on the various tissues of the mothers and their litters were observed light and electron microscopes. No external anomalies were observed in any of the rats born at the end of the 21st day, and there were no macroscopic defects in their organs either. While following xylene inhalation no structural defects in the kidney and pancreas was found, expansions in the smooth endoplasmic reticulum of the liver tissues, increases in the lysosomes, and defective mitochondrion structures were found in the pregnant and non-pregnant rats. It was noticed that xylene in particular caused structural defects in the liver of the fetus. Compared to the control groups, increases were observed in the activities of the AST, ALT, ALP, and Arginase enzymes in the liver.

Keywords: xylene, inhalation, rat, liver

An increased perinatal death risk has been described in infants of women working in the pharmaceutical laboratories, university research laboratories and pulp and paper industrial laboratories [1, 2, 3, 4].

Starting with the utilization of benzene and its methyl alkaline derivatives, it was observed that certain symptoms were seen in people exposed to them, and the rate of anomaly births increased. In teratological studies in mice, rats and rabbits there was little evidence that benzene, toluene or xylene were highly teratogenic. In particular, extensive information was obtained on the biochemistry and toxic effects of benzene.

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Studies on xylene and toluene were fewer [5]. Emphasis was given particularly to the teratogenic, embryotoxic, and carcinogenic effects of these substances [6, 7, 8, 9, 10, 11, 12].

Regarding xylene, which is extensively used in conventional routine or research laboratories, embryotoxic effects [13, 14], liver enzymes and structural changes [15, 16] were observed by administering it in various ways to laboratory animals in different doses and time periods.

The purpose of this study was to examine the structural changes occurring in the liver of pregnant and non-pregnant rats, plus the changes in the liver of their litters, by exposing them to xylene via inhalation at a daily atmospherical density of 11284 mg/m^3.

Materials and Method

A total of 17 female Wistar rats (Gülhane Military School, Institute of Laboratory Animals, Ankara, Turkey) weighing approximately 180–200 g were used. Seven female rats were kept with male rats for one night and subjected to vaginal smear test the next morning. Females having sperm in their smear were accepted being day "0" of pregnancy. All the rats were maintained on a standard rat pellet and tap water ad libitum. Starting from the 6th day of pregnancy, they were subjected to inhaling xylene (C8H10, Merck, Germany) at a density of 11284 mg/m^3 for 8 hours a day (7 day/week, total 21 days) until term. A separate group of non-pregnant rats (n=5) was subjected to inhaling xylene at the same concentration and for the same period. Exposure to the solvent was performed in inhalation chambers of 0.194 m^3 volume. We used modified inhalation chambers of Tahti et al. [17]. Samples were obtained via a charcoal tube connected to the outlet of the cabinet and were examined by gas chromatography [18]. The test subjects in the control group (n=5) inhaled only clean air. Six pregnant rats gave birth to 39 pups. Immediately after birth, tissue samples were taken from 5 newborn rats. Five rats from each group were killed by ether anaesthesia for light and electron microscopy. Blood samples were taken from the aorta of control and pregnant rats. Tissue samples for light microscopy were fixed in 10% formaldehyde embedded in paraffin and they were stained with hematoxiline-eosin. In addition semi-thin sections were stained with toluidin blue-Azur II and examined. Tissue samples for electron microscopy were fixed in 1/15 M phosphate buffered 2.5% gluteraldehyde solution (pH 7.2) at +4 °C and then postfixed at +4 °C in phosphate-buffered 1% osmic acid for 1 h and dehydrated through gradedethyl alcohol series. These specimens were embedded in Araldite Cy 212 and sections were cut with LKB ultramicrotome. Thin

sections were stained with lead citrate-uranyl acetate and then photomicrographs were taken by Jeol 100CX II electron microscope.

With regard to the liver tissue samples of the pregnant rats, the Reitman and Frankel method [19] was used for monitoring AST and ALT enzyme activities, the Bessey et al. method [20] for ALP enzyme activity, and, the TDMU method [21] for Arginase enzyme activity.

Results

The rats born approximately 21–22 days later weighed an average 5.7 g (4.8–7.5 g, total 39 pups), and did not have any external anomalies and anomalous organs. Administration of xylene by inhalation to pregnant rats on days 6 to 21 gestation did not adversely affect maternal body weight gain. Body weight gain of the non-pregnant rats did not differ significantly from the control values.

Histological observations

Compared to the control group, no structural changes were observed in the pancreas and kidney tissues of the litters under light and electron microscopy. Likewise, no effects or structural changes were determined in the pancreas and kidney tissues of the pregnant and non-pregnant rats.

In the liver tissue of the newborn rat, it was noticed in the light microscope that, compared to the control group, the membranes of hepatocyte nuclei had become disarrayed, heterochromatic nuclei were located in between, and, that there were quite a lot of vacuolization in the cells. At the electron microscope level, it was seen that the mitochondria in the hepatocyts had become deformed, swollen, elongated and that the rough endoplasmic reticulum in particular had dilated and in the hepatocytes a vacuolization like structure seemed to have appeared. In the cytoplasma of Kupffer cells, dense primary and secondary lysosomes were observed (Figs 1, 2). While at light microscope it was seen that the cytoplasm of the hepatocytes in the liver of the pregnant and non-pregnant animals were much more lightly coloured than to the control group. At electron microscope level, compared to the control group, it was determined that the mitochondria had concentrated particularly at the periphery of the hepatocytes and around the nucleus, that the number of lysosomes had increased, and, that there were numerous smooth endoplasmic structures having a web-like appearance with glycogen particles among them (Figs 3, 4).

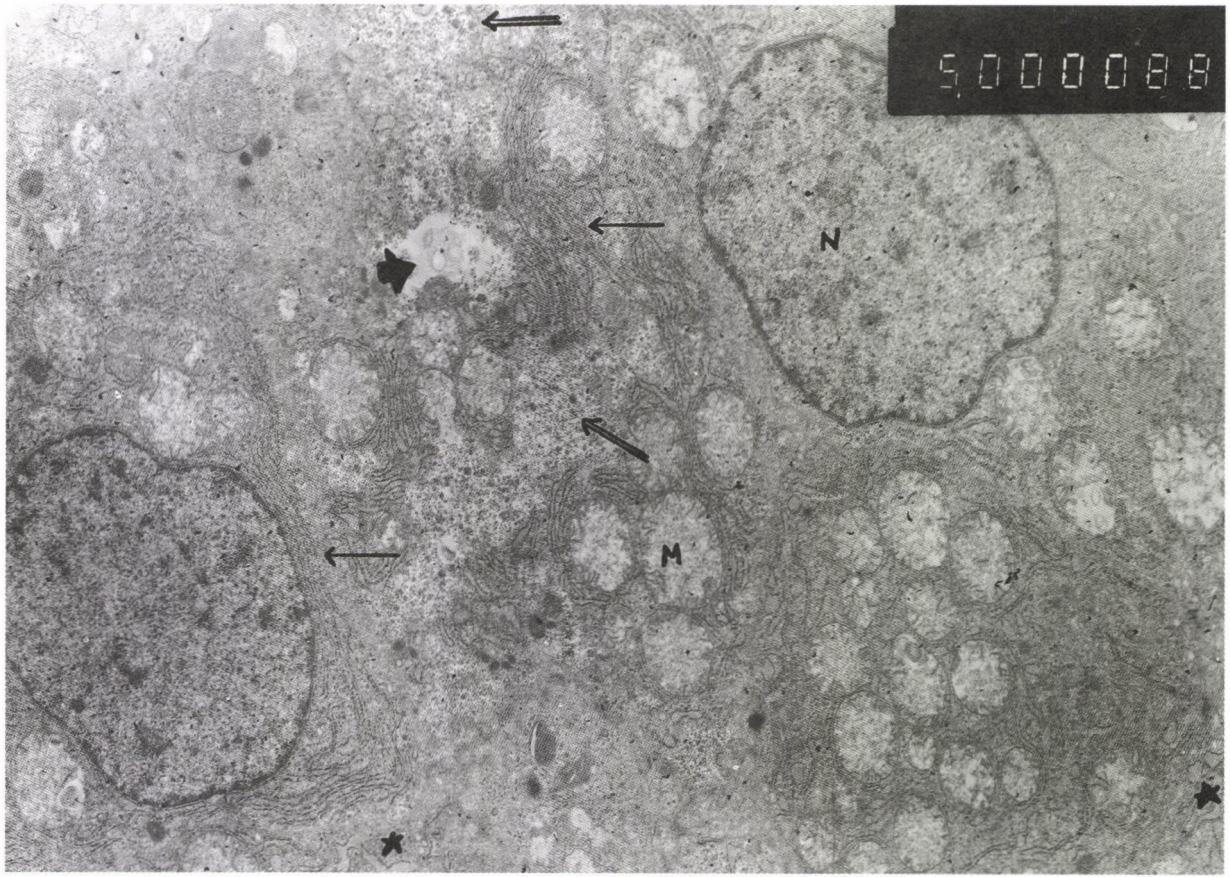

Fig. 1. Electron microscopic photomicrograph of hepatic tissue control group. Nucleus (N), mitochondria (M), rough endoplasmic reticulum (single arrows), glycogen granules (double arrows), bile canaculi (arrowhead) and smooth endoplasmic reticulum (*). Lead citrate-uranil acetate (original magnification 5000)

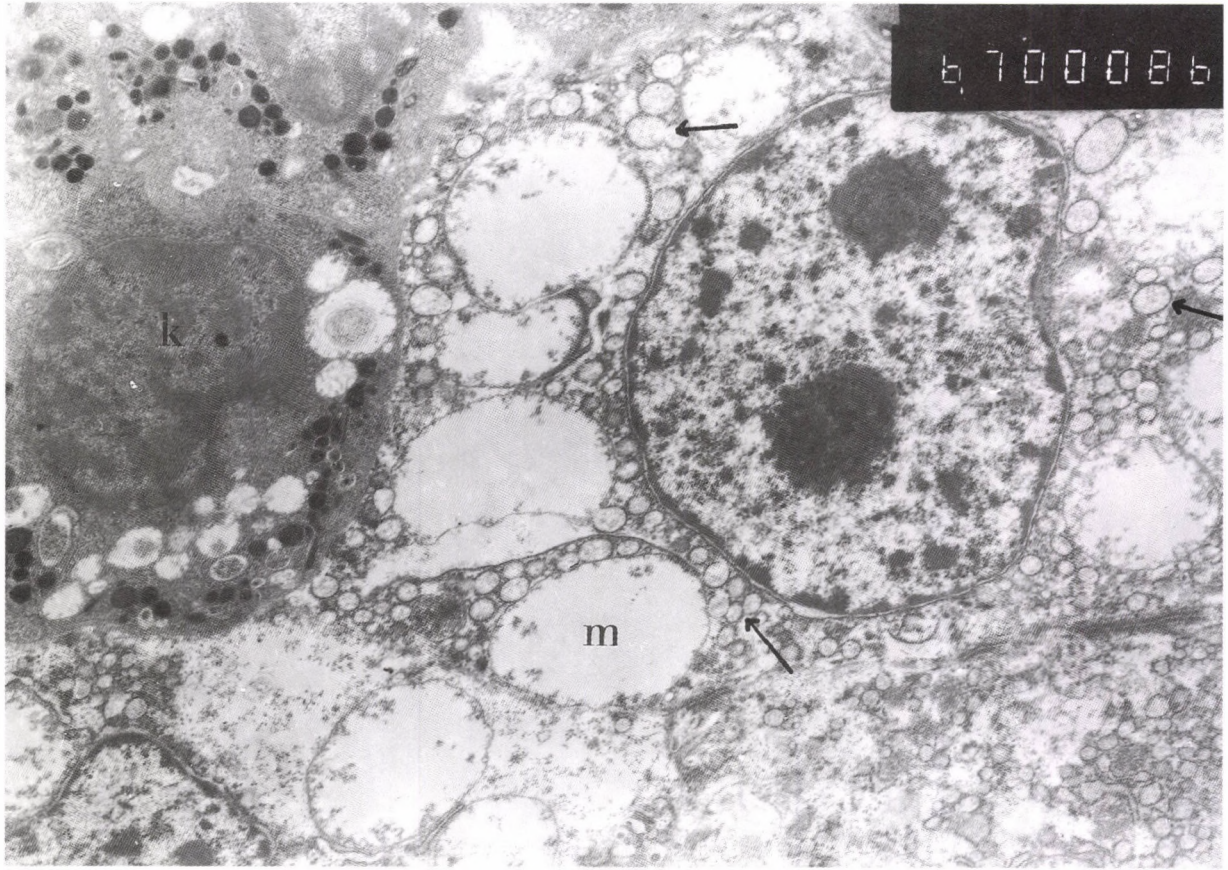

Fig. 2. Photomicrograph of newborn rat experimental group shows expanded mitochondria (M), dilated and vacuolized rough endoplasmic reticulum (arrows), Kuppfer cell (K) with lysosome granules in its cytoplasm. Lead citrate-uranil acetate (original magnification 6700)

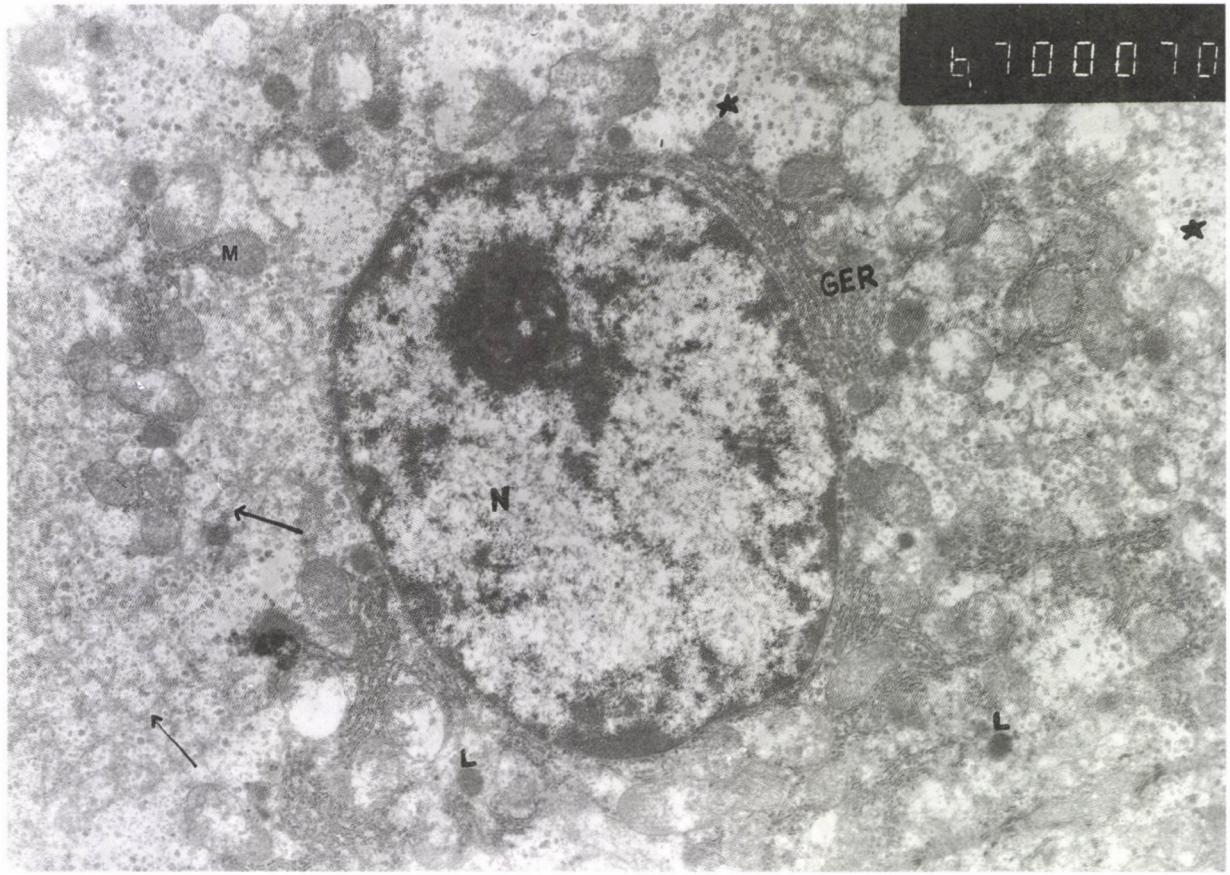

Fig. 3. Xylene exposed pregnant rat liver shows hepatocyte nucleus (N), rough endoplasmic reticulum (GER) derived from nucleus membrane, web-like dilated smooth endoplasmic reticulum (arrows), mitochondria (M), glycogen granules (*), lysosomes (L). Lead citrate-uranil acetate (original magnification 6700)

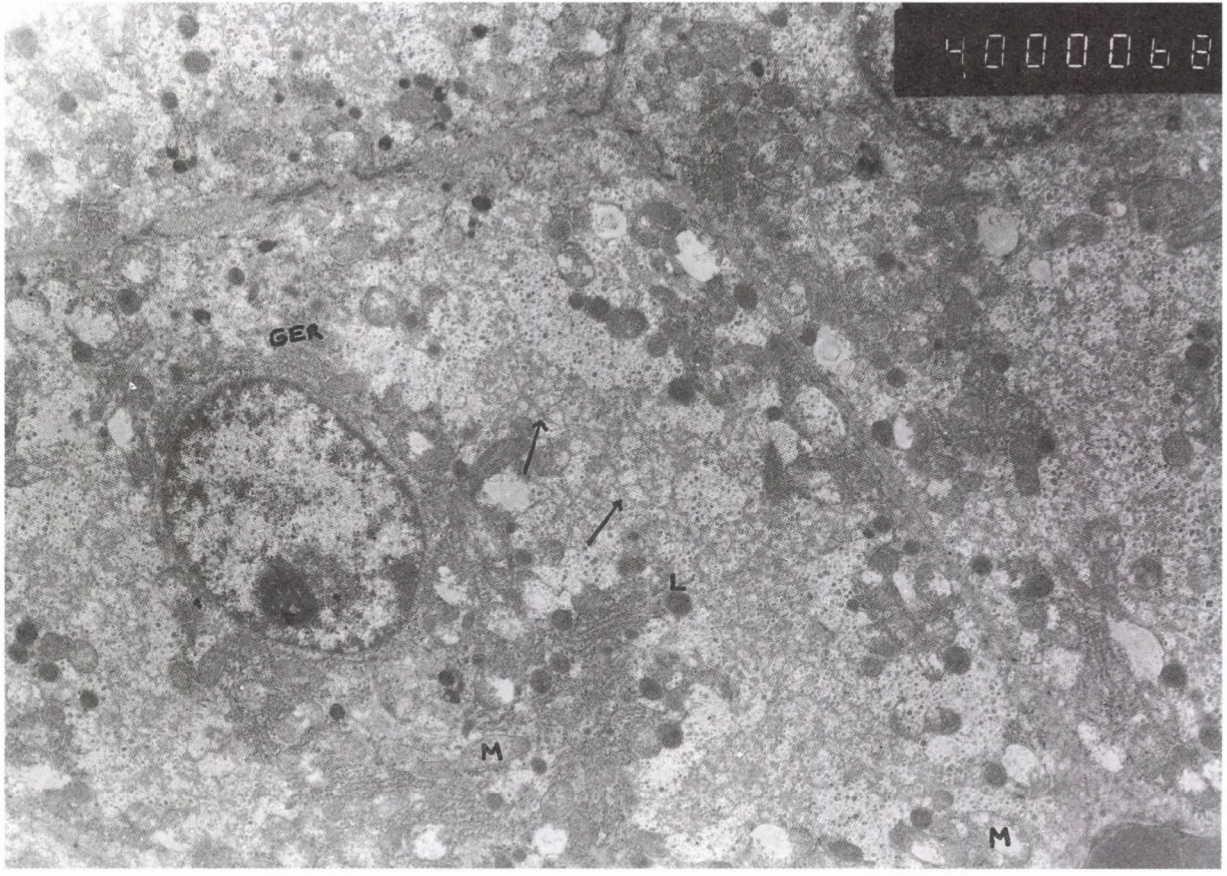

Fig. 4. Photomicrographs of the same group in Figure 3 shows increased number of lysosome (L) and concentration of various shape mitochondria (M) at the periphery of the hepatocytes and around the nucleus, rough endoplasmic reticulum (GER) and web-like smooth endoplasmic reticulum (arrows). Lead citrate-uranil acetate (original magnification 4000)

Table I

The effects of xylene inhalation on biochemical parameters in pregnant rats liver tissue

	Control (n=5)	Xylene (n=5)	Differences, %
AST (U/l)	28.49 ± 14.86	33.71 ± 2.48	18↑
ALT (U/l)	21.4 ± 14.27	25 ± 5.31	19↑
ALP (U/l)	4.5 ± 0.7	5.25 ± 2.06	17↑
Arginase (U/l)	83.63 ± 11.17	136.5 ± 2.39	63↑
Hb gr/dl	14.24 ± 1.27	13.38 ± 0.38	
HCt %	45 ± 4.24	42 ± 1.63	

From the biochemical aspect, it was determined that, compared to the control group, the AST activity in the liver had increased by 18%, the ALT activity by 19%, the ALP level by 17%, and the Arginase activity by 63%. In addition no significant increase in blood samples of the animals were detected in the Hb and Hct values (Table I).

Discussion

In view of the embryotoxic effect of aromatic substances we have investigated the toxic effects of xylene routinly used in our laboratory. No pathological deformities or abnormalies were seen in the pups of the pregnant rats which were exposed to xylene inhalation (2600 ppm) from day 6 of pregnancy to labour for 8 h a day. Ungváry et al. have reported a malfarmation rate of 1% in the pups of rats inhaling 3400 mg/m^3 xylene and a malformation rate of 6% when xylene was inhaled at a concentration of 1000 mg/m^3 [22].

In this study, we have investigated the effects of xylene on various tissues of pregnant, non-pregnant rats and pups at light and electron microscopic level. Structural effects of xylene on the liver were found to be similar to those reported previously [15, 16, 23, 24]. Compounds like xylene, toluene and benzene effect the enzyme system of the liver in particular [23]. In this study, the electron microscopic findings pertaining to the liver tissues of the test subjects which inhaled xylene at an atmospherical density of 11284 mg/m^3 (2600 ppm) were conforming to the structural changes defined in various literatures. Furthermore, in this study, similar findings which were not defined before had been obtained from the liver tissues of the infant rats. It was determined that xylene also affected the fetal liver tissue, and there were a significant amount of over expanded smooth endoplasmic reticulum in the hepatocytes, structurally deformed mitochondria, and granular endoplasmic reticulum which were expanded at certain points. It was

observed that in the pregnant and non-pregnant groups, while GER surronding the nucleus remained normal, the smooth endoplasmic reticulum had assumed a web-like form and there were moderately dense glycogen particles. Mitochondria concentrated around the nucleus and the sides of the hepatocytes, and, the number of lysosomes increased.

In previous studies realized on an experimental level, the cross of benzene, toluene, and xylene to placenta was observed in pregnant mice, and it was stated that the passage of the substance, labelled with radioactive C, to the brain, fat, liver, and kidney tissues of the mothers metabolites reached peak levels around 30 min to 1 hour. It was observed that the fixation of these compounds were lesser in fetal tissues compared to the mother [15]. It was determined that m-xylene in particular was trapped in the liver mainly [25].

In 1980, Ungváry et al. [12] and Tatrai et al. [15], by using luminous and electron microscopes, examined the changes occurring in the liver tissues of rats inhaling xylene at atmospherical densities of 1500, 3000, and 3500 mgr/m^3, respectively. They stated that, regarding the liver, the number of smooth endoplasmic reticulum vesiculae had increased, the nuclei distribution in the cells had changed and grown, and, that there was dilatation in the Golgi complex, and changes in the shapes of the mitochondria.

In a study made during 1990 [16], xylene was administered subcutaneously, intraperitoneally, orally, and by means of inhalation to mice, rats, and rabbits, and their liver tissues were evaluated from histological, enzymatic, and biochemical aspects. Liver sections were stained with PAS and Best-Carmin in order to observe the glycogen distribution, and it had been determined that there was the decreased in the glycogen amount of the test subjects which inhaled xylene at atmospherical intensities of 1500 and 4000 mgr/m^3, respectively. The following were observed at a microscopic level: 1) The amount of expanded smooth endoplasmic reticulum had increased in the centrolobular sections, and areas deprived of glycogen were observed in the hepatocyts, and 2) Mitochondria in various forms, autophagic fragments, and an expanded granular endoplasmic reticulum were determined. In particular, it was observed that the smooth endoplasmic reticulum, which was stretched at various points, gave an appearance similar to mezakson.

We contemplated that these structural changes occurring in the liver tissues of infant and adult rats may have occurred as a result of a defense mechanism against the toxic effects of xylene. Since the xylene we used in the experiment was a mixture of ortho-, meta, para-xylene as used in the market, we were unable to assess which isomer was related to the changes we had observed. However, previous studies have revealed that ortho-xylene was the most effective isomer [12].

In this study, it has been assessed that, compared to the control group, the liver AST, ALT, ALP, and arginase enzyme activities had increased, and, that there was a

decrease in hemoglobin and Hct values. In the study realized by Tahti et al. [17], it has been observed that the Hct values of rats subjected to inhaling toluene for short and long periods were lower, while their blood sugar values were higher compared to the control group. Meanwhile, they had also stated a statistically significant increase in the serum ASAT and ALAT values.

In conclusion, the embryotoxic effects of xylene and its impact on tissues are directly related to the dose and amount of inhalation. In our study, while no embryotoxic effects were observed, it was seen that the kidneys and pancreas were unaffected, and that structural changes occurred in the liver tissue. Therefore women in jobs with a risk of xylene exposure should be immediately removed from the hazardous environment when pregnancy is established.

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Measurement of the residual urine index in insulin-dependent and non-insulin dependent diabetic men with and without neuropathy

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Value of the residual urine index was evaluated in 40 individuals both insulin-dependent (IDDM) and non-insulin dependent (NIDDM) diabetic male patients with and without an objective evidence of neuropathy and in 20 age matched non-diabetic men serving as controls using post void bladder ultrasonographic technique. These studies revealed striking results in the neuropathic group. Both IDDM and NIDDM diabetic patients with neuropathy exhibited a significant ($P<0.005$) increase in residual-volume in comparison with the controls of the same age group and a direct correlation between residual urine retention and neurogenic bladder was found to be established thus suggesting a generalized massive hypotonia of the bladder in these patients. However, non of the two types of non-neuropathic diabetic patients showed significant difference in the above-mentioned parameters compared to that their respective controls. A non-significant association in the values of the study parameters between insulin dependent and non-insulin dependent diabetic men (with and without neuropathy) was also observed. These findings thus suggest a probable neuropathic involvement in the pathway of urinary tract in both IDDM and NIDDM diabetic men with neuropathy. The greater impairment of the values of residual urine index in these patients may be due to overall greater severity of neuropathy with sympathetic as well as parasympathetic damage irrespective of their type of diabetes.

Keywords: diabetes mellitus, neuropathy, residual urine index, ultrasonography

Diabetic patients suffer frequently from urologic disease. Disorders of the urinary tract in diabetic patients are similar in many ways to those of non-diabetics, but special factors account for distinct differences. Changes in the tissues, especially in the capillaries and larger blood vessels, alter functions in the organs involved, particularly the kidney is associated with a lessened ability of the tissue to combat infection.

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Thickening of the capillary basement membrane, arteriosclerosis, and arteriolosclerosis account for many of the problems in diabetes. Neuropathy involving both parasympathetic and sympathetic supply to the lower genitourinary system produces significant problems of the bladder and sexual dysfunction. The neuropathic changes in the genitourinary system chiefly affect the bladder and the sexual function. In diabetes the common lesion produces damage to the afferent or sensory part of the reflex arc resulting in a bladder of diminished or absent tone. This tends to produce incomplete emptying of the bladder [1, 10]. With marked loss of sensation, and a minimal rise of pressure of 5 to 10 cm even up to a volume of 1000 ml associated with a strong urge to void. These symptoms are often associated with incontinence [15].

There is a close correlation with other neuropathic changes in these individuals; i.e. diminished anal sphincter tone, absence of bulbocavernosus reflex, absence or diminution of knee jerks and ankle jerks, and neuropathic changes in the bladder [3, 6]. Although disturbances of micturition have long been recognized as a feature of diabetes [5, 9, 14], but the pathologic changes responsible for bladder dysfunction have not been extensively studied. Only a few studies of the pathologic changes in the autonomic nervous system in diabetes have been reported in the literature [2, 11, 29].

Alterations in capillary permeability, impaired axonal flow, and abnormal myo-inositol metabolism have been suggested as possible etiologic factors in the development of diabetic neuropathy [8, 27]. Diagnosis of urinary bladder dysfunction in diabetes mellitus has been facilitated during the past decade by increasingly sophisticated techniques for bladder evaluation [10, 13, 14]. Diabetic autonomic neuropathy affecting the gastrointestinal tract and vascular reflexes have also been reported [20, 23]. A bladder capacity of 500 ml is regarded as the upper limit of normal [18]. The earliest neurological deficit in diabetic patients with neurogenic bladder is said to be the loss of bladder sensation due to involvement of the afferent sympathetic nerves [22], and the increased capacity probably arises from defective perception of bladder filling. Peripheral neuropathic changes are also often sub-clinical while motor as well as sensory nerves may be affected [21, 25].

Neurogenic bladder dysfunctions are now established as a frequent complication of diabetes mellitus [12, 14] and are considered as a manifestation of autonomic neuropathy [17]. Vesicle ultrasonography is a simple technique for the determination of residual urine [29].

In the present studies we have used the vesicle ultrasonographic technique to evaluate residual urine index in both IDDM and NIDDM diabetic men (with and without neuropathy). The main aim of this study is therefore to provide data on the prevalence of neurogenic bladder dysfunction, as appreciated by incomplete voiding in these patients, and to determine possible relationship with the type of diabetes and the presence of manifestations of autonomic neuropathy.

Methods

The presence of residual urine (RU) was measured in 20 insulin dependent (IDDM) and 20 non-insulin dependent (NIDDM) diabetic men with and without an objective evidence of neuropathy and in 20-age matched non-diabetic men serving as controls by established postvoid bladder ultrasonographic technique [29].

All insulin dependent (IDDM) diabetics were aged 20–74 years mean 45.5, duration of diabetes 1–30 years mean 20, and non-insulin dependent (NIDDM) diabetics aged 17–78 years, mean 42.5 with known duration of diabetes 1–32 years, mean 21, male patients. The control subjects, 20 men aged 18–75 years, mean 41.7 were hospitalized patients without glycosuria or post-absorptive blood glucose levels above 5.0 mol/L; none had neurological abnormality at physical examination. All subjects were informed of the aim of the study. The presence of autonomic failure was determined by impotence, gastric atony (shown by contrast radiology) diarrhea (characterized by episodes of fecal incontinence and nocturnal bouts), loss of pupil motility, heart rate variation during deep breathing and orthostatic hyotension (a fall of at least 35 mm Hg in systolic arterial blood pressure in upright posture).

Ultrasonographic studies were all performed with a picker 80L immediately after vesicle voiding on an ATL ultramark 10 selector scanner using a 3.5 MHz or 5.0 MHz tranducer (Advanced technology labs. Inc., USA). When residual urine (RU) was detected, the largest transversal (T), longitudinal (L), and anterio-posterior (AP) dimensions (cm) were determined and an index of RU was calculated as AP×L×T. The presence of hydro-ureteronephrosis was determined in all subjects and an intravenous pyelogram (IVP) was performed and a post-void cystogram obtained.

Results

A comparison of the measurement of the residual urine (RU) index values in IDDM and NIDDM diabetic men with and without neuropathy and their respective controls is presented in Table I. This comparison revealed a significant ($P<0.005$) increase in the volume of residual urine in both IDDM and NIDDM diabetic men with neuropathy compared to their age matched non-diabetic control subjects. However, none of types of diabetics without neuropathy showed significant difference in these values and were within the normal limits. Clinical examination showed no signs and symptoms of hydro-uteronephrosis is any subjects, however, of all the diabetic men with neuropathy palpable painless bladder distentia was noted. Ultrasonography was repeated with an interval between 4 weeks and 2 months in all the subjects. No significant difference was observed between the two tests, however, residual urine was

found to be a permanent finding only when the residual urine index was above 30 in both IDDM and NIDDM diabetic neuropathic men (data not shown).

A comparison of the measurement of the residual urine index values between IDDM and NIDDM diabetic men with and without neuropathy is presented in Table II.

Table I

Comparative studies of the measurement of residual urine (RU) index values in insulin dependent (IDDM) and non-insulin dependent (NIDDM) diabetic men with and without neuropathy and in age-matched non-diabetic controls

Subjects		Residual urine index values (mean ± S.D)
IDDM without neuropathy	(n=10)	27*
IDDM with neuropathy	(n=10)	320
NIDDM without neuropathy	(n=10)	28*
NIDDM with neuropathy	(n=10)	350
Non-diabetic controls	(n=20)	25

n = Total number of subjects examined. IDDM and NIDDM (with and without neuropathy) values are compared with age-matched non-diabetic controls for t-test.
* = $P<0.005$.

Table II

Comparative studies of the measurement of residual urine (RU) index values between insulin dependent (IDDM) and non-insulin dependent (NIDDM) diabetic men with and without neuropathy

Subjects		Residual urine values (mean ± S.D)
IDDM with neuropathy	(n=10)	320
NIDDM with neuropathy	(n=10)	350
Statistical difference		Not significant
IDDM without neuropathy	(n=10)	27
NIDDM without neuropathy	(n=10)	28
Statistical difference		Not significant

n = Total number of subjects examined. IDDM and NIDDM (with and without neuropathy) values are compared with each other for t-test

Table III

Comparative studies of the significant residual urine index values, age, known duration of diabetes, and the presence of symptoms of autonomic neuropathy in insulin dependent (IDDM) and non-insulin dependent (NIDDM) diabetic men (with and without neuropathy) and in age-matched non-diabetic controls (data are shown as absolute values of mean and extreme values)

Subjects	Age (Years) (Mean)	Duration of Diabetes (Year)(Mean)	Residual urine index values (Number)	Autonomic neuropathy (Differential diagnosis)					
				Heart rate vibrations beat/min. (mean)	Orthostatic hypotension	Gastric atony	Nocturnal diarrhea	Impotence	Decreased pupil motility
IDDM without neuropathy (n=10)	20–74 (45.5)	5–25 (21.0)	2/10	0–45 (9.5)	1/10	0/10	1/10	1/10	2/10
IDDM with neuropathy (n=10)	18–68 (42.2)	1–30 (22.5)	9/10	0–40 (10.5)	9/10	8/10	10/10	9/10	8/10
NIDDM without neuropathy (n=10)	20–78 (46.5)	7–20 (22.0)	1/10	0.38 (9.8)	1/10	1/10	2/10	2/10	2/10
NIDDM with neuropathy (n=10)	17–70 (43.5)	1–32 (21.5)	10/10	0–36 (10.5)	10/10	10/10	8/10	10/10	9/10
Non-diabetic controls (n=20)	18–75 (41.7)	–	0/10	0–39 (10.2)	0/10	0/10	0/10	0/10	0/10

n = Total number of subjects examined

A non-significant difference in the values of the above-mentioned parameters was observed among these individuals, although these values were found to be slightly greater in NIDDM diabetic neuropathic men than their respective IDDM diabetic neuropathic group. These results indicate a neuropathic involvement in the pathway of micturition reflex in both IDDM and NIDDM diabetic men with neuropathy. The greater impairment of the residual urine index values in these patients may be due to over all greater severity of neuropathy in these individuals with sympathetic as well parasympathetic damage irrespective of the type of diabetes.

A comparison of the values of residual urine index calculated either by ultrasonography or with post-voidal cystogram during intravenous pyelography in all the diabetic subjects with neuropathy further confirmed incomplete voiding in these patients; No such residual volume at either test (intravenous pyelogram or ultrasonography) was noted in both types of non-neuropathic diabetics and in non-diabetic control subjects (data not shown).

A comparison of the values of significant residual urine index, age, known duration of diabetes and the symptoms of the presence of autonomic neuropathy in IDDM and NIDDM diabetic men (with and without neuropathy) and their respective controls is presented in Table III. Prevalence of residual urine was found to be increased in both IDDM and NIDDM diabetic men with neuropathy respectively compared to their respective controls. However both types of non-neuropathic patients showed more or less similar residual urine index values than non-diabetic control subjects. All the manifestations of autonomic neuropathy failure studies were found to be directly correlated with neurogenic bladder.

Discussion

The present data showed a significant residual volume in both IDDM and NIDDM diabetic patients having an objective evidence of neuropathy. However we could not found any imaging of residual urine in any of the non-neuropathic groups as well as non-diabetic control subjects. The presence of residual urine in both IDDM and NIDDM patients was strongly associated with the presence of autonomic neuropathy. These investigations have thus established an arbitrary index of residual urine (RU) by considering that the residual volume was significant only for and index above 30, since no control subject had an index exceeding 30.

Residual urine appears to be a frequent complication of both IDDM and NIDDM patients [29, 28, 7]. We observed it only in neuropathic patients. However absolute confirmation of these results need bladder catheterization to measure exactly the residual volume, but such a technique has already been rejected due to its septic risk

[24]. We have therefore confirmed our results by intravenous pyelogram thus suggesting a direct correlation between residual urine retention and the neurogenic bladder. In our results although incomplete voiding was found to be more frequent in both types of diabetic patients suffering from neurophathy, we also found it in some cases in both IDDM and NIDDM diabetic patients without neuropathy. Such a dissociation, has also been reported previously, for the cardiac autonomic abnormalities [30, 23, 19].

On the basis of our data and similar findings of the previous workers [23, 29], it is suggested that the somatic and autonomic nervous system could be effected separately during the evolution of diabetes mellitus. In our results, the association of neurogenic bladder with other manifestations of autonomic neuropathy further showed interesting observations where all the manifestations of autonomic neuropathy were found to be associated with residual urine. However these data should be interpreted cautiously because impotence [4], diarrhea [26], and orthestatic hypotension [16] could also be due to factors other than autonomic neuropathy. However our results regarding heart rate variation in both IDDM and NIDDM patients with and without neuropathy allows a definite interpretation. Our results did not show any significant difference in the heart rate variation between the diabetic subjects with and without neuropathy, despite a trend for lower values in the non-neuropathic group. At the same time, however, in some patients neurogenic bladder was observed in the absence of cardiac autonomic neuropathy. In conclusion the present findings confirm that incomplete voiding appears to be a frequent complication of diabetes mellitus, particularly in patients with neuropathy irrespective of their type of diabetes.

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The effects of artificial tear solutions on wound healing in full thickness corneal incisions

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Carbopols (carbomer, polyacrylic acid) are appropriate for ophthalmic use as an artificial tear in the form of viscous aqueous solutions. Carbopol 940 preparations were developed as long-lasting artificial tears for the relief of dry eye syndrome and traumatic injury.

We identified the 15 days local treatment effects of two artificial tear solutions by wound strength and histologic examination of the incision wounds of rabbit corneas by compairing these results with the controls.

Three layers of control corneas were regular. The untreated but wounded corneas epithelium and stroma were completely irregular. Both treated eyes had thinner epithelization in the incision site compared to control unwounded eye. Wounds treated with A preparation (viscotiers®) had vocuoles and numerous inflammatory cells and remarkable oedematous regions but B preparation (Thilo-Tears®) treated wounds had inflammatory cells and oedematous regions less than the other group. The wound strengths of gel treated wounds were bigger than those of controls.

A considerable result in wound strength and better wound healing was also obtained in B preparation treated group because of the arrangement of the pH and tonicity at the Thilo-tears® gel preparation.

Keywords: artificial tear solutions, carbopols, histology of corneal incision wounds, wound strength, rabbit

The term bioadhesion has been used in recent years to describe phenomena related to the ability of some synthetic and biologic macromolecules and hydrocolloids to adhere to biological tissues [2, 12]. Bioadhesive systems have been used for many years in dentistry and orthopaedics and for surgical applications [6, 10]. However,

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recently there has been also a significant interest in the use of bioadhesives in other areas such as soft tissue based artificial replacements and controlled release systems for local release of bioactive agents [3, 4]. Carbopols (carbomer, polyacrylic acid) are suitable for ophthalmic use as an artificial tear in the form of viscous aqueous solutions. Ophthalmic vehicles of carbopol 940 had an excellent appearance and clarity when compared with the other types of polymer [18]. Although many of viscolizers are used to reduce the surface tension significantly, their effect on ocular drug bioavailibity have been found to correlate to their surface activity [13]. Consequently, polyacrylate preparations were developed as long lasting artificial tears for the relief of dry eye syndrome and traumatic injury [19].

In the present study we identified the effects of two different compositions of carbopol as artificial tear solutions with/without arranged pH and tonicity on the corneal incisions wound strength and the histology.

Materials and Methods

Animals

Seventeen adult male and female New Zealand albino rabbits weighing 1.5± 0.5 kg were used. Animals were placed in separate cages. They were maintained under 20 °C temperature and daily light/dark cycle and fed with normal diet and vegetables for 15 days.

Corneal Wounds

A total of 17 rabbits were anesthetized with IP Ketalar+Rompun and topical propacain prior to wounding. Five mm long corneal full-thickness wounds were made horizontally with surgical blade on the limbus of right cornea, causing loss of the aqueous humor, under direct visualization through an operating microscope. Two sutures were placed at the middle of the incision for closing the wound. Wounded eyes of 12 rabbits were topically treated with two different artificial tear solutions. The topical therapy was started on the day after the surgery and after treatment for 14 days the wound healing was compared with the controls by histological techniques and by wound strength of corneal incisions.

Seventeen rabbits were studied; two groups 6 animals in each were treated by artificial tears and 5 animals served as untreated controls. In the first group 5 µl A preparation was applied on the right eyes twice a day and B preparation was applied in

equal volume and frequency to the right eyes of the second group. The left eye of the animals served as unwounded and untreated control.

Compositions of artificial tear solutions

A preparation: Viscotears® ophthalmic liquid gel (DISPERSA AG Hettlingen/Switzerland); Each gram contains poly-acrylic acid 2 mg, preservative cetrimide 0.1 mg.

B preparation: Thilo-tears® ophthalmic gel (Dr. Thilo Co. Gmbh, Alcon Couvreur, Belgium); Each gram contains carbomer 3 mg, mannitol 50 mg, preservative thiomersal 0.04 mg, sterile water 945.712 mg, pH: 7.5.

On completion of the treatment protocol all rabbits were killed with an overdose of intravenously administered pentobarbital sodium. Under visualization through operating microscope (Olympus SZ-PT), a 25 gauge disposible needle was inserted through the prelimbal clear cornea at the 6 o'clock meridian into the anterior chamber. The needle was connected by plastic tubing to a pressure transducer (P23 XL High Pressure Transducer) and a pump (Eyela Microtube Pomp MP-3). The sutures were cut with a microsurgical blade and removed with tying forceps. Buffered saline was pumped into the anterior chamber at a speed of 5 and the output of pressure transducer was recorded on a polygraph (Grass model 7).

Statistics

Statical analysis was carried out using Mann-Whitney U test.

Histology

Corneal structure was examined histologically at the end of the 14-day application period. Corneal wounds and control corneal pieces were cut into small pieces, fixed in 2.5% buffered gluteraldhyde for 2 hours and then postfixed in 1% osmium tetraoxide, dehydrated in serial alcohols and embedded in araldite. The thick sections of 0.5 μm were then stained with toluidine blue and examined under Olympus BH2 light microscope.

In this study animals were used in accordance with the Association for Research in Vision Ophthalmology (ARVO) resolution on the use of animals in research.

Results

At the end of the treatment; the control corneas and treated corneas were compared. Eye sections had three major corneal constituents in all groups: epithelium, stroma and endothelium. In three layers of unwounded and untreated eyes of controls were regular (Fig. 1). The wound region of wounded but untreated corneas were not healed. Epithelium layer was thinner and irregular, stroma was highly irregular (Fig. 2). A preparation and B preparation treated corneal wound histologies are shown in (Fig. 3a, 3b) respectively. Both treated eyes had thinner epithelization in the incision site compared to controls.

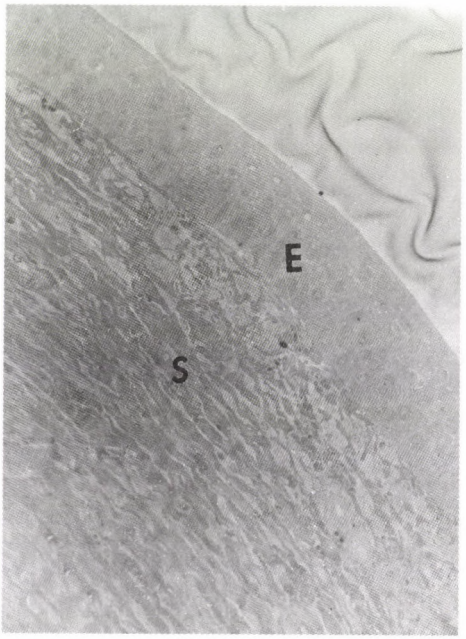

Fig. 1. The histology of normal cornea. Epithelium layer (E), stroma (S) in the control group. Toluidin blue × 400

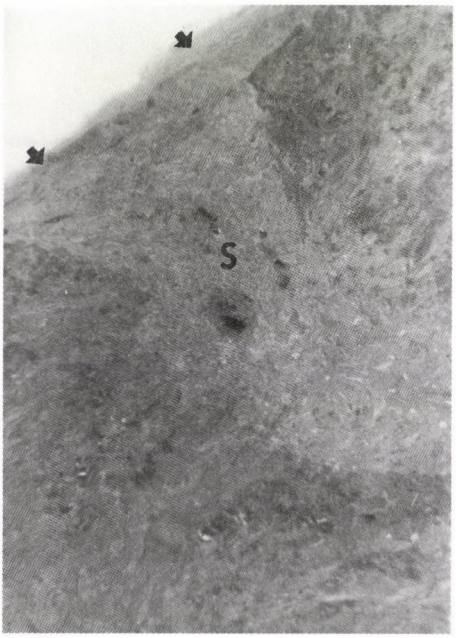

Fig. 2. The histology of untreated but wound cornea on the 15th day. Wound area has not completely healed. Epithelium layer was thinner and irregular, stroma was very much irregular (S). Toluidin blue × 400

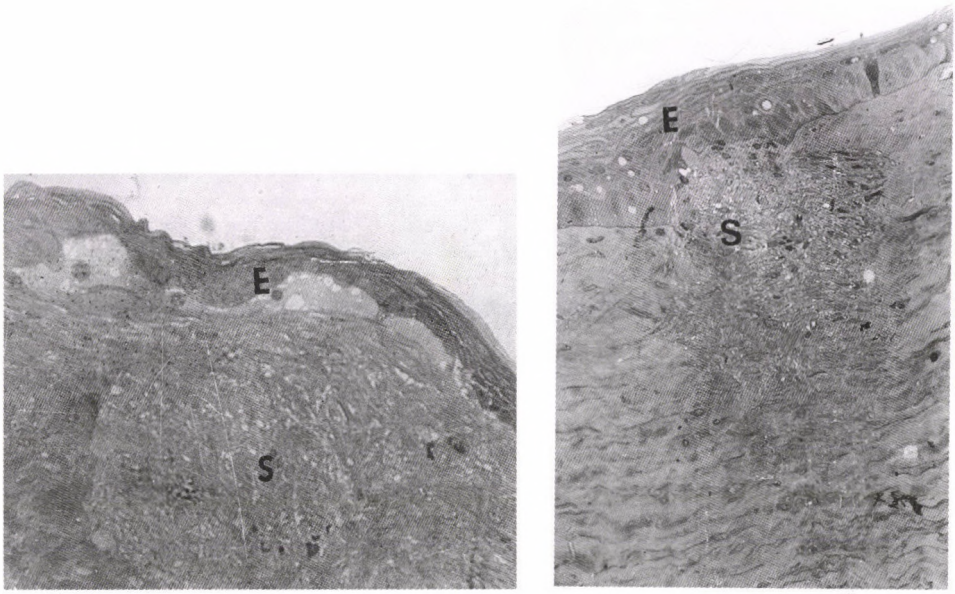

Fig. 3. The histology of wounded but treated with artificial tears on the 15th day. a. A preparation treated corneas: Although epithelization has begun in the incision site, cells from 2 or 3 layer (E). There is also a striking oedematous region between the epithelium and stroma (double arrows). In this region, numerous inflammatory cells (*) are observed together with small and big vacuoles (arrow). In the stroma, irregularly scattered collagenous fibers and among them inflammatory cells are found (S). Toluidin blue × 400. b. B preparation treated corneas: repaired stratified squamous epithelium (E) is observed in the incision site of cornea. Stroma is found out of to have lost regular fibrillary structure in the same region together with occasional inflammatory cell infiltration. Collagenous fibers seem to have an irregular structure. Toluidin blue × 400

There were oedematous regions, inflammatory cells and vacuoles between epithelium and stroma. B preparation treated wounds had inflammatory cells and oedematous regions less than A preparation treated group. The treatments increased the average intraocular pressure from 394 mm Hg to 626 when A preparation was administered for 15 days, and to 870 mm Hg when B preparation was administered for the same duration (Table I).

Table I

Full thickness corneal wound strength on the 15th day of operation

•	n	Wound strength (mm Hg) Mean±SEM
Controls (untreated)	5	394 ± 34.4
A treated preparation	6	626.7 ± 44*
B treated preparation	6	870 ± 34*$^{+}$

$^{+}p<0.005$ A preparation – B preparation.

*$p<0.005$ control-A preparation, control-B preparation

Treatments: 5 µl × 2/24 h

Discussion

The wound healing process is composed of various components. The studies on the healing of penetrating corneal wounds, either partial or full thickness, have included morphological, structural and biochemical analyses as well as evolution of the tensile strength. Biochemical and structural studies have included analyses of the types of collagens and proteoglycans, collagen cross-linking and organization of collagen fibrils in lamellae in regenerated stromal matrix [7]. One of them is the restoration of tensile strength through collagen synthesis. Collagen provides the strength and integrity for all the tissue repairments (skin, cornea etc.) which relies on the cross-linking and the deposition of collagen. The tensile strength of the healing wounds in situ is generally recognized as the reflection of the rate of repair [14, 15].

In our preliminary work we identified the local treatment effect of epidermal growth factor in the aqueous solution form on the corneal wound healing of alloxan diabetic mice [8]. Tear dilutes and washes the solution form of EGF (Epidermal Growth Factor) on topic applications of corneal surfaces. At the next step of research we identified the local treatment effects of EGF dosage forms on corneal and skin wound healing of mice. These results indicate that topical EGF treatment of alkali burned eyes with solution dosage form improved the incidence of corneal wound healing [9]. Contrary to these results the healing of skin wounds was better in the group treated with EGF in bioadhesive system [1, 5].

A proposal of this research was to lengthen the ocular contact time of drug and to improve the bioavailability of a therapeutic agents.

Polymers increase the viscosity of vehicle. Bioadhesion aspects of these compounds reduce the drainage losses of ophthalmic formulations thus they improve drug absorption and topical effectiveness of the drug [11].

Several polyanionic polymers are proposed for ophthalmic formulations. Consequently, polyacrylate preparations are developed as long-lasting artificial tears for the relief of dry eye syndrome and traumatic injury. Their efficacy is attributed to mucomimetic, rheological and lubricating properties of these high molecular weight polymers. Recently gels for antibiotic therapy have been formulated [17]. The claimed advantages of these commercially available products have good tolerance, prolonged contact time on ocular surface and good miscibility with lacrimal fluid.

Ludwig and coworkers used slit-lamp fluorophotometry to evaluate the precorneal kinetics of viscous eye drops containing carbopol 940 and fluorescent tracer in humans. They suggested that the ocular retention of the tracer depends on the concentration of the polymer instilled [16].

Ünlü and coworkers compared the patient acceptance of an artificial tear preparation of carbomer viscous solution form of two commercially available tear preparations in their research work. They reported that the patients with mild dry eye syndrome preferred PVP-containing artificial tears because of blurred vision with carbomer gels [18].

In the present study we identified the effect of carbopol 940 preparations which contain 2 mg/g carbomer and preservative cetrimide (A preparation) and 3 mg/g carbomer, thiomersal as preservative, sodium hydroxide to arrange pH and mannitol to arrange osmolarity (B preparation) on the cornea and corneal incision wounds by histologic and wound strength comparisons. Treatments with B preparation increased the wound strength and healing process seemed better than the other group.

Conclusions

According to these results we concluded that the pH, tonicity and preservatives of artificial tears must be carefully arranged to avoid blurred vision and delayed healing.

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Short-time predegenerated peripheral nerve grafts promote regrowth of injured hippocampal neurites

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Our previous studies revealed that predegenerated peripheral nerve grafts facilitated neurite outgrowth from the injured hippocampus and that this effect was particularly distinct when 7-, 28-, and 35-days predegenerated nerve grafts were used. It is recently known that a totally transected peripheral nerve exhibits biphasic neurite-promoting activity. The early phase lasts 7 days. The aim of the present study was to find whether short-time predegenerated (1–6 days) peripheral nerve grafts exert any neurotrophic effect and when this influence is maximal. Experiments were carried out on adult male Wistar rats. Sciatic nerves were totally transected and following l, 2, 3, 4, 5 and 6 days their distal stumps were implanted into the hippocampus. Control animals were treated with non-predegenerated sciatic nerve grafts. In all groups FITC-HRP was injected into the free end of graft six weeks following surgery. Special histochemic technique showed AChE-positive fibres inside the grafts of all examined groups. Fluorescence microscopic examination revealed the labeled cells in all examined groups, however their number was different in each group, depending on the predegeneration stage. They were most numerous at the fourth day of predegeneration.

The inability of adult CNS neurites to regenerate after injury is one of the most serious problems of modern neurobiology and medicine dealing with lesions of the nervous system. It has been thought to result from the formation of an unpermissive environment impeding the progression of growth cones in the site of a lesion [1]. By contrast, axons severed in the peripheral nervous system can successfully regenerate when they are associated with Schwann cells [22, 23]. Therefore under experimental

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conditions created by the grafting of peripheral nerve segments into the adult mammalian CNS, different types of mature CNS neurons can express regrowth of their injured axons into such grafts [2, 4, 5, 7, 9, 11, 14, 16, 17, 18, 21, 25].

The results of our previous studies showed that predegenerated peripheral nerve autografts facilitate outgrowth of injured central neurites more efficiently than non-predegenerated ones [16]. This influence was particularly evident when grafts were allowed to predegenerate for 7 as well as 28 or 35 days before the implantation into the hippocampus, it was therefore biphasic. The first phase lasts 7 days, then decreases and reaches the minimum at the 14th day after transection. The second phase of increased neurite promoting activity appears in 28 and 35 day after nerve transection [17]. Similar biphasic intensity of injured adult peripheral nerves' regenerative influence was observed *in vitro* [3]. Pretransected *vel* predegenerated peripheral nerve autografts were used for supporting CNS axonal regeneration [9] or as co-grafts in treatments in animal models of Parkinson's disease [7]. Peripheral nerves were predegenerated for these purposes for 3 days and 24 hours, respectively [7, 9]. The shortest predegeneration time in our previous studies was 7 days [16, 17]. If a shorter period of predegeneration were similarly effective, it could make the procedure simpler. It might be also promising in the potential probable therapeutic use of such technique in the future. The aim of the present study was to examine the neurotrophic activity of short-time (1-6 days)-predegenerated peripheral nerve grafts upon the injured hippocampal neurites in adult rats.

Materials and Methods

Experiments were carried out on 77 adult male Wistar C rats (body weight ~150 g). Animals were divided into seven equal groups – one control (C) and six experimental (D1–D6) ones. After intraperitoneal anaesthesia with chloral hydrate (420 mg/kg b.w.), the left sciatic nerve was exposed and totally transected on the level of the hip joint. In the control group a sham operation (no nerve transection) was performed. Then, the animals were kept in cages for, 1, 2, 3, 4, 5 or 6 days (groups D1-D6, respectively). Subsequently, they were reanesthetized and 1 cm long piece of distal stump of sciatic nerve was dissected. In the control group (C), the nerve segment was dissected immediately after the transection and was, thus, nonpredegenerated. The dissected nerve fragment was inserted into the glass cannula (1 mm i.d. and ~2 mm e.d.) filled with cold Ringer's solution for mammals.

The peripheral nerve segments were implanted into the hippocampus according to the method described by Lewin-Kowalik et al. [16]. Briefly, a hole was drilled in the skull (3 mm caudal and 3 mm lateral from bregma) and a 3 mm deep brain tissue injury

was made. Injury and implantation were made stereotaxically. One end of the graft was inserted into the injured brain and the caudal tip of the graft was laid over the skull bone, tied with 4–0 surgical silk, and attached to the skull by means of a two-component fibrin tissue glue Tissucol Kit (Immuno AG, Wien). During the whole experiment the animals received a standard diet and water *ad lib.*

Six weeks following implantation, the animals were reanesthetized and 10 µl of 0.5% solution of horseradish peroxidase conjugated with fluorescein isothiocyanate (FITC-HRP) was injected into the free end of grafts. Twenty-four hours later the rats were perfused transcardially with 5% sucrose in phosphate buffer (pH 7.2) followed by 2.4% formaldehyde buffered as above. Whole grafted brains were dissected from the skull, embedded in paraffin and 10 µm thick frontal sections were made by means of a rotatory microtome (Biocut, Jung). Slices were counterstained with hematoxyline-eosine (H-E) in order to verify the anatomical position of the grafts (Fig. 1), examined in the light and fluorescence microscope (Labophot 2, Nikon) and photographed. Frontal 10 µm thick frozen sections (Cryotom 620, Anglia Scientific) from one brain in each subgroup were subjected to a procedure visualising growing nerve endings on the basis of acetylcholinesterase (AChE) present in neurites growing into the grafts [12]. The absolute number of FITC-HRP positive cells were ascertained by means of a computer picture analysing system (SVIST-I-0032, Wikom, Poland). In each case cells were counted in the same volume of brain tissue, in every second section, in order to avoid double counting. The counting was executed by two independent searchers unaware of the kind of examined material. The results were subjected to statistical analysis using Student' t-test. Statistical significance was set at $p<0.05$.

Results

Fluorescence microscope examination revealed that FITC-HRP-positive cells were present in all examined brains (Photo 1). However, the number of cells was different in each group. The number of traced cells in each subgroup amounted to (mean±S.D.): **C** – 39.40±9.45; **D1** – 16.98±7.32; **D2** – 31.10±13.81; **D3** – 46.04± 21.69; **D4** – 97.07±45.48; **D5** – 48.70±19.55; **D6** – 20.54±9.63 (Fig. 2). Statistical analysis of these data revealed that differences between individual groups were in most cases non significant. Only in group D4 the number of traced cells was statistically higher than in all other groups. In group D5 the traced cells outnumbered only those in group C (Table I).

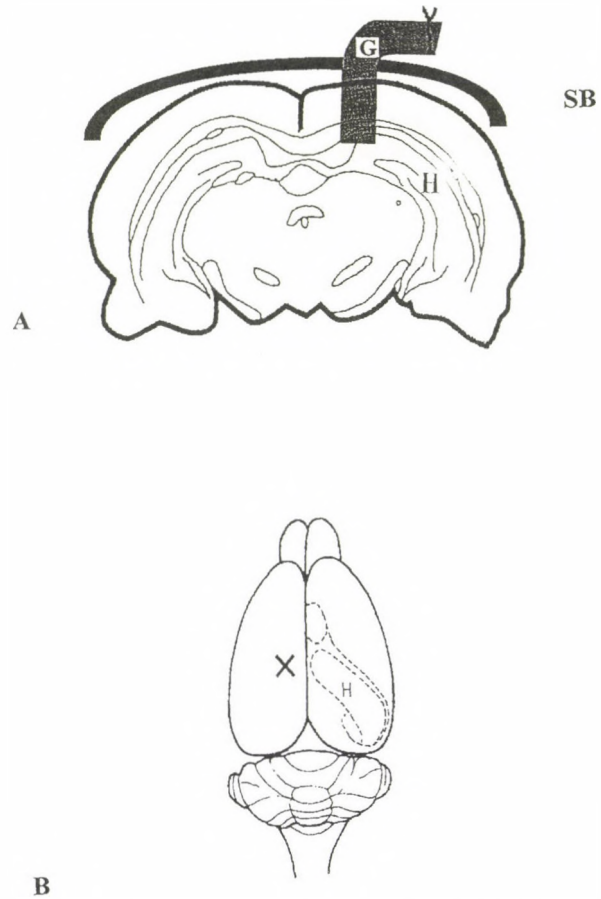

Fig. 1. Schematic diagram illustrating placement of implanted graft: **A** – schematic cross-section of the brain; **B** – general view of rat's brain; X – indicates the site of implantation; H – hippocampus, G – graft, SB – skull bone

Photo 1. FITC-HRP-positive cells in the hippocampus from the group D4 (×200)

Table I

Statistical (mean ±S.D.) comparison of groups. The value of the "p" coefficient indicated on the crossings of columns and rows, refers to the comparison of mean values from each group

C	D1	D2	D3	D4	D5	D6
39.40±9.45	16.98±7.32	31.10±13.81	46.04±21.69	97.07±45.58	48.70±19.55	20.54±9.63
C	n.s.	n.s.	n.s.	p<0.05	p<0.05	n.s.
	D1	n.s.	n.s.	p<0.05	n.s.	n.s.
		D2	n.s.	p<0.05	n.s.	n.s.
			D3	p<0.05	n.s.	n.s.
				D4	p<0.05	p<0.05
					D5	n.s.

n.s. – not significant

In the brains examined for presence of acetylcholinesterase-positive fibres nerve endings of ingrowing fibres inside the grafts in each group were found (Photo 2).

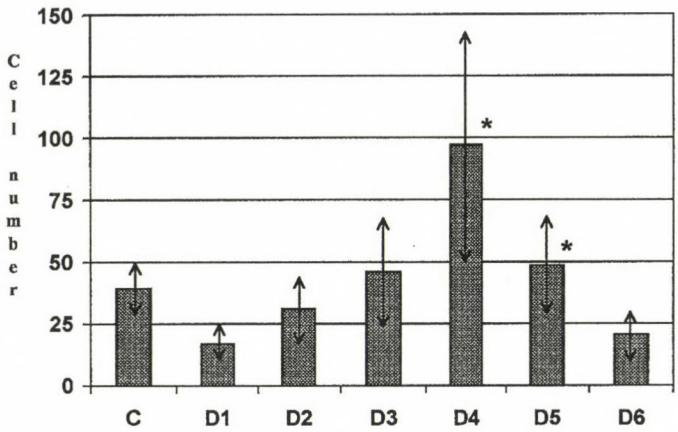

Fig. 2. Histogram showing a number of FITC-HRP-positive cells in individual groups six weeks following implantation. Asterisks show the groups which differ significantly from the control group

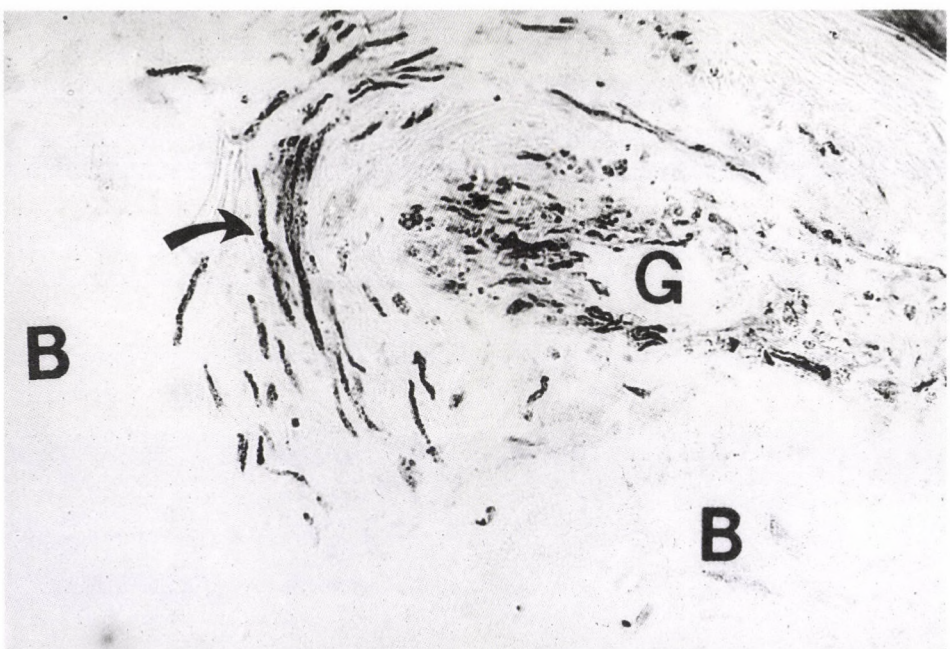

Photo 2. AchE-positive fibres present in the implanted graft (×200); G – implanted graft, B – surrounding brain tissue, arrow indicates AChE-positive fibres at the end of the graft

Discussion

In the present experiments the influence of short-time predegenerated peripheral nerve grafts upon regeneration was not constant but time dependent. During the first week it exhibited a biphasic intensity with peaks on day 4 and 7. The first peak was noticed in the present study, the second one we described in our former paper [17]. Numbers of traced cells are presented in Table I. A significant difference between groups D4 and D5 and a control group was found. However in the group D4 the number of traced cells was statistically higher than in group D5, and therefore we assumed that in group D4 the most intensive regenerating activity was present. The neurotrophic activity of peripheral nerve segments at 2, 5 and 7 days after excision was also examined by Blexrud et al. in the tissue culture [3]. They found the activity towards PC12 cells that increased linearly during the study period. Our results do not confirm these observations, but the differences may be due to different target cells and *in vivo* experimental conditions. Collier et al. [5] used sciatic nerve segments three days after excision for co-culture with embryonic ventral mesencephalic tyrosine hydroxylase-positive neurons. Increased number and growth of TH-positive neurons was noticed within a period no longer than 2 days of the nerve exposure. The authors suggest that a diffusible, soluble factor or factors from sciatic nerve can enhance the number and development of TH-positive neurons detected in cultures of embryonic ventral mesencephalon. The growth stimulating activity of peripheral nerve started in their experiments at the 4th day after transection, similarly to our observations. Short-time predegenerated peripheral nerve grafts were also used for supporting regeneration of respiratory neurons [9], peripheral nerve regeneration studies [18, 21] and as a so-called cograft in animal models of Parkinson's disease [7, 15]. Decherchi and Gauthier [9] stated that degenerated nerves were repopulated from central fibres similarly to that obtained using fresh nerve grafts implanted in the same area of the CNS at the level of descending respiratory pathways in the C2 spinal cord. The period of grafts' predegeneration was 3 days in vitro in cold saline. Their observations at this point are very similar to our results. Unfortunately, facilitation of neurite outgrowth from hippocampus after predegenerated grafts implantation reported in our earlier paper [16] has not been confirmed by Decherchi and Gauthier [9]. They supposed that the difference was due to a longer post-grafting time (2–4 months) than in our experiments (1 month or less). In previous series of our experiments we have documented that 6 week-postimplantation period was best for our purpose and we followed this protocol in the consecutive studies.

Twenty-four hour pretransected peripheral nerve grafts had no significant influence towards dopaminergic cells, but when they were cografted with chromaffin cells, they showed synergistic activity with the latter [6, 7]. However, this effect was

less prominent in mice receiving aging donor tissues compared with mice receiving young donor tissues [8]. Kordower et al. have shown that also in nonhuman primates the viability of grafted chromaffin cells can be enhanced four- to eightfold by cografting with fresh peripheral nerve segments [15]. On the basis of our results we can suppose that four- or seven-day-predegeneration should augment this effect, however the clinical utility of cograft approach remains questionable.

It is commonly believed that Schwann cells are important for nerve regeneration because they provide contact guidance and neurotrophic factors to axons [24]. This cells are known to increase the synthesis and release of substances with neurotrophic activity following nerve transection. In the majority of papers concerning this problem at least two kinds of neurotrophic factors have been described: the first one, with the activity blocked with anti-NGF antibodies, and the second, resistant to such treatment [5]. Ferguson et al. showed that in culture both NGF and non-NGF trophic activity are higher 3 and 4 days following transection than earlier ones [10]. Thus, changing metabolic activity of the living cells in the distal part of transected nerve is accompanied by fluctuating synthesis of known and unknown neurotrophic substances. The importance of Schwann cells in neurite regrowth was confirmed by experiments using cultured Schwann cells as grafts to the injured CNS. Martin et al. [20] implanted autologous Schwann cells at the site of acute compression lesion of the spinal cord. Such implant dramatically promoted the ingrowth of axons. The authors suggest that autologous Schwann cells which are readily available in the host, represent a promising material for grafts into the injured spinal cord. They suspect that only orphaned Schwann cells are conductive to neurite growth and that accompanying neurons downregulate some aspects of their growth-promoting ability. This is neither in agreement with our former and present results in the hippocampal formation nor with the observations of others [4,16]. This differences may be due to placing the grafts within different cell populations [20].

Nerve transection causes the cascade of time-dependent changes in distal as well as proximal part of the neuron. The microenvironment of predegenerated grafts for regenerating peripheral nerves also changes in time. Ochi et al. [22] have revealed that predegenerated acellular (freeze-thawing) bridges in the peripheral nerve showed the maximal influence on the length of a fibre after 4 weeks of predegeneration and the packing density of fibres after one week of predegeneration. Regeneration in the fresh acellular (0-day-nonpredegenerated) and 8 week predegenerated grafts was poor. In the acellular grafts only a scaffold of extracellular material remains. It has been suggested [13] that when the cells present in the grafts are freezed-thawed, some factors may be released to the basal lamina. The putative source of these factors are Schwann cells. However, the initial sharp rise of NGF protein occurs earlier than the Schwann cells proliferation starts. Liu et al. [19] suspect that during the first two days infiltrating

leukocytes may be the source of this growth factor. We observed that initiative NGF delivery had no influence on predegenerated peripheral nerve grafts activity towards central neurites.

Based on the results of our experiments it can be stated that the increase of neurotrophic activity of short-time predegenerated peripheral nerve grafts towards the hippocampal neurites appears for the first time at the fourth day following transection. We suppose that this is the moment when Schwann cells start to synthetize neurotrophic molecules. The identification of the molecular mechanism underlying this activity should be the main key for the development of further strategies for the repair of the damaged central nervous system.

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The effects of continuous light and darkness on the activity of monoamine oxidase A and B in the hypothalamus, ovaries and uterus of rats

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For assessing monoamine oxidase (MAO-A and -B) activities in the hypothalamus, ovaries and uterus, mature female rats were exposed to either continuous light or dark over 6 weeks. Confirming previous studies, continuous light induced constant estrus in all animals. The majority of animals kept under continuous dark during the six weeks remained mostly in diestrus with estrus appearing sporadically. The endocrinological function of the ovaries was disturbed by continuous light, which resulted in the development of polycystic ovaries, their morphological appearance being not significantly affected by continuous dark. Hypothalamic MAO-A activity was markedly reduced under the influence of both light ($p<0.05$) and dark ($p<0.01$). The activity of hypothalamic MAO-B was reduced only under the influence of dark ($p<0.01$). In the ovaries, no significant differences were detected in either MAO-A or -B activity as a result of these environmental manipulations. In the uterus of rats exposed to continuous light which developed polycystic ovaries, MAO-A activity was significantly lower ($p<0.05$) in comparison to either intact controls or rats exposed to continuous dark. These results demonstrate that chronic changes in photoperiodicity may considerably influence MAO-A activity and to a lesser extent MAO-B activity dependent on the tissue studied.

Keywords: dark, hypothalamus, light, MAO, ovaries, uterus

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Changes in the photoperiod as a stressor causes various disturbances in the functioning of the female rat reproductive system. Numerous authors [3, 7, 14, 16] have reported that continuous light induces the appearance of constant estrus-anovulatory syndrome characterized by polycystic ovaries and complete absence of ovulation. We have also previously reported [11] that female rats having been kept under continuous light or dark for two weeks, stopped cycling. Continuous light induced constant estrus in all animals. In the majority of animals kept under continuous dark during the six weeks, diestrus phase was present, while the estrus phase appeared sporadically. However, continuous dark does not induce significant morphological changes in the ovaries, as compared to animals exposed to the regular 12 hour light-dark schedule [11]. Hypothalamic dopamine (DA) and norepinephrine (NE) levels are significantly lower under the effect of either continuous light [12] or dark as compared to controls [10]. These changes in the hypothalamus affect the pituitary, which further interferes with the normal functioning of the ovaries and uterus.

It is known that MAO-A and MAO-B have different physiological functions and that they are variously distributed in the brain with different patterns of expression [13, 15]. The deamination of DA is catalyzed by both MAO-A and MAO-B, preferentially involving MAO-A [6, 17].

Keeping in mind the different physiological functions of MAO-A and MAO-B and their various distribution and expression patterns, as well as the previously mentioned effects of photoperiod manipulation, we were interested in determining the patterns of MAO-A and MAO-B activities in the female hypothalamo-reproductive organs-axis under the influence of continuous light and dark.

Materials and Methods

Virgin female Wistar rats randomly bred for 50 years at the Institute for Nuclear Sciences "Vinca", aged 3.5 months, with an average body weight 210 g were exposed to either continuous light or dark over 6 weeks. Compact fluorescent tubes, 40 W each, served as the light source; the intensity of illumination at the bottom of the non-reflective plastic cages used was 450 lux. The level of darkness under which the animals were kept, corresponded to that of a photo lab without any light source. Intact controls were kept under a schedule of 12 h of light (lights on 07:00 h–19:00 h) at 24–26 °C. All three groups of animals were fed with commercial rat pellets and had access to drinking water *ad libitum*. Vaginal smears were gently taken with luke warm water and plastic pipette tip on the day of sacrifice between 8:00 and 8:30 h, this manipulation not showing a stressful stimulus [12] and all animals were decapitated with a guillotine (Harvard Apparatus) between 11:00 and 12:00 h. The hypothalamus

was dissected by the method of Glowinski and Iversen [10], the optic chiasma delimiting the anterior part of the hypothalamus, the anterior commissure being the horizontal reference and the mammillary bodies delimiting the posterior part of the hypothalamus. The ovaries and uterus were also removed, placed on an ice-cold microscope slide and cleaned of surrounding tissue. The excised tissues were frozen at –70 °C until assayed for MAO-A and -B activities as described by Radojičić et al. [21]. Briefly, the tissues were then defrosted and homogenized in cold 1 mM potassium buffer at pH 7.8. Homogenates were then centrifuged at 600×g for 10 min to remove cell debris and nuclei. The supernatants were decanted and used for the enzyme assay. Radioactive monoamine substrates purchased from Amersham, U.K.[5-hydroxy (G-^{3}H) tryptamine creatine sulphate for MAO-A and benzylamine-hydrochloride (methylene-^{14}C) for MAO-B] were added to the samples in concentrations of 50, 100 and 200 μM (specific activity of 2 μCi/μmol), followed by the extraction of labeled metabolites into organic solvent for the estimation of radioactivity [5], performed with a 1219 Rackbeta, LKB Wallac Liquid Scintillation Counter. Radioactive materials were purchased from Amersham (England). MAO activity was expressed as V_{max}, in nmol of substrate metabolized per hour of incubation per ml (nmol/h/ml). V_{max} values were calculated from linear regression analysis of triplicate determinations with three different substrate concentrations (50, 100 and 200 μM).

The Student's t-test was used for statistical analysis. A value of $p<0.05$ was considered significant.

Results

In all animals exposed to continuous light, polycystic ovaries developed. Animals exposed to continuous dark had ovaries with smaller corpora lutea, than those found in intact controls [11].

As shown in Table I, MAO-A activity was significantly reduced in the hypothalamus of rats exposed to either continuous light ($p<0.05$) or dark ($p<0.01$), while MAO-B activity was markedly decreased only in animals exposed to continuous dark, compared to corresponding controls ($p<0.01$) (Fig. 1).

It may be seen from Table I and Figure 2 that ovarian MAO-A and -B activities in rats exposed to either continuous light or dark remained unchanged as compared to controls.

In the case of the uterus, only MAO-A showed significantly reduced activity ($p<0.05$) in rats exposed to continuous light when compared to the controls. There were no significant changes in uterine MAO-B activity in either experimental group when compared to corresponding controls.

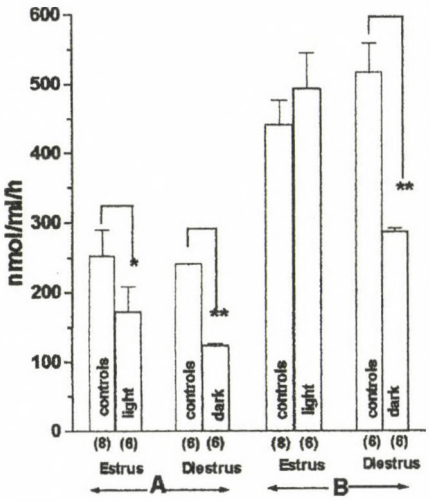


Fig. 1. The activities of MAO-A and MAO-B in the hypothalamus of controls and rats exposed to continuous light or darkness

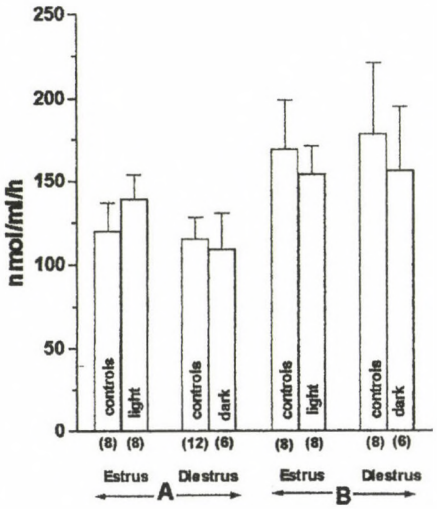
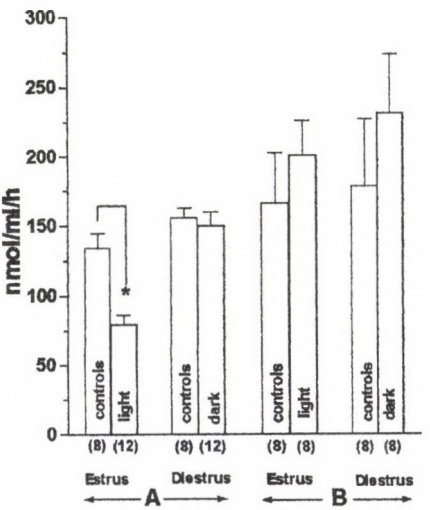


Fig. 2. The activities of MAO-A and MAO-B in the ovaries of controls and rats exposed to continuous light or darkness



Bars represent Mean±SEM
*p<0.05
**p<0.01
( ) N° of rats

Fig. 3. The activities of MAO-A and MAO-B in the uterus of controls and rats exposed to continuous light or darkness

Table I

The activities of MAO-A and MAO-B in the hypothalamus, ovaries and uterus of rats exposed to continuous light or dark and their intact controls

Groups of animals	MAO-A (nmol/ml/h)	MAO-B (nmol/ml/h)	Groups of animals	MAO-A (nmol/ml/h)	MAO-B (nmol/ml/h)
	Estrus			Diestrus	
Hypothalamus					
Controls	252.0±38.0	440.0±36.0	Controls	240.0±0.6	515.0±43.0
Light	172.0±36.0*	492.0±53.0	Dark	123.0±3.0**	287.0±5.0**
Ovaries					
Controls	120.0±17.0	169.0±30.0	Controls	115.0±13.0	178.0±43.0
Light	139.0±15.0	154.0±17.0	Dark	109.0±22.0	156.0±39.0
Uterus					
Controls	134.0±11.0	166.0±37.0	Controls	156.0±7.0	178.0±49.0
Light	79.0±7.0*	201.0±25.0	Dark	150.0±10.0	231.0±43.0**

*$p<0.05$; **$p<0.01$ compared to corresponding controls

Discussion

As the aim of the present study was to explain if the reduced hypothalamic DA (reduction of 34.1%) and NE (reduction of 24.5%) levels seen in rats exposed to continuous light [12], might result from increased degradation, we determined MAO-A and -B activities in the hypothalamus of both experimental and corresponding control groups of rats. Bearing in minded that MAO-A activity is an indicator of catecholamine (CA) degradation, our results, showing significantly lower MAO activity in the hypothalamus of rats exposed to either continuous light or dark, as shown in Figure 1, indicate that reduced hypothalamic CA content under the influence of changes in photoperiodicity [12] seems not to be the consequence of degradation by MAO-A. Previously we have reported [11] that, in rats exposed to continuous light which induced the appearance of polycystic ovaries, the serum concentration of estradiol was much higher than in intact controls, possibly being responsible for the reduced hypothalamic MAO-A activity. This effect of estrogen on MAO-A activities is supported by the results of Ortega-Corona et al. [20] which showed that estradiol benzoate administration in ovariectomized female rats decreased hypothalamic MAO-A activity. In rats exposed to continuous light in the present study, hypothalamic MAO-B activity remained unchanged, whereas in rats exposed to continuous dark it was significantly reduced compared to controls, as shown in Figure 1. The reduced

hypothalamic MAO-B activity in rats exposed to continuous dark is unclear and requires further study. Many studies have indicated the involvement of CA in the regulation of ovarian metabolism. CA concentration in the ovaries has corresponded to serum pituitary and steroid hormone levels, whereby NE concentration was higher by far in the preovulatory follicle than in the newly formed corpus luteum [1, 2, 4, 18]. It is also known that CAs are involved in ovarian hypertrophy [4, 8, 9, 19]. Unchanged CA concentrations in polycystic ovaries of rats exposed to either continuous light or dark, as compared to intact controls, confirm the link between ovarian CA levels as well as their morphological and physiological changes. The correlation between the enzymatic pattern of MAO-A with endocrine functioning and morphological changes during the estrous cycle has been confirmed [22].

MAO-A and -B activities in ovaries of rats exposed to continuous light or dark are unchanged in respect to controls, as shown in Figure 2, which is in agreement with CA levels in ovaries observed under the same experimental conditions [12].

In the uterus, MAO-A activity was significantly decreased in rats exposed to continuous light. However, in animals exposed to continuous dark, both MAO-A and -B activities remained unchanged as compared to controls, as shown in Figure 3. These differences in enzyme activity between the rats, exposed to continuous light and those exposed to continuous dark, might result from more complex changes in the endocrinological functioning of polycystic ovaries in the rats exposed to continuous light.

In summary, it may be suggested that:

1. Lowered hypothalamic DA and NE levels, previously observed under the same experimental conditions [12] are more likely to be consequence of lowered CA synthesis than a degradation by MAO-A. In addition, MAO-A activity responded in the same direction, regardless of the type of the stimulus applied (light or dark), whereas MAO-B activity reacted differently, indicating differential physiological activities of both enzymes.
2. Unchanged MAO-A and -B activities in ovarian tissue correspond to unchanged NE content in the ovaries of either continuous light or continuous dark exposed rats.
3. Significantly lowered uterine MAO-A activity, under the influence of light or dark, also correlates to uterine NE content, which suggests that synthesis is reduced in this case rather than NE being increasingly degraded.

To confirm our view that changes in photoperiodicity lead to a reduced CA synthesis in the hypothalamus and uterus, further studies will be concerned with the activity of CA synthesizing enzymes.

Acknowledgement

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The protective effect of *Ginkgo biloba* extract on CCI_4-induced hepatic damage

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The aim of this study was to evaluate the protective effect of Ginkgo biloba extract on CCI_4-induced hepatic damage in rats. Hepatic malondialdehyde, glutathione and hydroxyproline levels and histopathologic alterations in liver specimens were assessed. 200 mg/kg/day Ginkgo biloba extract were given orally to the animals for 10 days, then a single dose of 2 ml/kg b.w. carbon tetrachloride was, administered intraperitoneally. Ginkgo biloba extract treatment reduced hepatic malondialdehyde levels significantly ($p<0.05$), but did not alter glutathione ($p>0.05$) and hydroxyproline levels ($p>0.05$). The light and electron microscopic findings showed that Ginkgo biloba extract limited the CCI_4-induced hepatocyte necrosis and atrophy. These results suggest that this extract may protect the hepatocytes from carbon tetrachloride-induced liver injury.

Keywords: Ginkgo biloba, CCI_4, liver, lipid peroxidation, microscopy

Carbon tetrachloride (CCI_4) is known to stimulate lipid peroxidation and cause fatty degeneration and centrilobular necrosis in liver [5]. The peroxidative effect of CCI_4 has been shown by increased malondialdehyde (MDA) production in rat liver microsomes [11].

Cytolysis is the main liver damage due to haloalkanes, but only in the CCI_4-induced liver model, have been steatosis and fibrosis analysed and the related results reported [17]. Furthermore, there are many studies regarding the enhancement of lipid peroxidation process that can lead to increased collagen production [4]. Collagen

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production is also represented by its rather unique constituent amino acid hydroxyproline [13].

Glutathione (GSH) is a tripeptide present in all mammalian cells which participates in many metabolic processes, among which, the protection of cells against free radicals and toxic metabolite belongs to the most important ones. Many substances cause hepatic damage by decreasing GSH levels [3]. It has been reported that reduction of cellular GSH is accompanied by lipid peroxidation [12].

Ginkgo biloba is a popular ornamental tree in many parts of the world and a member of the Ginkgoaceae family. Ginkgo biloba extract (Gbe) is known to be efficient in preventing diseases associated with free radical generation. It is a potent in vitro free radical scavenger since it easily reacts with superoxide radicals [18]. Likewise, Gbe reduces the free radical-induced lipoperoxidation generated by the NADPH-Fe^{3+} system in rat microsomes [19]. In vivo studies on animal models have confirmed the antiradical properties of this extract [6].

The aim of the present study was to investigate the protective effects of Gbe on CCl_4-induced damage in rat liver in terms of hepatic MDA, GSH and hydroxyproline levels. Histopathological alterations were also studied by light and electron microscopy.

Materials and Methods

Gbe used in this study was obtained from Abdi Ibrahim Company, Istanbul, Turkey. The extract contains 24% flavonoids and 6% terpene lactones.

Male Wistar rats weighing 150–200 g were housed under conditions of 12 h light/12 h dark cycle, on standard laboratory diet and tap water *ad libitum*. Rats were divided into three groups. Group 1 served as control and these animals did not receive any treatment. Groups 2 and 3 received a single dose of CCl_4 injection (2 ml/kg). CCl_4 diluted with olive oil, was injected intraperitoneally and the rats were killed after 24 h. Group 2 received 200 mg/kg/day Gbe orally and group 3 received the same amount of 0.9% sodium chloride solution, for 10 days before CCl_4 administration. All animals were killed by an overdose of panthotal sodium anaesthesia. The livers were excised; rinsed in saline; and then dried; and frozen immediately in liquid nitrogen. The specimens were stored there until used. For light microscopic evaluation, tissues were fixed in 10% buffered formaline and embedded in paraffin. The sections were stained with both haematoxylin-eosin and periodic-acid schiff (PAS). For electron microscopy, tissues were fixed with 2.5% gluteraldehyde and osmium tetroxide. The ultrathin sections were stained with uranyl acetate and lead citrate and examined under Carl Zeiss EM.

Liver MDA levels were estimated by the method of Uchiyama and Mihara [20]. The breakdown product of 1,1,3,3-tetraethoxypropane was used as standard and the results were expressed as nmol of MDA/g liver.

Total liver nonprotein sulfhydril groups (TNPSH) were assayed by the method of Ellman [10]. This was used as a measure of reduced liver glutathione, which constitutes most (>95%) of the liver TNSPH. The results are expressed as μmol of GSH/g liver.

Liver hydroxyproline levels were estimated by the method of Woessner [21]. The hydroxyproline values were determined from the L-hydroxyproline standard curve and given as μg hydroxyproline/mg liver.

The data were expressed as mean±S.D. The results were analysed by using one-way ANOVA and Mann-Whitney U test. $p<0.05$ was considered as significant.

The protocol was reviewed and approved by the animal Care Committee and Surgical Research Center of Gazi University Medical School.

Results

Hepatic MDA, GSH and hydroxyproline levels of all groups are shown in Table I.

MDA formation in liver significantly increased after CCl_4 administration ($p<0.05$). This increase was suppressed by Gbe treatment ($p<0.05$).

Hepatic GSH and hydroxyproline levels decreased in the CCl_4 group in comparison with the control group, but the differences were not statistically significant ($p>0.05$). Gbe treatment had no effect on the GSH and hydroxyproline levels in the CCl_4-treated liver.

Table I

Effect of Gingko biloba extract on hepatic malondialdehyde, glutathione and hidroxyproline levels in CCl_4-treated rat liver

Groups	n	nmol MDA/ g tissue±SD	μmol GSH/ g tissue±SD	μg HP/ mg tissue±SD
Control	6	57 ± 7	8.4 ± 0.7	0.19 ± 0.04
CCl_4-treated	6	77 ± 11.2*	7.4 ± 1.4	0.1 ± 0.06
Gbe+ CCl_4-treated	6	48.2 ± 12.4*	8.0 ± 0.6	0.17 ± 0.06

*$p<0.05$, significantly different from the control

CCl_4, Carbon tetrachloride

Gbe, Ginkgo biloba extract

MDA, Malondialdehyde

GSH, Glutathione

HP, Hidroxyproline

The morphological changes in CCl_4 group (group 3) include marked necrosis, congestion and atrophy of the hepatocytes (Fig. 1a and Fig. 1b). Gbe treatment reduced these alterations. In Gbe-treated group, the perivenular hepatocytes have clear cytoplasm and small inactive nuclei (Fig. 2a). There were granules which were positively stained with PAS and they vanished after diastase treatment (Fig. 2b). Electron microscopic evaluation showed that the perivenular cells with clear cytoplasm were degenerated hepatocytes (Fig. 2c). However, the peripheral hepatocytes were normal in this group (Fig. 2d).

Discussion

In this study the protective effect of Gbe on CCl_4-induced hepatic damage in rats was evaluated.

The toxicity of CCl_4 depends on the metabolic activation of trichloromethyl radical ($^{\circ}CCl_3$), which consequently facilitates lipid peroxidation [14]. It has been well established that CCl_4 is cleaved to $^{\circ}CCl_3$ by the mixed function oxidase system in liver microsomes, and $^{\circ}CCl_3$ radical reacts with unsaturated fatty acids to form lipid peroxides in liver [1].

Lipid peroxidation was measured by MDA formation, using the method of thiobarbituric acid reaction. In CCl_4-treated rats, the hepatic MDA levels were markedly increased ($p<0.05$). Gbe treatment reduced the MDA levels dramatically ($p<0.05$) when compared to MDA levels of untreated rats. This result is in agreement with the previous studies which attributed this effect to its free radical scavenging activity [6, 18, 19].

GSH is an important constituent of cellular protective mechanisms against a number of noxious stimuli including oxygen derived free radicals. Moreover, reduced GSH also acts as a radical scavenger by itself and as a detoxicant in eliminating different electrophonic compounds of exogenous and endogenous origin mediated by glutathione S-transferase [7]. A slight decrease in liver GSH content was detected after CCl_4 administration, but this difference was not statistically significant ($p>0.05$). Also, no effect of Gbe on GSH levels in CCl_4-treated rat liver was detected ($p>0.05$). Nakashima also found that the contents of water-soluble antioxidants in liver were not altered at 24 h after CCl_4 administration [16]. In an earlier study, CCl_4 administration did not significantly decrease the GSH levels in taurine-treated rat liver [8]. It can be suggested that Gbe may have direct suppressive effects on the process of lipid peroxidation rather than the formation of GSH in rat liver.

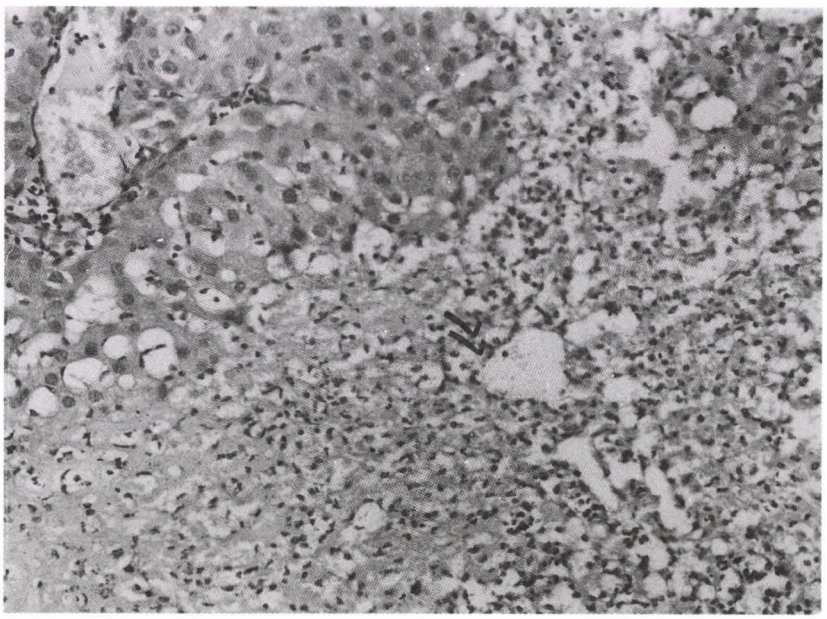

Fig. 1a. A light microscopic view in CCl_4-treated group. Necrosis, congestion, and hepatocellular atrophy in the perivenular area (arrows), HE×200

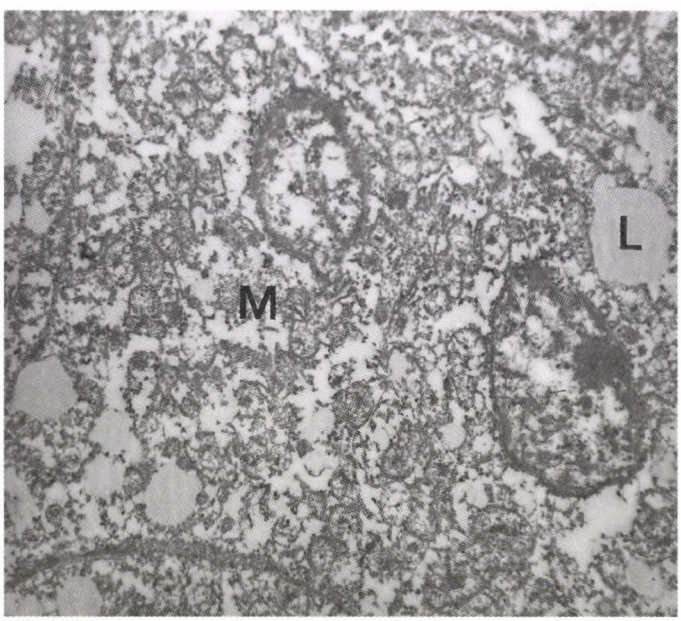

Fig. 1b. An electron microscopic view in CCl_4-treated group. Degenerated cytoplasm and organelles. Mitokondrion (M), Lipid droplets (L), ×3000

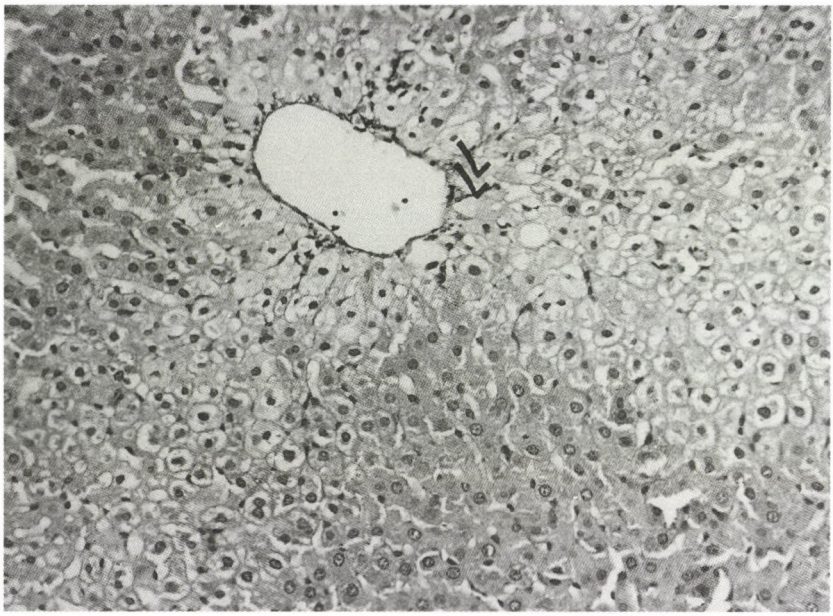

Fig. 2a. A light microscopic view in Gbe-treated group. Hepatocytes having larger, paler cytoplasm located in the perivenular area (arrows) compared to adjacent hepatocytes, HE×200

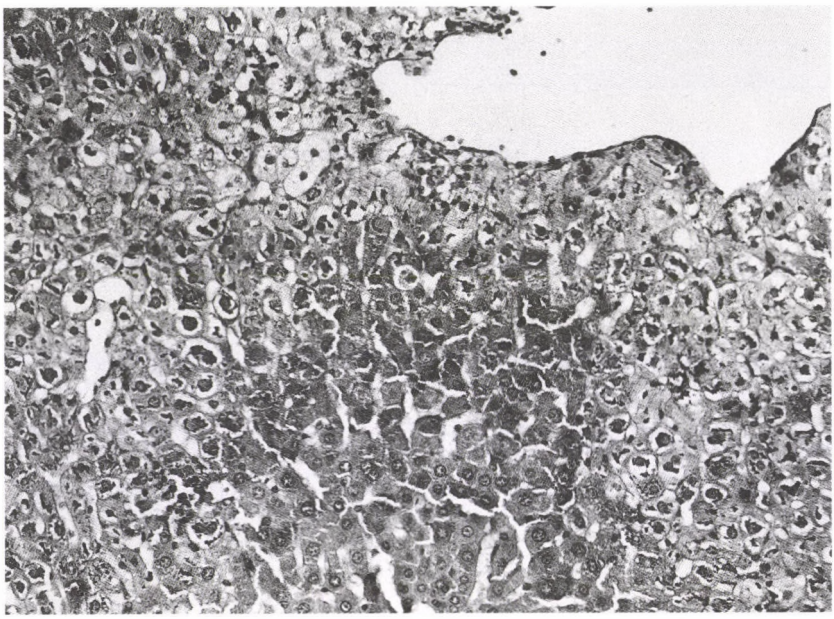

Fig. 2b. A light microscopic view in Gbe-treated group. PAS stained hepatocytes which have glycogen droplets, PA5×400

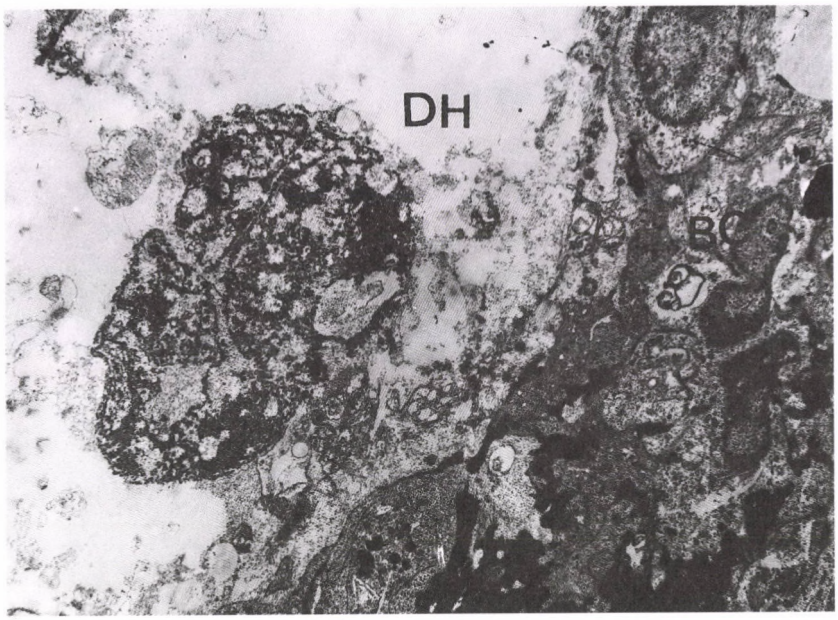

Fig. 2c. An electron microscopic view in Gbe-treated group. Degenerated hepatocyte (DH) in perivenular area, all organelles were lost, electron-dense material in extracellular region (thick arrow) and some blood cell (BC), Uranyl acetate, lead citrate, ×3000

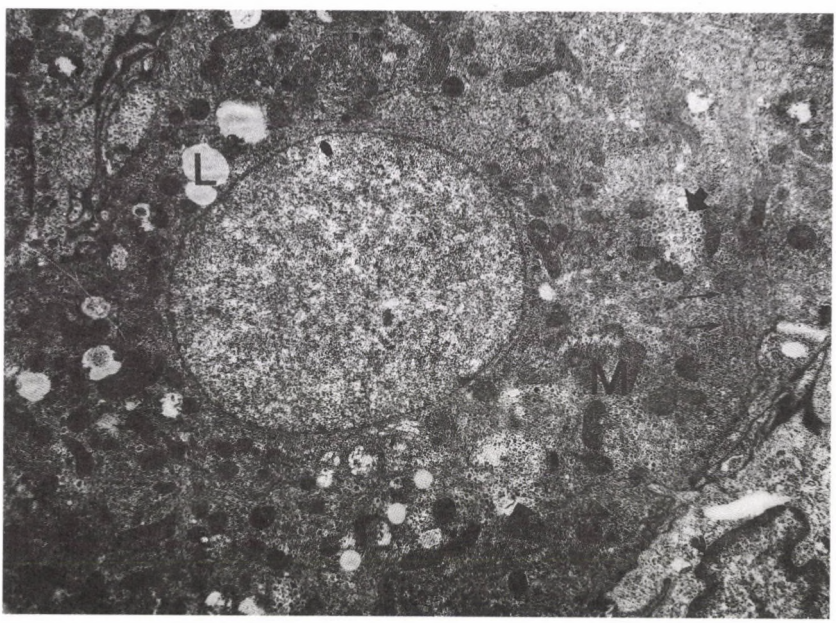

Fig. 2d. An electron microscopic view of peripheral hepatocyte in Gbe-treated group. Mitokondri (M), Granular endoplasmic reticulum (thin arrow), glycogen granules (thick arrow), Lipid droplets (L), Uranyl acetate, lead citrate, ×3000

The effects of Gbe are attributed to the presence of flavonoidic compounds in this extract, which exhibit potent free radical scavenging activities [18]. Flavonoidic compounds have been used as protective agents against free radical-mediated toxic liver injury in rats [15]. Several studies have demonstrated the antioxidant activity of Gbe in preventing oxidative degradation of rat liver microsomes [9] and in reducing lipid peroxidation of human liver microsomes [2].

According to the results of the present study, the hydroxyproline levels and the microscopic evaluation of the CCl_4-treated liver did not suggest any fibrotic alterations. This may be due to the fact that the exposure time of liver to CCl_4 was short. However, this time is sufficient for the formation of lipid peroxidation and hepatocellular damage. The microscopic findings suggest that the Gbe pretreatment prevents the rapid progress of the CCl_4-induced necrosis and limits the damage to the perivenular area. In conclusion, these data suggest that the administration of Gbe may prevent the oxidative tissue damage in CCl_4-treated rat livers.

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In memoriam Professor Peter Bálint (1911–1998)

Professor Peter Bálint, M.D. Ph.D. (Chem.) former head of the Department of Physiology at the Semmelweis University of Medicine (Budapest), chief editor of the Acta Physiologica Hungarica, member of the Hungarian Academy of Sciences deceased after a long illness on 17 April 1998. Peter Bálint was born on 26 August 1911. His way to Physiology was not straight. He first studied medicine at Budapest. After getting his degree he felt necessary to study chemistry and received a Ph.D. degree also in this. Then he went back to clinical medicine with a sustained interest in laboratory work. His first publications concerned clinical chemistry; the first of them was "Identifizierung der Pentose in vier Fällen chronischer Pentosurie" (Identification of pentose in four cases of chronic pentosuria), which appeared in the prestigious "Biochemische Zeitschrift" in 1934. After the second world war his interest turned to nephrology. He was specially interested in the genesis of azotaemia – it should not be forgotten that in these years there was no artificial kidney yet and chronic renal disease was a lethal diagnosis.

Nephrology had strong traditions in Hungary but at that time also got new impetus from the epoche forming work of Homer W. Smith. Peter Bálint was among the first in Hungary who discovered the possibilities in applying this methodology in various pathophysiological conditions and published papers in this field for nearly four decades. Being a pioneer in clearance methodology in Europe he later established the limits of this methodology under various experimental conditions, especially in acute renal failure. The "shock kidney", the acute renal failure which follows and its possible prevention was his main interest from the nineteen-fifties until the eighties.

Professor Bálint was working hard on establishing international connections in the field of nephrology in a political period in which such connections were not at all favoured. It's partly his merit that the scientific isolation of Hungarian physiologists did not last long. His extraordinary gift for speaking languages (he was equally fluent in Hungarian, German, French, English, had a good working Russian knowledge and was able to communicate in Italian and Spanish) certainly helped him in this respect. Under his directorship the Department of Physiology became a center of nephrology studies and some members of this Department became well-known investigators in this field.

Attila Fonyó

Acta Physiologica Hungarica, Volume 85 (4), pp. 287–290 (1997/98)

Physiology in the Oeuvre of a Prominent Hungarian Medical Scientist – Endre Hőgyes

E. **Monos***, Mária **Faragó****

Fig. 1. A portrait of Dr. Endre Hőgyes (1847–1906)

Endre Hőgyes, one of the most prominent and internationally-renown leaders in the field of medical research, especially the treatment of rabies, *was born one hundred and fifty years ago* in Hungary. E. Hőgyes had started his medical and research carrier in 1870. In 1889 he had become vice-president of the Royal Hungarian Society of Natural Sciences and was elected as a member of the Hungarian Academy of Sciences (MTA) and that member of the National Council of Public Health. Scientific carrier of E. Hőgyes has always been closely linked to physiological sciences. E Hőgyes made a significant contribution to different areas of physiological sciences; his most important scientific publications in this field deal with renal physiology, respitary mechanics, cerebellar function, and associated eye movement. Endre Hőgyes was the first to

* Emil Monos, Professor of Physiology at the Semmelweis University of Medicine, President of the Hungarian Society of Physiology
** Mária Faragó, Tutor of Physiology at the Semmelweis University of Medicine, Member of the Biotech Group of Janssen-Cilag (Pharmadivision of Johnson & Johnson)

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organize Hungarian physiologists into a community. The "Special Physiological Conferences" were initiated within the Hungarian Royal Society of Natural Sciences in 1891. As a token of appretiation, Hungarian physiologists and other medical professionals have announced the year of 1997 as a memorial year of Dr. Endre Hőgyes.

Endre Hőgyes, one of the most prominent and internationally-renown leaders in the field of medical research, *was born one hundred and fifty years ago* in Hungary (30 November, 1847) Hajdúszoboszló – 8 September, 1906, Budapest). The last decades of the 19th century witnessed an epoch of revitalisation within Hungary after the Compromise of 1867. The research work of Endre Hőgyes made him one of the leading scientists of that period, since he had outlined the principles of medical research, based on natural sciences. The impact of his research and social activities in the development of medical physiology in Hungary during those days cannot be overestimated. The present commemoration is merely a brief summary of some aspects of his multidimensional heritage and a modest token of appreciation and gratitude from his successors.

Endre Hőgyes graduated at the Medical Faculty of Pest University of Sciences in 1870. His research began under the guidance of Professor Kálmán Balogh at the Department of Theoretical Medicine, then at the Department of Pharmacology. In 1874, Dr. Hőgyes accepted an invitation to work at the Department of General Pathology and Pharmacology, Ferencz József University of Kolozsvár. Later, he was twice elected Dean. During his years spent at Kolozsvár, Dr. Hőgyes demonstrated his eminent scientific and organizational skills: in 1876 he founded the Kolozsvár Society of Medical and Natural Sciences.

In 1889 Dr. Hőgyes became vice-president of the Royal Hungarian Society of Natural Sciences; and in the same year he was elected as a member of the Hungarian Academy of Sciences (MTA).

In 1883, Dr. Hőgyes became a director of the Department of General Pathology and Medicine at the University of Pest. Later, in 1890, he founded and became head of the Pasteur Institute. From 1888 until his death, Dr. Hőgyes was editor and publisher of the Medical Weekly Gazette (Orvosi Hetilap), and since 1889 he was a member of the National Council of Public Health, an important establishment of that period.

In 1893, Dr. Hőgyes was elected as president of the Hungarian Society of Medical Literature Publishers. By that time, he had written a monumental book entitled, Memorial Volume On the Past and Present of the Medical Faculty of Budapest Royal University of Science (Athenaeum Ltd., Budapest, 1896).

In Hungary, streets and schools are named after Endre Hőgyes. Since 1991, a Hőgyes Commemorative Medal has been annually awarded by the Scientific Council of Health Sciences to eminent Hungarian scholars. There is a wide variety of papers and a

valuable monograph in appreciation of the work of Endre Hőgyes (Z. Alföldy, Sós J.: The Life and Work of Endre Hőgyes, Akadémiai Kiadó, Budapest, 1962). Appreciation of the fine heritage left by Endre Hőgyes is the moral duty of all generations of physicians.

The work and carrier of Endre Hőgyes has been linked to physiological sciences by several ways. The most important fields are as follows:

1.) The first years of his professional career, spent under the guidance of Professor Kálmán Balogh (28 September, 1835 Szolnok – 15 July, 1888 Budapest), who was an outstanding personality of the period following the War of Independence (1848–49), were devoted to studying physiology. Between 1860–1862, as a young assistant professor, Dr. Balogh commenced his work under the supervision of an eminent professor of physiology, János Czermak, who spoke no Hungarian (Prague, June 17, 1828 – Leipzig, September 16, 1873).

After the leave of Professor Czermak, Dr. Balogh continued his work at the Department of Physiology, Pest University, and in a few years, following in the tradition of Samuel Rácz (Pest, 1744, March 30 – Pest, 1807, February 24), he published the second textbook on physiology in Hungarian, entitled "Human Physiology" (Pest, 1862). That same year, MTA (Hungarian Academy of Sciences) awarded the book with a Grand Prize. Professor Balogh was the first person to introduce modern principles of separate teaching in pathophysiology, pathohistology, and pharmacology.

2.) Endre Hőgyes completed his first scientific experiments and publications in the field of physiology. Since 1872, he had been submitting a series of original publications in the field of renal circulation, physiology and the pathophysiology of tubular function, respiratory mechanics, cerebellar reflexes, and mechanism of associated eye movements. The results of the latter research were summarized in a work of three volumes entitled "On Neurologic Mechanisms of Associated Eye Movements I–III" (MTA, Budapest, 1880–85). Endre Hőgyes is regarded as the first internationally-recognized Hungarian renal physiologist.

Later, he moved his focus of attention to microbiology and the treatment of rabies. His modification of Pasteur's anti-rabietic vaccination is still valid. His work in this field was awarded with a Grand Prize by the Hungarian Academy of Sciences.

3.) Due to his outstanding work, Endre Hőgyes became a leading personality at the Section of Physiology within the Royal Society of Natural Sciences, which was founded in 1841. On the occasion of the 50th anniversary of the Society, Endre Hőgyes presented a valuable chapter in the "Memorial Volume On the 50-year Anniversary of the Royal Hungarian Society for Natural Sciences" summarizing the activities of the Section. (IV. Physiology, KMTT, Budapest, 1892, pp. 63–74).

4.) In an attempt to help organizing Hungarian physiologists into a community and to achieve recognition of their activities, the so-called Special Physiological Conferences were initiated within the Society of Natural Sciences in 1891. The contribution of Endre Hőgyes as vice-president of the Society cannot be overestimated. At the first meeting of the Conference Dr. Hőgyes was the chairman. The protocol of the meeting, which took place at the Department of Physiology /Eszterházy St. 5/ at 5 p.m. on 27. Nov, 1891, has recently been found *(Hippocrates* 10: 46–56, 1993). The following scholars were present: Béla Lengyel, Nándor Klug, Árpád Bókai, Géza Mihalkovics, Károly Laufenauer, László Udránszky, Pál Plósz, Adolf Ónodi, Ottó Petrik, and István Csapodi.

Béla Lengyel, as secretary of the meeting, "*had opened the Meeting and summarized the goals of the Conferences, as a site for informal scientific meetings for physiologists, where they can discuss the recent achievements in physiology or related sciences in the form of original presentations or overview of the literature. He suggested to accept these principles and to hold the meetings accordingly. For successful organizational work, a chairman and a secretary should be elected*". Endre Hőgyes suggested Professor Nándor Klug, director of the Department of Physiology, as chairman and Ernő Jendrássik, who is still internationally-recognized for developing a maneuver for eliciting the patella reflex, as secretary.

The scientific oeuvre of Endre Hőgyes is a valuable heritage for both Hungarian and other European physicians. His creativity and talent attracts adoration and respect among our contemporaries. Our country, and all Hungarian physicians, cannot but be proud of the heritage of Endre Hőgyes. Consequently, Hungarian medical professionals have announced this year, 1997, as a memorial year in honour of Dr. Endre Hőgyes.

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Changes in transthoracic electrical impedance during endotoxemia in dogs

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Our aim was to investigate the role of hematocrit (H) and respiration in transthoracic electrical impedance during endotoxemia.

Transthoracic electrical impedance at end-expiratory apnea (Z_0) and at end-inspiration (Z_{max}), H values, and extravascular lung water level (EVLW), estimated by means of gravimetric analysis and the impedance method, were measured in splenectomized and mechanically ventilated dogs. In endotoxemia, there were increases in Z_0, Z_{max}, H and the respiratory frequency. In the splenectomized dogs, both impedances slightly increased without any significant change in H. In the ventilated dogs, Z_0, and Z_{max} increased similarly, while H increased. In the splenectomized, ventilated dogs, no changes were found in the impedances or H. The EVLW values showed that there was no serious edema in the endotoxemic groups.

The results suggest that Z_0 increased mainly in association with the increase in H. We conclude that the noninvasive measurements of the changes in impedance can be used for continuous monitoring of the fluid and gas shifts in the thorax.

Keywords: transthoracic electrical impedance; noninvasive; respiration; hematocrit; endotoxemia

The basal transthoracic electrical impedance (Z_0) is well documented to be a very sensitive noninvasive measure of fluid shifts in the thorax, and it is a very important factor in calculating the stroke volume [13]. The different tissues in the thorax (bone, fat and muscle) may contribute as constant factors to Z_0. However, there are also dynamically changing parameters (resistivity of the blood, and intrathoracic gas and fluid volumes). Any change in these parameters would be expected to cause a change

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in Z_0. There is an exponential relationship between the blood resistivity and the hematocrit (H) *in vitro* [9]. As Z_0 is determined, in part, by the blood impedance or resistivity, it seems likely that Z_0 is also affected by H. Previous experiments suggested different relationships between Z_0 and H [19, 23, 25]. Other reports demonstrated that there was a linear relationship between changes in lung volume and in Z_0 during breathing [2, 3, 4, 10, 12]. As Z_0 increases during inspiration and decreases in expiration, measurements of this parameter can be used to monitor the respiratory changes. Thus, the impedance measured at end-inspiration (Z_{max}) was utilized to estimate the respiratory effects of endotoxemia, and Z_{max}–Z_0 was regarded as a measure of respiratory changes.

Monitoring of changes in Z_0 can be used to detect pulmonary edema and the body fluid balance in animals [5, 14, 18, 22] and in humans [7, 11, 17]. The presence of endotoxin in the blood is known to induce profound changes in the systemic and pulmonary circulations. Earlier studies [5, 24] revealed that there was an altered ratio of air to fluid volume and lung resistivity in severe sepsis. This can be attributed to pulmonary edema, atelectasis and the altered hemodynamics in the lung.

We designed a series of experiments to investigate the effects of endotoxemia induced by a prolonged, slow infusion of a low dose of endotoxin on the transthoracic impedances. The aims were to study 1.) the changes in Z_0, Z_{max} and H, 2.) the role of H in Z_0 changes, 3.) the effects of respiration on changes in Z_0 and Z_{max}, and 4.) the extravascular lung water level (EVLW).

Materials and methods

Animal procedure

Mongrel dogs of either sex were anesthetized with sodium pentobarbital (30 mg/kg iv) and anesthesia was maintained with small supplementary doses (2–5 mg/kg/h). The average body weight (BW) was 13.8 kg (range: 11–16 kg). The animals were placed on heating pads to maintain the body temperature at about 37–38 °C. Polyethylene catheters were introduced into the femoral artery to record arterial blood pressure (BP) and to take blood samples. A catheter in the femoral vein was used to infuse endotoxin, and another one inserted through the jugular vein was used to measure central venous pressure (CVP), and in some experiments pulmonary arterial pressure (PAP). Splenectomy was performed from a midline laparotomy and the abdominal wall was then closed. The intubated animals were ventilated during the course of experiments by means of a ventilator (Harvard Apparatus, South Natick, MA). Two current band electrodes and two spot voltage electrodes were positioned on

the neck and the thorax. The experiments had been approved by the Ethical Committee for the Protection of Animals in Scientific Research of Szent-Györgyi Albert Medical University and the NIH guidelines on the use of experimental animals.

Measurements

The impedance cardiography system employed to measure the thoracic impedances (Z_0 at end-expiratory apnea and Z_{max} values at end-inspiration) and cardiac output (CO) was described earlier [1]. Stroke volume was calculated according to the empirical equation of Kubicek et al. [13]. BP, PAP and CVP were measured continuously with Statham P23Db transducers and recorded on a Beckman R611 Dynograph recorder. Arterial blood P_{O_2}, P_{CO_2} and pH were measured with a blood gas analyzer (Radiometer, Copenhagen), and hematocrit (H) values were measured with a microhematocrit centrifuge. The white blood cell count (WBC) was also determined. Total peripheral resistance (TPR) was calculated from BP, CVP and CO. At the end of the experiments the lungs were removed and weighed for the gravimetric analysis (wet weight/dry weight, WW/DW). Z_0 values at the end of experiments were used according to Spinale et al. [22] ($dEVLW_{imp}$, ml/kg) to estimate EVLW.

Experimental groups

Four protocols were performed in this study:

I. 1. *The control group* (*CONT*, n=8) received a saline infusion. 2. The endotoxin group (*ETX*, n=9) received a 2-h infusion of *Escherichia coli* endotoxin (serotype O55:B5, Sigma) dissolved in 1 ml/kg of saline: a total dose of 7.25 μg/kg during 45 min, and thereafter 6.25 μg/kg during 75 min.

II. 1. *The splenectomized control group* (*SPL CONT,* n=5) received a saline infusion 1 h after splenectomy. 2. The splenectomized endotoxin group (*SPL ETX,* n=6) received endotoxin at the same rate as in the *ETX* group 1 h after splenectomy.

III. 1. *The ventilated control group* (*RESP CONT,* n=6) received a saline infusion. 2. The ventilated endotoxin group (*RESP ETX*, n=9) received an endotoxin infusion at the same rate as in the *ETX* group.

IV. 1. *The ventilated-splenectomized control group* (*RESPL CONT,* n=5) received a saline infusion 90 min after splenectomy. 2. The ventilated-splenectomized endotoxin group (*RESPL ETX,* n=6) received an endotoxin infusion at the same rate as in the *ETX* group 90 min after splenectomy.

In the control groups, the volume and timing of the saline infusion were the same as for the endotoxin infusion.

In some experiments (n=6) oleic acid (37.5 or 75 μl/kg) was infused into the right atrium during 3 min to test the impedance cardiography system as a means of estimating EVLW [16]. In other supplementary experiments (n=6), mechanically ventilated dogs were used to study the effects of changes in tidal volume (V_T) and the respiratory rate (f) on Z_0 and Z_{max}.

Statistical analysis

Nonparametric tests were used for the statistical analysis [21]. Data are given as means, mean percentage values, medians, and the 25th and 75th percentiles [8]. The Mann-Whitney rank sum test and the Wilcoxon signed rank test were used for intergroup and within-group comparisons, respectively. The Spearman rank-order correlation coefficient was used to test association. A value of $P<0.05$ was considered significant. Statistical analyses were performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany).

Results

In the present protocol, the main effects of endotoxin on the hemodynamic parameters in the *ETX* and *RESP ETX* groups were slight increases in BP and CO at the beginning of endotoxin infusion. However, BP thereafter decreased significantly in all endotoxemic groups. CO did not change significantly until the end of the infusion in the *ETX* group, but it decreased significantly in the other three endotoxemic groups. After the endotoxin infusion, BP increased toward the baseline value in all groups. PAP decreased in all the endotoxemic groups, to a greater extent in the *ETX* and *SPL ETX* groups, and less so in the *RESP ETX* and *RESPL ETX* groups. TPR decreased significantly at the end of endotoxin infusion, then increased to the control value. WBC decreased to almost the same level in the four endotoxemic groups and returned toward the baseline values at the end of the experiments (data not shown).

The basal Z_0 values (time 0) varied in the different groups (*CONT* 41.6; *ETX* 39.1; *SPL CONT* 41.2; *SPL ETX* 46.6; *RESP CONT* 51.5; *RESP ETX* 47.4; *RESPL CONT* 40.6; and *RESPL ETX* 42.3 ohm). There was a marginally significant difference between the *ETX* and *RESP CONT* groups. The basal H values were similar in six of the eight groups (*CONT* 43; *ETX* 40.l; *RESP CONT* 39.8; *RESP ETX* 40.7; *RESPL CONT* 40.6; and *RESPL ETX* 42.8%), but differed in the two splenectomized groups

(*SPL CONT* 48.2; and *SPL ETX* 48.6%). There were no significant differences between groups in BW (11–16 kg) or in the mean distance between the voltage electrodes (24–29 cm).

Oleic acid infusion was characterized by a dose-dependent decrease in Z_0 and an increase in $dEVLW_{imp}$ (Fig. 1A). The EVLW values measured with the impedance method and gravimetric analysis increased significantly in response to oleic acid (Fig. 1B).

After endotoxin infusion, both Z_0 and Z_{max} increased significantly up to the end of infusion (Fig. 2A). Z_0 then decreased toward the baseline value, while Z_{max} decreased only slightly in the *ETX* group. In the *SPL ETX* group, there were only moderate increases in Z_0 and Z_{max}, and the impedances then returned to the baseline levels (Fig. 2B). At the end of infusion, the percentage changes in Z_0 and Z_{max} were almost the same as in the control group. In the *ETX* group, Z_{max}–Z_0 gradually increased up to the end of infusion and remained elevated up to the end of the experiments (Fig. 2A insert); in the *SPL ETX* group, there was an initial increase (Fig. 2B insert).

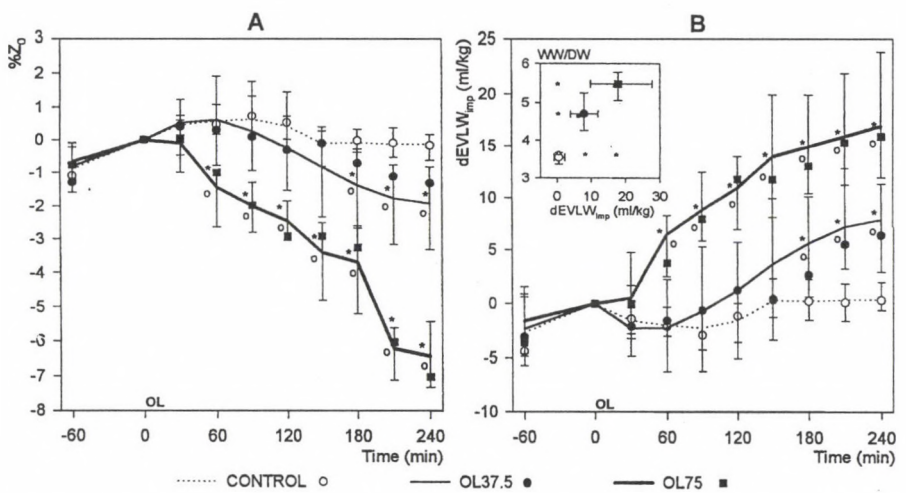

Fig. 1. Changes in transthoracic electrical impedance ($\%Z_0$) (A) and in EVLW estimated with the impedance cardiography system ($dEVLW_{imp}$) (B), and the relationship between EVLW values measured with the impedance method and gravimetrically (insert) in the control and oleic acid groups. Values are mean percentage changes ($dEVLW_{imp}$: mean values) (lines), medians (symbols) and 25th and 75th percentiles. o Significantly different from baseline value. * Significantly different from control group. OL: oleic acid 37.5 or 75 μl/kg

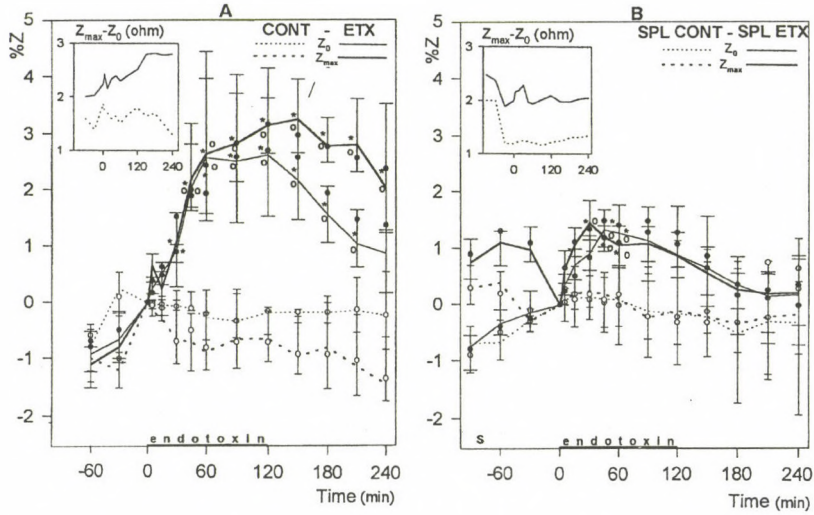

Fig. 2. Percentage changes in basal (Z_0) and maximum (Z_{max}) transthoracic electrical impedance in the *CONT, ETX* (A) and *SPL CONT, SPL ETX* (B) groups, and in Z_{max}–Z_0 (inserts). Values are mean percentage changes (lines), medians (symbols) and 25th and 75th percentiles. o Significantly different from baseline value. * Significantly different from control group. S: splenectomy

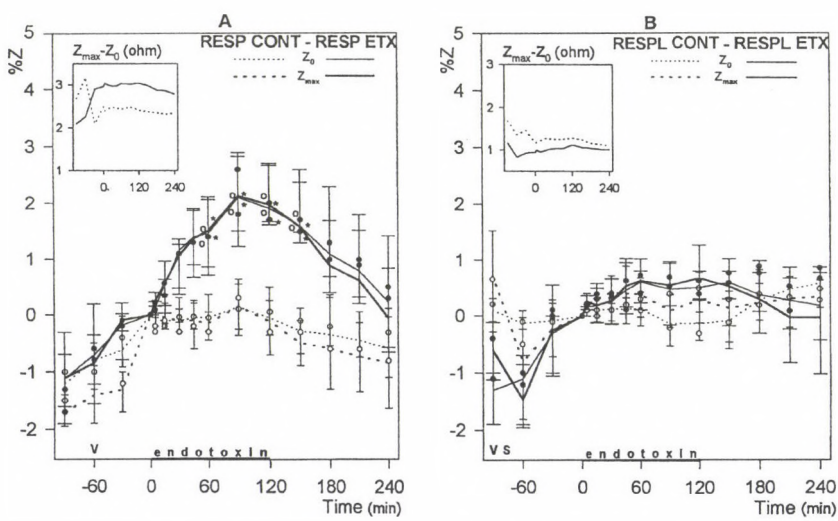

Fig. 3. Percentage changes in basal (Z_0) and maximum (Z_{max}) transthoracic electrical impedance in the *RESP CONT, RESP ETX* (A) and *RESPL CONT, RESPL ETX* (B) groups, and in Z_{max}–Z_0 (inserts). Values are mean percentage changes (lines), medians (symbols) and 25th and 75th percentiles. o Significantly different from baseline value. * Significantly different from control group. V: ventilation. S: splenectomy

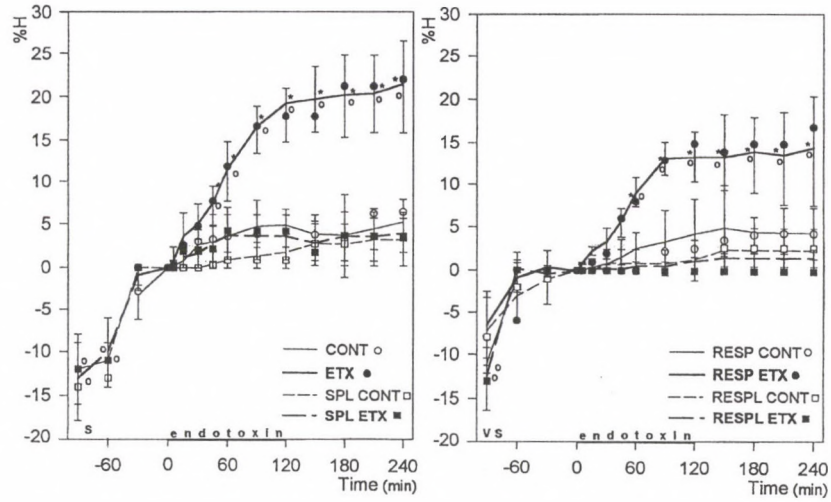

Fig. 4. Percentage changes in hematocrit (H) values in the control and endotoxemic groups. Values are mean percentage changes (lines), medians (symbols) and 25th and 75th percentiles. o Significantly different from baseline value. * Significantly different from control group. V: ventilation. S: splenectomy

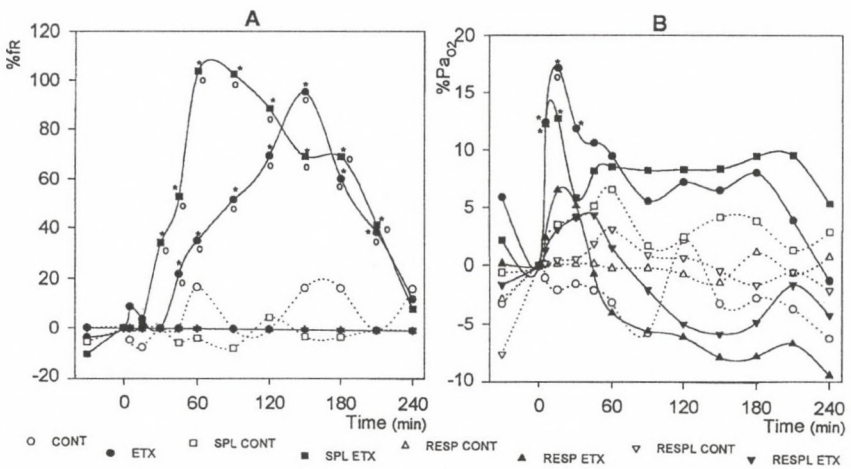

Fig. 5. Percentage changes in respiratory frequency (f_R) (A) and arterial blood P_{O_2} (Pa_{O_2}) (B) in the control and endotoxemic groups. Values are mean percentage changes. o Significantly different from baseline value. * Significantly different from control group

Z_0 and Z_{max} increased in the *RESP ETX* group to the same extent, but more slowly and to a lower value than in the ETX group (Fig. 3A). There were no significant changes in Z_0 and Z_{max} in the *RESPL ETX* group (Fig. 3B). Further, no changes in Z_{max}–Z_0 were observed in the two ventilated endotoxemic groups (Fig. 3A, B inserts).

There were significant increases in H in response to endotoxin infusion only in the *ETX* and *RESP ETX* groups (Fig. 4).

During the endotoxin infusion, f_R increased significantly in the *ETX* and *SPL ETX* groups, and returned to the baseline value at the end of the experiments (Fig. 5A). Of the blood gas parameters, the initial increase in Pa_{O_2} was the most marked effect of endotoxin infusion in the *ETX* and *SPL ETX* groups (Fig. 5B). Pa_{CO_2} gradually decreased in the *ETX* and *SPL ETX* groups, but it remained unchanged during the infusion, and then it slightly increased in the *RESP ETX* and the *RESPL ETX* groups. There were significant falls in pH in the *RESPL ETX* and *RESP ETX* groups, but no significant changes in the *ETX* and *SPL ETX* groups (data not shown) were found.

Serious edema did not occur in the different groups, as shown by both types of EVLW measurements (Fig. 6).

In the supplementary experiments (n=6), changes in V_T (Fig. 7A) and f (Fig. 7B) were applied, and Z_0 and Z_{max} were measured in mechanically ventilated dogs. Both Z_0 and Z_{max} increased, but the changes in Z_{max} in response to increasing V_T were more pronounced, and a rise in Z_{max}–Z_0 was observed only in these dogs (Fig. 7A).

Discussion

We investigated the effects of infusion of a low dose of endotoxin on the changes of Z_0, Z_{max} and H, and the role of H and respiration in the impedances. Our results suggest that both H and respiration have some kind of a role in the changes of the two impedance parameters during endotoxemia.

Our earlier results [1] demonstrated a high scatter in the basal Z_0 values. This variability in Z_0 may have been caused by variations in chest configuration, tissue mass, tissue resistivity and body fat [15]. In the current experiments, in the *SPL CONT* and *SPL ETX* groups, splenectomy resulted in high baseline H values. Some authors have emphasized that percentage changes in Z_0 provide more reliable estimates of fluid balance than do the absolute values [17, 20, 22]. Therefore, not only the changes in the impedance, but also those in the other parameters were given as percentages of the basal values.

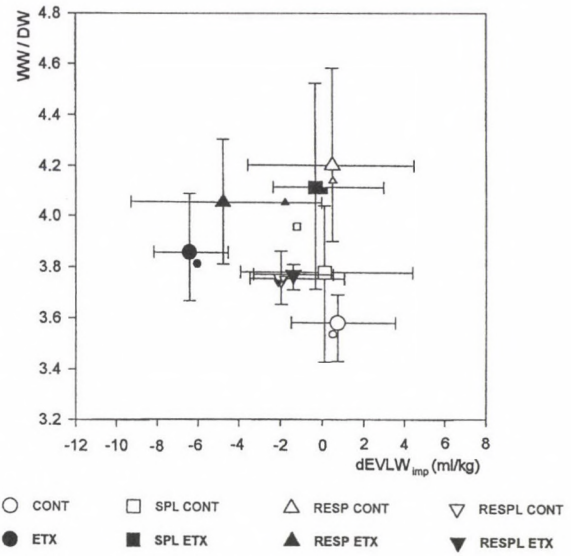

Fig. 6. Plot of extravascular lung water estimated by gravimetric analysis (WW/DW) and by the impedance method (dEVLW$_{imp}$). Large symbol: mean value. Small symbol: median value. Vertical and horizontal lines: 25th and 75th percentiles

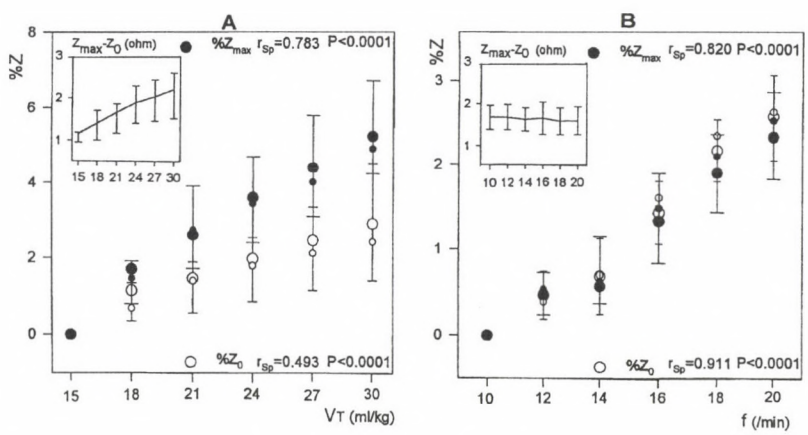

Fig. 7. Effects of tidal volume (V_T) (A) and respiratory rate (f) (B) on basal (Z_0) and maximum (Z_{max}) transthoracic impedance, and on Z_{max}–Z_0 (inserts) in mechanically ventilated dogs (supplementary experiments). Large symbol: mean value. Small symbol: median value. Vertical lines: 25th and 75th percentiles. rs_p: Spearman correlation coefficient

Many investigators have examined the relationship between Z_0 and H, but opinions as to the relationship between the two parameters differ. Thomas et al. [23] showed that there was a positive correlation between Z_0 and H in humans. Luepker et al. [14] observed a direct linear relationship between Z_0 and H, whereas Quail et al. [19] demonstrated an inverse one in dogs. In our second protocol, we studied the change in Z_0 during endotoxemia after removal of the spleen. It is obvious that endotoxemia failed to increase H in the splenectomized dogs, though it increased Z_0 slightly. In the *RESP ETX* group, both Z_0 and H increased. These results indicate the importance of H in the changes in Z_0. However, the small increase in Z_0, in spite of the unchanged H in the *SPL ETX* group, reveals that other factors may also be involved. We postulate a possible effect of changes in respiration, as suggested by the increases in the respiratory frequency and in the arterial blood P_{O_2} in the *ETX* and *SPL ETX* groups. Obviously, these changes did not occur in the ventilated dogs.

Earlier reports demonstrated that changes in Z_0 reflected changes in lung volume during breathing [2, 3, 4, 10, 12]. In our experiments, the Z_{max}–Z_0 was used as a measure of respiratory changes. The supplementary experiments in mechanically ventilated dogs (n=6) showed that this parameter increased more in response to increasing V_T than to the change in f. In the *ETX* group, Z_{max}–Z_0 gradually increased up to the end of endotoxin infusion; in the *SPL ETX* group, it increased at the beginning of the infusion. There were, of course, no changes in the two ventilated-endotoxemic groups (*RESP ETX* and *RESPL ETX*).

Endotoxin induces lung permeability changes and pulmonary edema [26]. Many investigators have found Z_0 to be a noninvasive measure of fluid volume changes [5, 7, 11, 14, 17, 18, 22]. Pomerantz et al. [18] and Hemstad et al. [11] demonstrated that Z_0 decreased before any changes in compliance or arterial blood P_{O_2} occurred in dogs. Fein et al. [7] had difficulties in differentiating between mild and severe edema. In our experiments involving the infusion of two doses of oleic acid, we observed a dose-dependent decrease in Z_0. We therefore estimated EVLW from the Z_0 changes by the method of Spinale et al. [22]. We concluded that serious pulmonary edema did not occur in the endotoxemic groups, as indicated by WW/DW, measured by means of gravimetric analysis, by dEVLW, measured by the thoracic impedance method, and by the observed hemodynamic changes. Bernstein [6] suggested that in septic patients the reduced Z_0 may be attributed to dilated muscle and cutaneous blood vessels. On the other hand, Young and McQuillan [24] found that a small Z_0 in sepsis may be related to pulmonary edema and atelectasis due to the changed lung resistivity. Sepsis changed the blood flow distribution in the thorax and the hemodynamics of the lung via the pulmonary hypertension. In our experiments, the endotoxemic model caused only a mild-pulmonary hypotension, and no serious septic responses occurred, as demonstrated by the observed hemodynamic changes.

Our results indicated that endotoxemia induced with a prolonged, slow infusion of a low dose of endotoxin increased Z_0, Z_{max} and H and changed the respiration. The increase in Z_0 can be attributed mainly to the increase in H, and the change in respiration may be reflected in changes in Z_{max}–Z_0. The thoracic impedance method has been used successfully to detect changes in respiration and to estimate EVLW changes in the thorax. The transthoracic electrical impedance proved to be a sensitive noninvasive parameter of changes in the intrathoracic fluid and gas volumes, and in the resistivity of the blood.

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Intrinsic neuronal time delays can be compensated in cat visual cortex and frog tectum with regard to motion analysis

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Motion detection is an essential biological property of vertebral brain. In order to localize moving objects exactly, intrinsic time delays of the neuronal network must be compensated for. Invariance of position with regard to velocity of a stimulus due to a negative spatial shift is one option for compensation. Experimental results found in the present study support the view that negative spatial shift occurs in the visual cortex of the cat and the tectum of the frog. An order of 30% of the visual neurons may be suited to compensate intrinsic time delays.

Keywords: motion detection, time delay, compensation, plasticity

Motion perception is a basic property of the neuronal network which is essential for organisms in order to determine its own position and the coordinates of objects. In humans impaired colour vision or stereoscopic vision are compatible with a normal and inconspicious life. On the contrary, if motion perception is impaired, it is almost impossible to pour a cup of tea without overflow and crossing a road can be a dangerous adventure for a person. Neither apparent (phi) motion, nor motion after-effects were noticed by a patient with bilateral temporo-occipital brain damage [16]. Additionally, the velocity of a moving objects was extremely underestimated. Stroboscopic flickering of about 8 Hz frequency also abolished these phenomenons in experimental situations [13].

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One condition for determining the position of a visual object in space is to combine the external and internal coordinate system [5]. The most ideal situation would be, if the two systems were spatially congruent in both coordinate systems. In reality, however, this is not possible, because of an intrinsic biological time delay of the neuronal network (neuronal conduction time, delay of synapses). The spatial difference between internal and external system of an object moving with a certain velocity must be corrected. Options are either to measure speed or to warrant invariance with regard to velocity and shape. One key question is: Are neurons able to compensate physiological time delays during the motion detection process? The following paper suggests a basic theoretical model and gives experimental evidence for compensation capabilities of visual neurons as a basic principle of motion detection in mammals and amphibians. Both cat and frog have sets of motion sensitive visual neurons, which are well characterized and therefore these species are particularly suited for analysis of basic principles of motion detection.

Material and Methods

Experimental

Animals and anaesthesia

Anaesthesia in 5 adult cats (*Felis domestica*, 2–4 kg body weight) was induced with ketamine and xylazine (0.25 ml KetalarR i.m., 0.25 ml RompunR i.m.) and atropine (1 mg i.m.). The animals were then anaesthetized with nitrous oxide (70% N20:30%02) and aliquots of barbiturates (Nembutal^R, approx. 1 mg/kg/h). In addition, a local anaesthetic (Xylocaine^R 2%) was administered during preparation. Muscle relaxation was achieved with gallamine (Flaxedil^R 20 mg initially, then 10 mg/kg/h). Nutrition and fluids were given via an indwelling venous catheter (vena salvatella). Respiratory data were adapted by controlling end-expiratory CO_2. ECG, body temperature and an EEG of the frontal cortex were continuously recorded during the experiment. The animal was put into a stereotactic apparatus and the head of the animal was fixed. The bone above areas 17 and 18 and the dura mater were carefully removed and the brain protected with agar. All animals were sacrificed after approximately 3 days (Nembutal^R 1000 mg i.v.) and the brain was removed for histological verification of electrode positions using current-induced lesions.

Three adult frogs (*Rana esculenta*) were positioned between cotton gauze pads moistened with amphibian Ringer solution on cork board. Narcosis was introduced with an amphibian anaesthetic (MS 222) and was supplemented by xylocaine. Animals

were relaxed with repeated doses of curare. The bone above the tectum was removed carefully by means of a milling tool. Amphibian Ringer solution was used repeatedly to protect the brain. The acute experiment lasted about 2 days. The animals were then sacrificed by decapitation.

Data acquisition and analysis

Single units were recorded from cat's visual cortex using an array with up to 8 independent microelectrodes (4 to 10 MOhm), which allowed separate recording of 8 electrodes. The array was fixed to a microelectrode manipulator (Microdrive 50-11-5) and adjusted to an anterior-posterior direction above areas 17 and 18. In the experiments with frogs a single microelectrode was positioned and driven with a micromanipulator (Microdrive 50-11-5).

Each receptive field was plotted by hand on a tangential screen and their positions were delineated on the screen. Light bars were moved with different velocities forward and backward. The trajectories of the light bars, which were also drawn on the screen, were adjusted in order to stimulate each neuron subsequently. Visual field representations extended up to 30 degrees of visual angle. The geometric arrangement of both the neuronal stimulation and the data acquisition system was maintained constant during a series of recordings.

Action potentials were digitalized using a window discriminator (Window discriminator WPI 120) and stored on an IBM compatible personal computer. The data were then analysed off-line and presented descriptively on the basis of the PSTHs (Peri-Stimulus-Time-Histogram). The latency of psth peaks was separately determined for both forward and backward motion. The principle of psth analysis and definitions are shown in Fig. 1. In order to visualize the time delay in this example, each time axis included one complete half-cycle of forward or backward motion. The bin width was held constant for analysis at 20 ms.

Theoretical considerations

The total latency (T) of responses to a moving bar in the psth is the sum of the conduction time (t) and a low pass time constant of higher order (t') and the time tb, which the bar needs to reach the edge of the receptive field which is located at distance ds from the starting position of the stimulus [5]. The latency of the psth (T) is measured as the onset of response for defined experimental geometrical conditions (cf. Fig. 1). If two bars are moved with different velocities, we get two total latencies as follows:

$$1: T_1 = t_1 + t' + t_{b1} \qquad T_2 = t_2 + t' + t_{b1}$$

As the distance from starting position to the receptive field (ds) is given by the experimental condition and the velocity of bar (v) is determined by the investigator, tb can be replaced by the quotient ds/v as follows:

$$2: T_1 = ds/v_1 + (t+t') \qquad T_2 = ds/v_2 + (t+t')$$

If different increasing stimulus velocities are used subsequently, a systematic temporal shift (time delay, t_L) of the responses towards the direction of the stimulus is expected (cf. Fig. 1). If we assume full compensation, the time delay t_L should tend asymptotically to zero and, consequently, the spatial shift (S_{sp}), given by the equation $S_{sp}=v*t_L$, will be independent of stimulus velocity. Equations 2 have therefore to be corrected for the time delay. Hence, the total latencies T_i for different bar velocities V_i are given by

$$3: T_i = ds/v_i + t' + t + t_{Li} = [ds + (t'+t+t_{Li})*v_i)]/v_i$$

If we introduce a logarithmic scale, we obtain

$$4: \ln T_i = \ln ([ds + (t+t'+t_{Li})*v_i]/v_i)$$

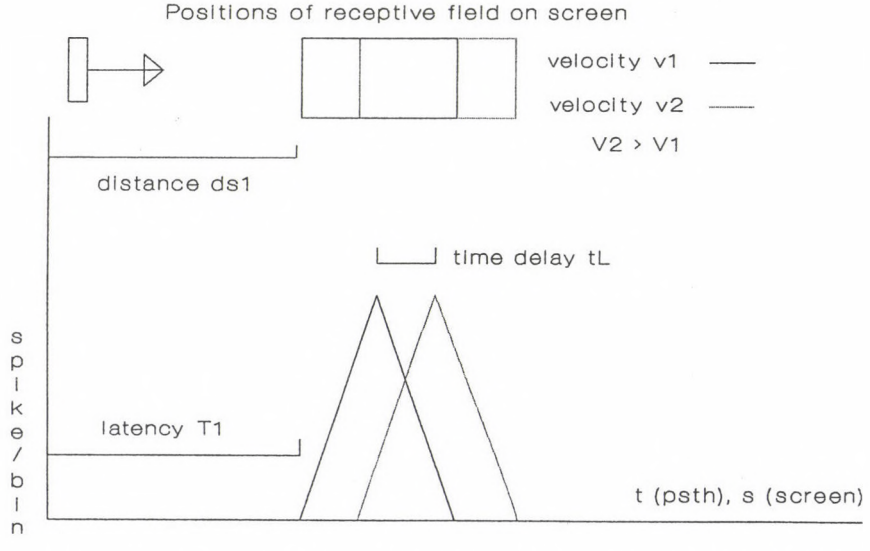

Fig. 1. Definition of PSTH parameters. The receptive field is shifted towards the direction of the stimulating light bar as the velocity V of the bar is increased. If this time delay (tL) is small compared the increase in speed, the spatial shift (Sp=v*tL) could be invariant with regard to velocity. The position on the screen corresponds to the position on the visual cortex

If the factor $(t+t'+t_L)$ tends to zero, i.e. if the time delay is negative and completely compensates conduction time constant, the result is given by equation 5.

$$5: \ln T_i = \ln (ds) - \ln (v_i)$$

Equation 5 can be represented as a straight line in a double-logarithmic coordinate system. The point of intersection depends on the geometrical design (ds). The degree of compensation would sufficient, if the slope is exactly –1. Neurons which show a slope greater than –1, but smaller than zero, will be partially capable to antagonize the spatial shift, as $v*t_L$ will increase, if higher velocities are used. As the velocity increases, localization of moving objects will be impaired gradually. If the slope is smaller than –1, overcompensation should be supposed and, as a consequence, the spatial shift will show negative values. These cells would be suited for complete compensation of intrinsic biological time delays.

Results

Figure 2a, b and c show original recordings of single area 17 cell stimulated by a light bar with velocities of 10, 15 and 30 deg/sec, respectively. The velocity in the second recording (2b) was increased 5 deg/sec and produced a relative time delay t_L of 225 ms compared to psth 2a. Interestingly, this neuron compensated time delays better at highter velocites, as the delay was reduced to approximately 17.5 ms, if the bar was applied with 30 deg/sec. As a consequence, the relative position of the receptive field on the cortex was slightly shifted towards the direction of the stimulating light bar at a velocity of 15 deg/sec, but was not substantially shifted at a velocity of 30 deg/sec. The extent of this spatial shift (S_{sp}) depends on both time delay t_L and velocity of the bar. It is evident that the velocity of the stimulus influenced the variable anterior-posterior position of the receptive field on the visual cortex of the cat.

Figures 3 and 4 show the alteration of the latency of the PSTH peaks as a function of stimulus velocity of 8 cells simultaneously recorded in cat visual cortex, respectively. Within the range of stimulus velocities used, the cells appear to fulfill the theoretical conditions for full or partial compensation. With regard to forward motion 6 out of 18 area 17/18 cells investigated in this study showed a slope smaller than –0.9 in the log-latency versus log-velocity plot. Thus, about 1/3 of area 17/18 cells may be suited for complete or partial compensation of intrinsic time delays.

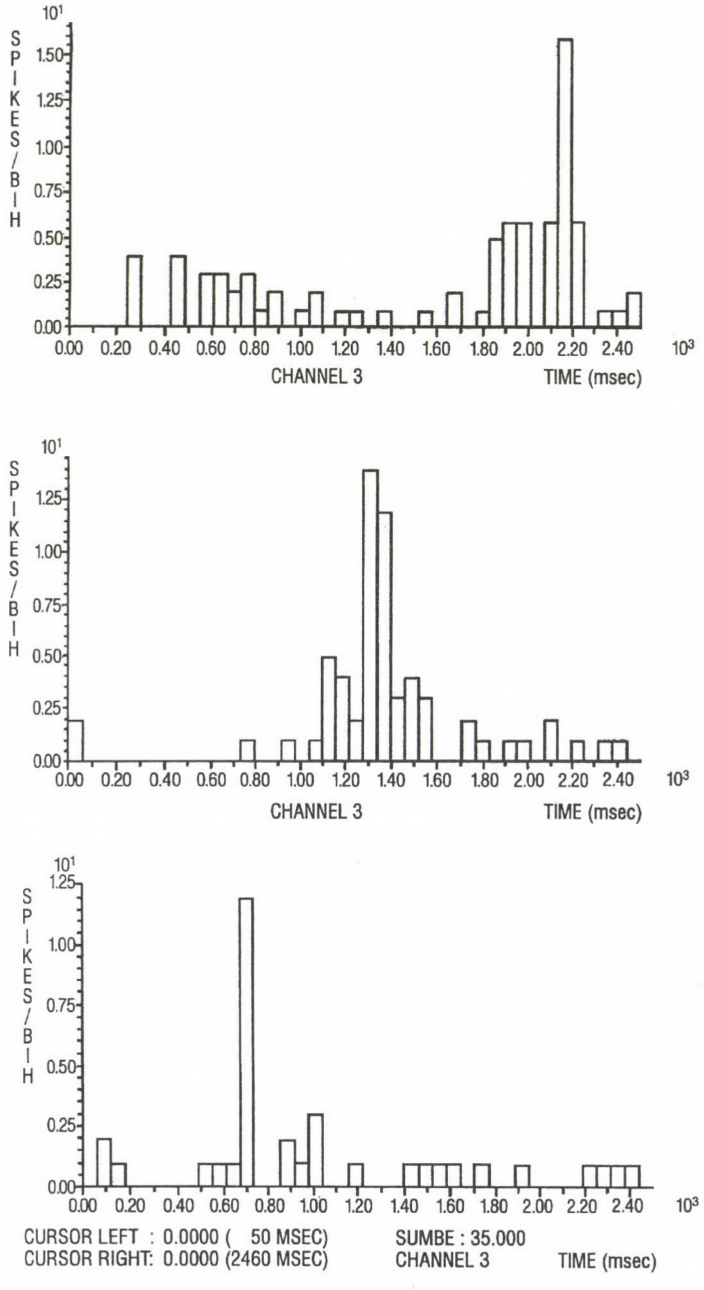

Fig. 2a/b/c. Original recording of an area 17 cell in cat visual cortex at 3 different velocities. Time axis was normalized at 2500 ms. Velocities of light bars were 10 (a), 15 (b) and 30 (c) deg/sec, respectively. With regard to peak maximum at a velocity of 10 deg/sec (2150 ms) the neuron produced time delays of approximately 225 ms (15 deg/s) or approximately 17,5 ms (30 deg/sec)

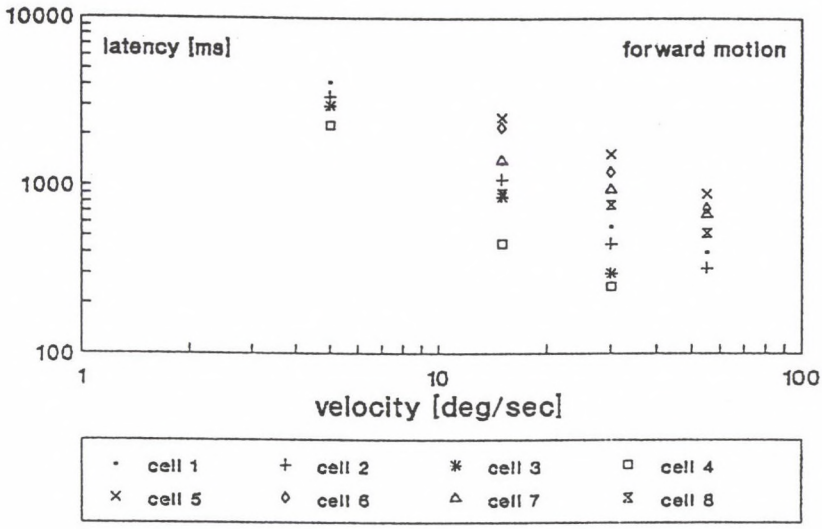

Fig. 3. Latencies of 8 simultaneously recorded neurons in cat visual cortex as a function of stimulus velocity. The cells presented in Fig. 2 appear to be suited for compensation of the time delay

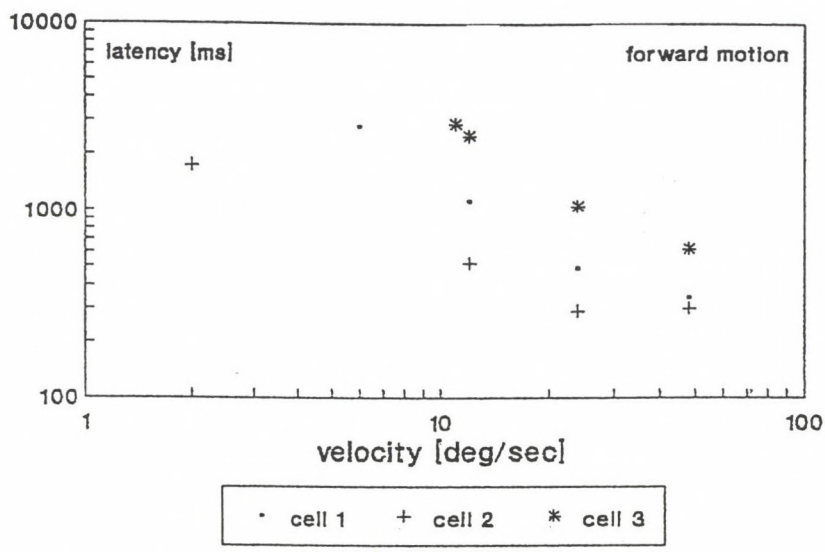

Fig. 4. Latencies of 3 simultaneously recorded cells in cat visual cortex as a function of stimulus velocity. Neuron No. 3 appears to be suited for compensation of time delays within the range of stimulus velocities used

Table I

Distribution of slopes in the log-latency vs. log-velocity plots of 18 visual neurons in cat primary visual complex

	Slope>–0.9	-0.9<=slope<=–1.1	Slope<–1.1
Forward motion	5	7	6
Backward motion	6	9	3

Figure 5 represents the latency versus velocity plot of a single cell in frog's tectum. For relatively low velocities the cell is compensating the time lag sufficiently, but however, it seems that the capability for compensation is diminished with increasing bar velocities. This finding is identical with a variable spatial shift. In Fig. 6 the latencies of another tectal neuron are presented as a function of velocity. This cell obviously is able to process velocities up to 100 deg/sec.

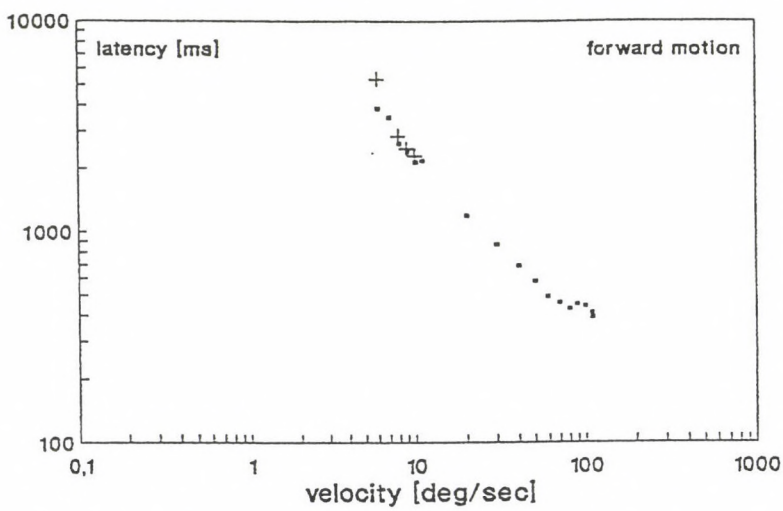

Fig. 5. Latency of a single cell in frog's tectum as a function of stimulus velocity. The neuron is suited for compensation up to 100 deg/sec (crosses represent repeated recordings)

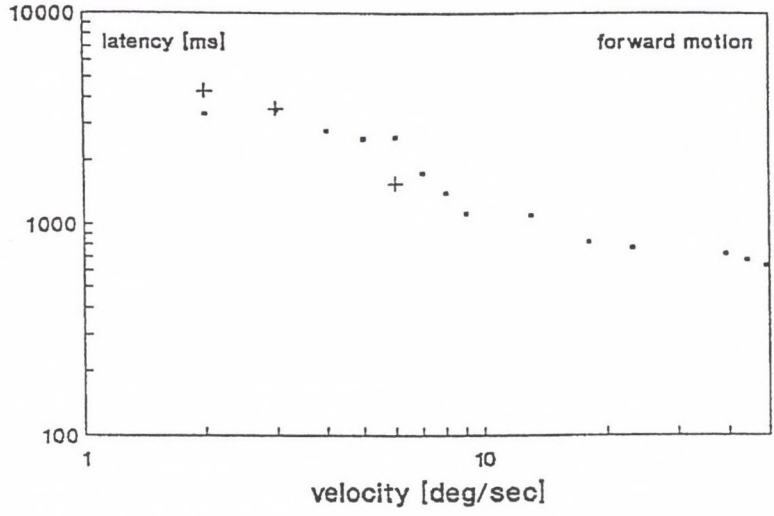

Fig. 6. Latency of a single neuron in frog's tectum as a function of stimulus velocity. The neuron is only partially suited for compensation of the time delay up to approximately 10 deg/sec (crosses represent repeated recordings)

Discussion

The results indicate that about 1/3 of cells in cat's visual cortex may be capable to compensate completely the intrinsic time delay as they produce a negative spatial shift. Moreover, the results show that neurons in frog's tectum are, at least in principle, suited for compensation. Dinse and Koch [2] and Koch et al. [6], recording with an array of eight microelectrodes, showed that the apparent velocity ($V_{app}=dX_{rf}/dt_{PSTH}$; dX=distance between receptive fields on screen, dt=time interval between psth peaks) between two receptive fields during a sweep of activity along a vertical trajectory could well exceed the velocity of the stimulus. Of course, one prerequisite is that responses of the single cell are able to "run forward" in comparison with the velocity of the stimulating light bar. This result is clearly verified in the present work. Of course, motion perception is not the property of a single cell. On the contrary, Reichardt [12] postulated that motion detection in insects, for example, is based on the "team work" of neighboured ommatidia. Similarly, motion perception in vertebrates is the result of interaction of an array of visual receptive fields or cortical cells which are stimulated subsequently. Jensen and Martin [5] found that particularly the Clare-Bishop-Area (Postero-medial lateral suprasylvian area, PMLS) was responsible for compensating

time lags. Areas 18 and 19 were not or only partially able to render the spatial shift invariant in respect to increase of velocity. However, in the present study it is shown that not only PMLS structures may be able to minimize the time delay but also the striate visual cortex. In real life, it is essential for animals to produce this "run forward" in order to catch a prey, i.e. to combine their own coordinate system and the system of the background. This processing could only be achieved for a certain range of velocities up to a cut-off level for an individual neuron.

Most of the models of motion processing postulate a series of receptive units which are characterized by their position Px and time tx {S1, t1; S2,t2;...). These units feed direction and velocity sensitive subunits [10]. The time intervals $dt=t_{x+i}-t_x$ could be used as a basic information for motion analysis [15]. The level of subunits is followed by a neural integrator not yet specified. The resulting signal will be proportional to the velocity of the stimulus. As the spatial distance of the receptive units is given, the system could use the temporal distance t between the two or more units to get the velocity-dependent factor. Complex stimulus-background and inter-area interactions influence motion detection [11]. According to Orban et al. [11] global motion differences are computed in V2 of the monkey by so-called antiphase direction selective cells, which deliver information to layer 4 cells of V5. Such interactions may also be postulated for the visual system for the cat and frog and could be responsible for motion analysis on a finer scale. Livingstone [7] postulated different channels for colour detection, shape detection and motion detection in humans, which correspond to the blob, parvo-interblob and magno system, respectively. The latter might include the equipment to compensate the intrinsic time delays of biological systems in order to achieve invariance of the coordinates of the object with regard to velocity and shape.

The position of the visual receptive field is variable depending on the velocity of a stimulus. This finding clearly refers to the notion of neuronal plasticity. According to the results presented in this paper, motion detection and short-term plasticity appear to be close related properties of the visual and probably of the neural system. The original model of Mountcastle and Darian-Smith [9] for receptive fields in somesthesia defined both constant position and size of the receptive field in the neural space. However, cortical maps are nowadays considered as dynamic features and can be altered by external influences such as drugs (for review see Garraghty and Kaas [4]). The data of this study underline that receptive field position is decisively influenced by stimulus velocities.

The extrapolation of the results to motion analysis processes in man is complex as experimental methods are not identical. However, psychophysical studies give hints that separate systems for colour and motion analysis exist in man [8]. Moreover the concept of plasticity with regard to motion detection appears also valid in human, as experiments with visual evoked potentials have shown [3]. More sophisticated neurophysiological methods such as PET (positron emission tomography) or fMRI

(functional magnetic resonance tomography) revealed that moving gratings preferentially stimulated extrastriate regions, which an indication for a more complex motion processing in man compared to cat or frog [1, 14]. It can therefore be supposed that compensation of time delays in humans involves both striate and extrastriate areas.

In conclusion, the localization of moving objects in space is based on the ability of the neuronal network in the visual cortex of the cat and the tectum to compensate biological intrinsic time delays. About 1/3 of cat visual neurons even produced a negative spatial shift and, therefore, overcompensated time delays.

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Adherence of peripheral blood neutrophils in X-ray operators

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The studies were conducted among 39 people (29 women and 10 men) operating X-ray equipment in radiology institutes and comparisons were made in the following schemes: A/ Group of persons operating the X-ray equipment (39 persons) and control group (18 persons). B/ A subgroup of persons that had been operating radiology equipment for >5 years (29 workers was separated) and the results were compared with those for the subgroup of persons working <5 years (10 workers). C/ Among the workers a subgroup of women (29) was separated and the results were compared with those for the subgroup of men (10 men). The following investigations were carried out: 1./ The absolute number of neutrophils in peripheral blood. 2./ Neutrophil adherence to fibres. 3./ Evaluation of CD 11b/CD 18 adhesion molecules expression on the surface of neutrophils. 4./ Evaluation of fibronectin concentration in serum.

In the group A of the employees examined who operated X-ray units compared with the control group, the statistically significant decrease in the percentage of peripheral blood neutrophils showing adherence was observed and statistically significant decrease in median value and decrease of "Peak" value from the curve of the CD 18 adhesion molecule expression was detected and amounted to respectively: 91.0±4.26% adhering neutrophils in workers operating X-ray units and 94.8±2.98% in control group; values of median 120±4 in workers and 123±3 in control group in logarythmic scale; values of "Peak" 120±4 in workers and 123±3 in control group in logarithmic scale. Our own testing results show that the adherence properties of the peripheral blood neutrophils taken from X-ray operators are independent of the length of employment period and of the sex of the persons tested.

Keywords: X-ray, neutrophils

Adherence of peripheral blood neutrophils as element of the function of those cells may undergo changes under multidirectional actions of both extra- and intracellular factors [9, 10, 11]. Recent reports indicate that adherence properties of

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neutrophils may be influenced by inflammatory cytokines, e.g. TNF = tumor necrosis factor [3, 12, 17, 20], IL-1 = Interleukin-1 [20], G-CSF = granulocyte colony stimulating factor [25], as well as by fibronectin [7] and glycoproteins of basement membrane of vascular wall, such as e.g. thrombospondin [23] and laminin [2]. Clinical and experimental evidence has shown the importance of so-called family of adhesion molecules on the surface of leukocytes in early phase of leukocyte adherence to endothelial cells in vivo [15]. Many of the above-mentioned factors influence the adherence just through the mechanism depending on the complex family of adhesion molecules on the surface of leukocytes, being called leukocyte adhesion glycoproteins of CD11/CD18 group, included among leukocytic beta 2 integrines which are stored inside neutrophils and then in response to various activators are being shifted onto the surface of those cells [1, 9, 11, 21]. Moreover, it is to be stressed, that the adhesion molecules being most important for the function of adherence neutrophils are CD11b/CD18 molecules previously called Mol or Mac-1 [15, 24].

The aim of this study was to estimate neutrophil adhering properties in the light of expression of CD11b/CD18 adhesion molecules on the surface of those cells, in persons who operate X-ray units.

Materials and Methods

The studies were carried out among 39 people (29 women and 10 men, between 21 and 68 years of age, 37.5 mean age) operating X-ray equipment in radiology institutes. Their length of employment in operating X-ray equipment was at least 1 year and varied from 1 year to 35 years (average length 11.6 years). Exceeding the admissible standard of ionizing radiation dose kept as obligatory in radiology departments, was not observed in any person. A control group, identical to the studied one with respect to age and place of residence, was formed by 18 healthy persons (14 women and 4 men) not working in radiology institutes. In this group there were no deviations from norm in the range of basic laboratory test and there was no detectable infection during the month preceding the study.

Studies were conducted on the aforenamed persons and comparisons were made in the following schemes: A/ Group of persons operating the X-ray equipment (39 persons) and control group (18 persons). B/ Among the workers operating X-ray equipment there was separated a subgroup of persons that had been operating radiology equipment for >5 years (29 workers) and the results obtained were compared with those for the subgroup of persons working <5 years while operating radiological equipment (10 workers). C/ Among the workers operating the X-ray equipment a

subgroup of women (29 women) was separated and the results of tests obtained were compared with those for the subgroup of men (10 men).

Blood was collected from the elbow vein (in the morning between 7 and 9 a.m.) from persons operating X-ray equipment and qualified for the control group with the purpose of testing neutrophils and blood serum. The following invetsigations were carried out: 1./ The absolute number of neutrophils in peripheral blood was determined by means of the "Cytron Absolute" automatic device of the "Ortho Diagnostic" firm. When operating this unit a method of flow cytometry was used, based on analysing the data obtained by a laser beam. The results were expressed with reference to 1 ml of blood. 2./ Adherence to fibers [14]. After determining the number of neutrophils in heparinized whole blood samples, the blood was passed through adherence columns (Pasteur pipettes, in which 80 mg of scrubbed polyacrylonitrile-anilane-fibres were packed). The number of neutrophils in the filtrated blood was determined as an average value of 3 measurements done simultaneously for each person examined and the percentage of neutrophils adhering to the column was calculated (index of adherence). 3./ Evaluation of CD 11b/CD18 adhesion molecules expression on the surface of peripheral blood neutrophils was tested by the flow cytometry [16]. 100 µl whole blood was placed in polystyrene test tube and monoclonal antibodies coupled with fluorescein isothiocyanate (FITC), 10 µl each, against CD11b and CD18 integrines of Daco company, were added. After mixing, the samples were incubated in the dark at room temperature for 30 min. Next, into each test tube the portions of solution, 2 ml each, used for erythrocytolysis, were added, mixed and left for 10 min at room temperature. The test tubes were centrifuged (400 g, 5 min) and supernatants removed. Then the cell suspension was washed twice, adding Cell Wash of "Becton Dickinson" Company at 0 °C, 1 ml each time. After the second washing into the test tubes portions of 0.25 ml Cell Wash were added. The so-prepared cell suspension was subjected to dichromatic analysis in the "Cytron Absolute" flow cytometer of "Ortho-Diagnostic" Company, with the argon laser of 488 nm light wavelength. Each time minimum 10,000 cells were analysed. The histograms received were examined by ImmunoCount II computer program. The expression magnitude of the molecules tested was estimated and represented by; median, "Signal" and "Peak" values of the fluorescence curve (Fig. 1). 4./ Fibronectin concentration in serum was evaluated by the nephelometric method [4] using Behring Nephelometer Analyzer and N Antiserum to Human Fibronectin of Behringverke Diagnostic AG. A chemical method of instrumental analysis based on measurement of intensity of light dispersed by the suspension of sparingly soluble substances and compared with the intensity of light obtained from standard solution was applied in this unit. Results were expressed in g/l.

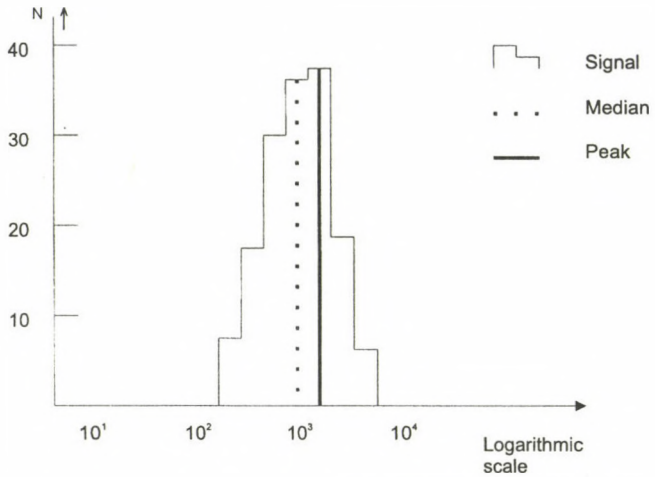

Fig. 1. Scheme of fluorescence intensity curve. N = cells number, Signal = total luminous intensity of the cell population tested, Median = central value of luminous intensity of the cell population tested, Peak = luminous intensity group cells with the largest number

The results were statistically analyzed by calculating the arithmetic means ($\bar{X}$) and standard deviations (SD). Comparison of the means in both tested groups was preceded by verifying the hypothesis of variances equality [18]. Fischer F-test was used for the difference of two variances. The statistical significance was estimated by Student's *t*-test for independent data and Cochrane and Cox test [18]. The difference between arithmetic means was considered significant at the significance level $p<0.05$.

Results

In workers operating radiological equipment, in any of the group/subgroups the number of neutrophils in peripheral blood did not differ significantly as compared with the control group/subgroups and amounted to respectively: group A 2 901±1528 per 1 ml and 2 666±1219 per 1 ml; subgroup B 1 603±1697 per 1 ml and 2 363±682 per 1 ml; subgroup C 2 573±1 002 per 1 ml and 3 859±2 320 per 1 ml, respectively.

In the group A of the employees examined who operated X-ray units compared with the control group, the statistically significant decrease in the percentage of peripheral blood neutrophils showing adherence was observed. The results are presented in Table I.

Table I

Adherence index of peripheral blood neutrophils investigated in persons operating X-ray equipment and in controls

Investigated group/subgroup		Adherence index %		Statistical significance of differences between means		
		$\bar{X}$	±SD	t/C	p	
Workers operating X-ray equipment (total group)	N=39	91.0	±4.26	o C	3.8775	<0.05
Control group	N=18	94.8	±2.98	C 0.05	2.0722	
Subgroup operating X-ray equipment over 5 years	N=29	91.5	±4.30			
				t°	1.5386	n.s.
Subgroup operating X-ray equipment below 5 years	N=10	89.6	±3.71			
Subgroup of women operating X-ray equipment	N=29	91.03	±4.01			
				t°	0.0187	n.s.
Subgroup of men operating X-ray equipment	N=10	91.00	±5.16			

N = group size, C = by Cochrane and Cox's test "C", t = by Student's *t*-test, n.s. = not significant

In the subgroup B concerning the employment period and the subgroup C concerning sex there were no significant differences concerning adherence index when the results were compared with adequate control subgroups, Table I.

Among workers of radiology institutes in comparison with the control group and in the additionally isolated subgroups no statistically significant differences in the range of median values, "Signal" and "Peak" from the curve of the CD11b adhesion molecules expression on the surface of peripheral blood neutrophils were found. The values of median in the group A amounted to 179±10 in the logarithmic scale and 183± 15 in the logarithmic scale; in the subgroup B, 178±11 in the logarithmic scale and 181 ±3 in the logarithmic scale; in the subgroup C, 178±11 and 180±6 in the logarithmic

scale, respectively. The values of the "Signal" were respectively: group A, 516 865± 275 595 in the logarithmic scale in the group operating radiologic equipment and 487 475±232 377 in the logarithmic scale in the control group; subgroup B, 550 456± 306 684 in the logarithmic scale in employees working for >5 years and 419 450± 117 870 in the logarithmic scale in employees working for <5 years; subgroup C, 457 018±180 141 in the women and 690 418±417 998 in the logarithmic scale in the men operating X-ray equipment. The values of the "Peak" amounted to in the group A, 180±10 in the logarithmic scale in persons who operate X-ray units and 183±16 in the logarithmic scale in the controls; in subgroup B, 179±12 in the logarithmic scale in those operating for >5 years and 182±5 in those operating for <5 years; subgroup C, 179±11 in the logarithmic scale in women and 181±6 in the logarithmic scale in the men operating X-ray equipment, respectively.

In the group A of persons operating the radiology equipment as compared with the control group a statistically significant decrease in median value from the curve of CD18 adhesion molecule expression on the surface of peripheral blood neutrophils was observed, Table II. In separated subgroups: B, based on employment period and C, considering sex no statistically significant differences were found concerning the median value, Table II, part A.

Table II

Glycoprotein expression of CD18 group on neutrophil surface in workers operating X-ray equipment and in controls

Investigated group/subgroup Part A		Values of median from the curve of the CD18 adhesion molecule expression in logarithmic scale		Statistical significance of differences between means		
		$\bar{X}$	±SD	t/C	p	p
Workers operating X-ray equipment (total group)	N=39	120	±4			
				t^o	2.9511	<0.05
Control group	N=18	123	±3			
Subgroup operating X-ray equipment over 5 years	N=29	120	±5	C^o	0.2130	n.s.
Subgroup operating X-ray equipment below 5 years	N=10	120	±1	C	2.0939	0.05
Subgroup of women operating X-ray equipment	N=29	120	±4			
				t^o	0.0383	n.s.
Subgroup of men operating X-ray equipment	N=10	120	±5			

Table II (*Cont.*)

Investigated group/subgroup Part A		Values of peak from the curve of the CD18 adhesion molecule expression in logarithmic scale		Statistical significance of differences between means		
		$\bar{X}$	±SD	t/C	p	p
Workers operating X-ray equipment (total group)	N=39	120	±4	t°	2.6830	<0.05
Control group	N=18	123	±3			
Subgroup operating X-ray equipment over 5 years	N=29	120	±5	C°	0.5510	n.s.
Subgroup operating X-ray equipment below 5 years	N=10	121	±2	C 0.05	2.1068	
Subgroup of women operating X-ray equipment	N=29	120	±4	t°	0.03721	n.s.
Subgroup of men operating X-ray equipment	N=10	120	±5			

N = group size, C = by Cochrane and Cox's test "C", t = by Student's *t*-test, n.s. = not significant

Among the workers of investigated group of radiology institutes as compared to the control group and in the additionally isolated subgroups no statistically significant differences have been found in the range of "Signal" value from the curve of the CD18 adhesion molecule expression on the surface of peripheral blood neutrophils. They were in the group A, 341 395±169 821 and 325 226±146 738 in the logarithmic scale; in the subgroup B, 326 874±187 831 and 279 107±78 775 in the logarithmic scale; in the subgroup C, 305 663±119 685 and 445 020±247 294 in the logarithmic scale, respectively.

The decrease of "Peak" value from the curve of the CD18 adhesion molecule expression on the surface of peripheral blood neutrophils from the persons operating X-ray equipment compared with the control group was detected, Table II. There was no significant difference between the respective values of the "Peak" when both the subgroup B and C were estimated, Table II, part B.

Persons operating X-ray equipment as compared with control group and additionally isolated subgroups did not show any statistically significant differences in the range of fibronectin concentration in blood serum and amounted to respectively: group A, 0.207±0.090 g/l in the workers operating X-ray units and 0.197±0.060 g/l in the controls; subgroup B, 0.199±0.095 and 0.230±0.073 g/l; subgroup C, 0.191±0.084 and 0.253±0.095 g/l.

Discussion

It has been known for some time that the number of peripheral blood neutrophils can influence the adherence, particulary when the number of neutrophils is very high [6]. Previous studies have shown a decrease in the total leukocyte, absolute lymphocyte and absolute neutrophil counts in humans after radiation [13, 22]. In the present study we have not found statistically significant differences in the total number of the peripheral blood neutrophils among the people operating X-ray equipment in radiology institutes as compared to analogous cells from control group and in investigated subgroups. It means that those numbers lay inside the interval of physiological values, thus the effect of the neutrophil number on index of adherence can be excluded.

In our study a statistically significant decrease in the capacity of neutrophil adherence in persons operating X-ray equipment as compared to control group was found. Irrespective of the factors influencing the adherence properties of neutrophils the finding of a lower capacity of neutrophil adherence in investigated persons may suggest some changes in cellular surface properties of the peripheral blood neutrophils. However, adherence studies give no information if the changes in the surface properties of neutrophils are the consequence of the influence of plasma factors [6], or if they are caused by intraneutrophilic metabolic disturbances [6, 9].

During inflammation leukocytes are attracted by chemotactic stimuli, resulting in migration of cells from the blood stream to the inflamed tissues. As an initial event, the leukocyte adhere to the endothelial blood vessel lining, thereafter diapedesis occurs [8, 9, 15]. A central role in neutrophils adherence and activation, developing as a result of stimulation by chemotactic factors as well as with contribution of inflammatory cytokins is played by the complex of the surface leukocytic glycoproteins of the CD11/CD18 group [9, 11].

In studies carried out in the rats there was evidence indicated on the molecular mechanisms that the adhesion and migration of leukocytes was elicited by radiation. It was observed that immunoneutralization of the common beta subunit (CD18) of the leukocyte adhesion glycoprotein CD11/CD18 had a profound effect on attenuating leukocyte adherence after irradiation [19].

Our own testing results obtained by evaluation of the adhesive CD18 particles expression on the surface of peripheral blood neutrophils in X-ray operators indicate its attenuation revealed by the median reduction in the expression curve for those particles and their "Peak" values as compared with the control group. Thus they constitute on a molecular level an additional confirmation of the attenuation observed for the peripheral blood neutrophil adherence to fibres in the tested group. However, they do not allow to determine which factors initiate those processes, i.e. whether they are of intra- or extracellular origin. It may only be supposed that this factor is not the

fibronectin, multifunctional adhesive glycoprotein, the concentration of which in the investigated group in serum has not changed. The results of these studies are in accordance with the earlier findings [5] which revealed that the neutrophils stimulated, originating from the tissues irradiated, are not able to increase the expression of the surface CD11b/CD18 molecules.

Moreover, our own testing results show that the adherence properties of the peripheral blood neutrophils from X-ray operators are independent of the length of employment period and of the sex of the persons tested because in the subgroups isolated no statistically significant differences were observed concerning the adherence index and the expression evaluation for the surface CD11b/CD18 adhesion molecules.

Conclusion

Observed attenuation of peripheral blood neutrophils adherence was confirmed on a molecular level by reduction in the expression of CD 18 adhesion molecule on the surface of those cells in the workers who operate X-ray units and was independent of the length of employment period and of the sex of the investigated persons.

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Purified extracts from short-time-predegenerated rats' sciatic nerves promote the regrowth of injured hippocampal neurites

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Our previous studies revealed that purified extracts (submicrosomal fractions) obtained from peripheral nerves predegenerated for 7-, 28-, and 35-days facilitated neurite outgrowth from the injured hippocampus. It is recently known that totally transected peripheral nerve exhibits biphasic neurite-promoting activity. The early phase lasts 7 days. The aim of the present study was to find whether extracts obtained from short-time predegenerated (1–6 days) peripheral nerves exert any neurotrophic effect and when this influence is maximal. Experiments were carried out on adult male Wistar rats. Sciatic nerves were totally transected and following 1, 2, 3, 4, 5 and 6 days their distal stumps were homogenized and centrifuged. Extracts were implanted into the hippocampus by means of autologous connective tissue chambers. Reference groups were treated with extracts from non-predegenerated nerves, NGF solution or fibrin (groups C, NGF and B+F, respectively). In all groups FITC-HRP was injected into the extracranial end of chamber six weeks following surgery. Histochemic technique showed AChE-positive fibres inside the chambers of all examined groups. Fluorescence microscopic examination revealed the labeled cells in all examined groups, however their number was different in each group. They were most numerous at the fourth day of predegeneration.

Keywords: sciatic nerve, hippocampal neurites, peripheral nerve degeneration, neurotropic effect, Wistar rat, NGF

It is commonly known that axons in the adult mammalian central nervous system (CNS) do not regenerate spontaneously although recent pieces of evidence indicate that they have some ability for initial sprouting [1]. Numerous research teams are looking

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for a method useful in promoting and supporting the CNS cells regeneration. The brain and spinal cord are treated for instance with fetal tissue implants, peripheral nerve grafts, genetically modified cells, hormones, tissue extracts and neurotrophic factors [6, 7, 13]. A variety of tissue extracts as well as growth factors were introduced into the CNS not only in order to stimulate regrowth of injured fibres, but also to assess their neurotrophic activity in respect to different neuronal populations [17, 19, 23]. One of the principal problems as far as the latter experiments are concerned was to find an adequate supplying system. In 1979 Lundborg and Hansson made use of performed autologous chambers for the study of peripheral nerve regeneration [25]. These tube-shaped chambers were called pseudo-synovial or mesothelial. On the basis of our previous work we developed a method for making connective autologous tissue chambers as well as a way of their implantation into the CNS [4, 5]. The content of the implanted chambers consisted of fibrin mixed with purified extracts from distal stumps of rats' sciatic nerves predegenerated for 7, 28 and 35 days. Such extracts revealed the ability to stimulate regeneration in lesioned hippocampal neurites [23]. Our recent studies have indicated that short-time-predegenerated peripheral nerve grafts have also some regeneration ability [24]. Therefore we decided to check whether tissue extracts derived from such nerves are able to promote the regrowth of injured hippocampal neurites.

The aim of the present paper has been to ascertain whether submicrosomal fractions obtained from distal stumps from short-time-predegenerated (1–6 days) rats' sciatic nerves exert any neurotrophic effect and whether this eventual influence resembles that of short-time-predegenerated sciatic nerves grafts.

Materials and Methods

Experiments were carried out in 169 male Wistar C rats (b.w. ~150 g). Seventy animals were donors of nerves used for the preparation of submicrosomal fractions. They were divided into seven equal groups. Ninety-nine rats were used as recipients of implanted chambers, filled with appropriate submicrosomal fractions or reference solutions.

Preparation of submicrosomal fractions

Subsequently to an intraperitoneal anaesthesia with chloral hydrate (420 mg/kg), both sciatic nerves were totally transected at the level of the hip joint. One to six days later the animals were sacrificed by decapitation and distal stumps of the transected nerves were dissected and placed in cold Ringer's solution for mammals. The seventh

group formed a control; in this case nerves were collected without predegeneration. Submicrosomal fractions were prepared according to the method described by Sieroń et al. [31]. The dissected distal nerve stumps were rinsed in 0.9% NaCl and then homogenized in 2 ml of a homogenizing buffer per 1 g of nerve tissue. Homogenates were filtered, centrifuged twice and subsequently ultracentrifuged. All manipulations were performed at 0 °C (273 K) in the presence of a protease inhibitor PMSF. The total protein concentration in the final extracts was determined by the method of Bradford [9].

Preparation of the autologous connective tissue chamber and the surgical procedure

Recipients were anaesthetized as above and pieces of a silicone tube (10 mm long, 2 mm e.d.) were implanted subcutaneously on their backs. Four weeks later, the tubes were removed. During the period of implantation a connective tissue layer grew around the tube, thus forming a connective tissue chamber. Subsequently one end of the chamber was cut off and the connective tissue was pulled down. Through the free end of the silicone tube, the chamber was filled by means of a microsyringe with 20 µl of tissue extract, 20 µl of fibrinogen and 5 µl of thrombin solution. After few minutes its content became jelly-like because of fibrin formation. The whole chamber assumed the form of a 'sausage', one end of which was tied with 4–0 surgical silk. With the help of this thread, the chamber was drawn into a glass cannula (moistened inside with cold Ringer's solution for mammals) and its protruding end was cut off in order to assure contact between the brain tissue and the content of the chamber [4, 5].

Animals were divided into nine groups. Six of them were grafted with chambers containing submicrosomal fractions obtained 1–6 days after nerve transection (groups 1D–6D, respectively). The other three formed reference groups. The first reference group (the control – C) got chambers with a fraction obtained from non-predegenerated nerves. In the second one (B+F), chambers were filled with fibrin and buffer used for homogenization only. The third-one (NGF), contained 20 µl of NGF solution inside the chamber (Table I).

The chambers were implanted into the hippocampus according to the method described by Barski et al. [4, 5]. Briefly, a hole was drilled in the skull (3 mm caudal and 3 mm lateral from bregma) and a 3 mm deep brain tissue incision was made. The cutting as well as the subsequent implantation were made stereotaxically. The cut end of the chamber was inserted into the injured brain (at a depth of 3 mm) and the caudal tip of the chamber was laid over the skull bone and attached to it by means of a two-component fibrin tissue glue Tissucol Kit (Immuno AG, Wien). During the whole experiments the animals received a standard diet and water *ad lib.*

Table I

Contents of implanted connective tissue chambers

Group	Protein		Fibrinogen		Thrombin	
	Volume (µl)	Concentration (µg/ml)	Volume (µl)	Concentration (mg%)	Volume (µl)	Concentration (U.I./ml)
B+F	–	–	20	300	5	500
NGF	20	2000	20	300	5	500
C	20	780	20	300	5	500
1D	20	1410	20	300	5	500
2D	20	1640	20	300	5	500
3D	20	1840	20	300	5	500
4D	20	1790	20	300	5	500
5D	20	1830	20	300	5	500
6D	20	1800	20	300	5	500

Histology

Six weeks following implantation, the animals were reanaesthetized and 10 µl of 0.5% solution of horseradish peroxidase conjugated with fluorescein isothiocyanate (FITC-HRP) was injected into the free ends of the chambers. Twenty-four hours later the rats were perfused transcardially with 5% sucrose in phosphate buffer (pH 7.2) followed by 2.4% formaldehyde buffered as above. Whole grafted brains were dissected from the skull, embedded in paraffin and 10 µm thick frontal sections were made by means of a rotatory microtome (Biocut, Jung). Slices were counterstained with hematoxylin-eosin (H–E) [3] in order to check the anatomical position of the grafts. Subsequently the specimens were examined by means of a light and fluorescence microscope (Labophot 2, Nikon) and photographed. Frontal 10 µm thick frozen sections (Cryotom 620, Anglia Scientific) from one brain in each group were subjected to a procedure visualizing growing nerve endings. This was performed on the basis of acetylcholinesterase (AChE) presence in neurites growing into the chambers [16]. The number of FITC-HRP-positive cells were ascertained by means of a computer picture analyzing system (SVIST-I-0032, Wikom, Poland). Cells were counted in the same volume of brain tissue in each case in every second section in order to avoid double counting. The counting was executed by two independent searchers unaware of the kind of examined material. The results were subjected to statistical analysis by means of the Student's *t*-test. Statistical significance was set at $P<0.05$.

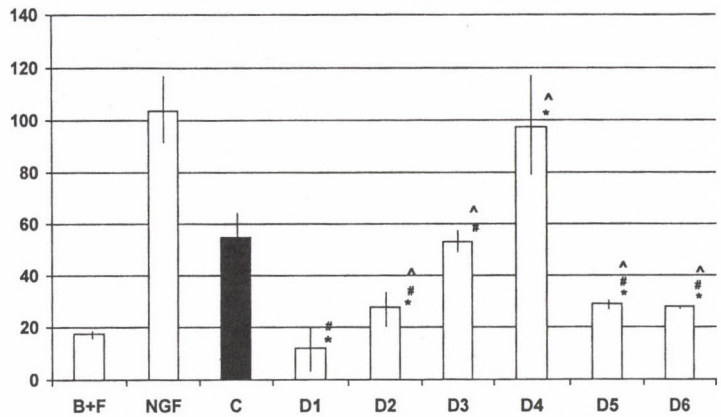

Fig. 1. Histogram showing a number (mean ±S.D.) of FITC-HRP-positive cells in individual groups six weeks following implantation. × – show the experimental groups which differ significantly from the B+F group. # – show the experimental groups which differ significantly from the NGF group. * – show the experimental groups which differ significantly from the C group

Results

In all experimental groups the protein concentration was significantly higher than in the control one (Table II).

Table II

Protein concentration (mg per 1 g of fresh tissue) in the submicrosomal fractions obtained from short-time-predegenerated rats' sciatic nerves

Control	D1	D2	D3	D4	D5	D6
1.4±0.32	2.43±0.81	3.17±0.44	3.45±0.56	3.34±0.47	3.42±0.50	3.38±0.44

Fluorescence microscope examination revealed that FITC-HRP-positive cells were present in all examined brains (Photo 1).

We found the following numbers of FITC-HRP-positive cells in the respective individual groups (mean+S.D.): B+F – 17.66±0.50; NGF – 103.64±12.42; C – 54.83± 19.73; 1D – 12.00±7.47; 2D – 27.67±5.90; 3D – 53.10±4.76; 4D – 97.35±38.98; 5D – 29.00±2.03; 6D – 27.90±0.14. In most cases the statistical differences between individual subgroups were significant (Table III).

Photo 1. FITC-HRP-positive cells present in the hippocampus from the group D4 (x200)

Table III

Statistical (mean± S.D.) comparison of groups

B+F 17.66±0.50	B+F							
NGF 103.64±12.42	p<0.05	NGF						
C 54.83±9.73	p<0.05	p<0.05	C					
D1 12.00±7.47	n.s.	p<0.05	p<0.05	D1				
D2 27.67±5.90	p<0.05	p<0.05	p<0.05	p<0.05	D2			
D3 53.10±4.76	p<0.05	p<0.05	n.s.	p<0.05	p<0.05	D3		
D4 97.35±18.98	p<0.05	n.s.	p<0.05	p<0.05	p<0.05	p<0.05	D4	
D5 29.00±2.03	p<0.05	p<0.05	p<0.05	p<0.05	n.s.	p<0.05	p<0.05	D5
D6 27.90±0.14	p<0.05	p<0.05	p<0.05	p<0.05	n.s.	p<0.05	p<0.05	n.s.

The value of coefficient "p" indicated on the crossing of columns and rows, refers to the comparison of mean values from each group

n.s. – not significant

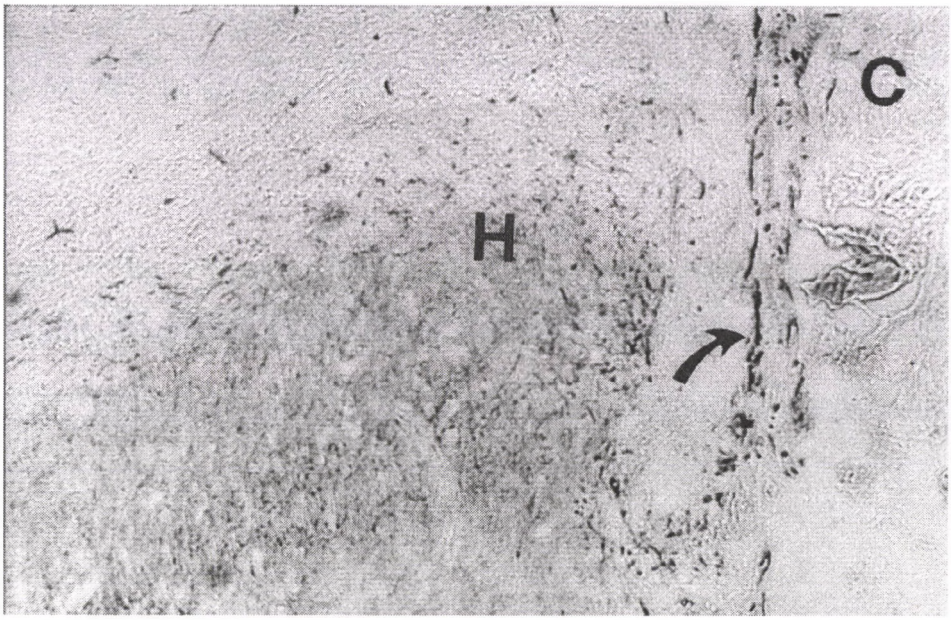

Photo 2. AchE-positive fibres present in the implanted connective tissue chamber (x100) **C** – implanted chamber; **H** – hippocampus; **arrow** shows the AChE-positive fibre

The acetylcholinesterase-positive fibres were found inside the chambers in all examined brains (Photo 2).

Discussion

On the basis of the results presented above we can state that tissue extracts (postmicrosomal fractions) obtained from short-time-predegenerated distal stumps of rats' sciatic nerves contain a soluble factor or factors which is or are responsible for the promotion of regrowth of injured hippocampal neurites. Our previous studies concerning the influence of short-time-predegenerated peripheral nerve grafts on the regrowth of injured hippocampal neurites revealed similar patterns of changes of their neurotrophic activity [24]. This ability of the tissue extracts obtained one and two days following transection was lower than immediately after peripheral nerve transection. On the third day it started to rise reaching the maximal value on the fourth day after transection. The number of traced cells was very similar to the results obtained in NGF-treated groups, so we can presume that at least a part of this effect is due to this neurotrophin. It is well established that some components of connective tissue and

extracellular matrix such as different types of collagene, fibronectine and fibrine posses also some regenerative activity in the peripheral as well as the central nervous system [2, 18]. Moreover, it was proved that the presence of fibrine matrix is indispensable for starting nerve outgrowth [10, 18, 29]. For that reason we decided to add a fibrin solution to our sciatic nerve extracts. In order to check whether the regenerative effect of the chambers was the result of their own connective tissue and fibrin activity or the influence of the introduced nerve extract, the group B+F was created. We found that brains grafted with chambers containing no tissue extracts exhibited only a very slight regenerative reaction (Table III). It can be therefore hipothesized that a part of the neurotrophic activity of purified extracts obtained from short-time-predegenerated sciatic nerves depends neither on the influence of the connective tissue forming the chambers nor the fibrin contained therein.

In the last decade a variety of substances have been tested in order to recognize their ability to promote and support the regrowth of central axons [11, 13, 27, 28]. Different extracts from nerves or target tissues were recently used to stimulate the regrowth and survival of central neurons [8, 12, 17, 19, 26, 30, 32, 33). The main aim of our investigation is to recognize the mechanism by which peripheral nerves stimulate the regeneration in the CNS as well as re-create this mechanism in the brain. The results of our previous experiments [20, 21, 22, 23] have suggested that at least a part of the regenerative activity of predegenerated peripheral nerve grafts is due to their soluble components and can be transferred into the CNS by means of an appropriate delivery system. These studies were focused on the neurotrophic activity of nerve grafts and their purified extracts after at least 7 days of predegeneration. In this study we have tried to investigate whether the short-time-predegenerated peripheral nerves also contain soluble factors able to promote the survival of injured CNS neurites. It also seemed important to find out how this neurotrophic activity has been changing in time. Górka et al. found, by means of one- and two-dimensional gel electrophoresis, that the content and the composition of proteins obtained from short-time-predegenerated rats' sciatic nerves showed significant changes over time, mostly on the 4th, 5th and 6th day after nerve transection [14, 15]. The changes in neurite growth-promoting activity found in our present experiments seem to follow changing metabolism of the cells present in the distal part of the cut sciatic nerve. The highest neurotrophic activity was observed at 4th day after nerve transection, however it was not statistically different from the influence of NGF itself (Table III). To differentiate NGF and non-NGF trophic substances released by sciatic nerves, anti-NGF antibodies should be used. These investigations will be a target for our future studies. The results of our experimental study indicate that the highest neurite-regrowth-promoting activity appears in the peripheral nerve 7 days following transection [21, 23].

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Activity of cathepsins in rat's spleen due to experimentally induced pancreatitis

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The aim of this study was to establish and quantify the changes of the level of cathepsin B, D and L in the spleen during experimental pancreatitis. The experiment was carried out in 115 male Wistar rats, randomly divided into three groups: intact (n=15), injected with 0.9% NaCl solution into the common bile pancreatic duct (n=50) and injected with 5% sodium taurocholate into this duct to induce acute pancreatitis (n=50). After 2, 6, 12, 24 and 48 hours rats were anaesthetised, and blood was taken for amylase determination from the heart, and the spleen was removed. Alpha-amylase level in the blood serum samples was measured by enzymatic method. Cathepsin activity was established by spectrophotometric methods using substrates which form coloured complexes when they react with these proteases. The specific free fraction activity of cathepsin B, D and L in the spleen changed during the course of experiment, but there was no correlation between their activity and the intensity of pancreatitis established by serum amylase level.

Keywords: cathepsins, spleen, experimental pancreatitis, rats

Acute pancreatitis [AP] is a severe disease causing multiorgan insufficiency including impairment of liver, kidney, cardiac and brain function, as well as the generation of microembolics. The essence of disturbances in AP is to break off tissue inhibitors and plasmatic barriers, which leads to the activation of trypsinogen. Lysosomal cathepsins play a decisive part on this process [10]. It has been proven that there is a direct relationship between the onset of vascular disease and the level of cathepsins, too [7, 8]. Complication of AP may include not only local pancreatic necrosis with pseudocyst or abscess formation, but extrapancreatic manifestations such

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as pulmonary, renal, hepatic, endocrine, and coagulation abnormalities. Coagulation abnormalities associated with AP are usually represented as thrombophlebitis or widespread microthrombi in the venous circulation, but splenic parenchyma complications and splenic vein thrombosis during pancreatitis are potentially life threatening [5, 9, 14, 16, 25, 27]. The aim of our experiment was to establish and quantify the changes of the level of cathepsin B, D and L in the spleen during the course of experimental pancreatitis.

Materials and Methods

The experiment was carried out with 115 male Wistar rats. Weight varied from 250 to 350 g. The animals were fed with standard diet. They had been given only water 24 hours before operation. Rats were randomly divided into three groups: **A** – group of intact animals was not operated and used to mark initial biochemical parameters (15 rats), **B** – an other group of animals which were injected with an 0.9% NaCl into the common bile-pancreatic duct (50 rats), and **C** – the experimental group of animals which were injected by retrograde way with sodium taurocholate into the common bile-pancreatic duct to induce acute necrotic pancreatitis (50 rats).

The Bioethical Committee of University Medical School of Lublin approved the experimental protocol. Animals were anaesthetised by ketamine injection in a dose of 4 mg/kg body mass. Cutting in the medial line opened the peritoneal cavity. After laparotomy an injection needle 0.5×16 mm was inserted into the common bile-pancreatic duct via the proximal part of duodenum (Aho's method) [1]. The hepatic duct was closed near to the hilus by soft, small surgical forceps. The lean ligature (2–0) was put around the bile-pancreatic ostium. A needle and a duct wall were tightened by a lean ligature.

Animals in group A remained intact, in group B they were injected with saline by retrograde way, and in group C they were injected intraductally by 5% solution of sodium taurocholate (Sigma, Chemical Co., St. Louis, Missouri) in a dose 0.1 ml/100 g of body weight. After 2, 6, 12, 24, 48 hours rats were anaesthetised again, and thoracotomy was performed taking blood for amylase determination from the left ventricle of heart. Then the animals were given an overdose of ketamine, and the pancreas with spleen were removed during laparotomy.

The samples were taken for histological examinations. Then the spleen was frozen at the temp. of –20 °C.

Alpha-amylase [EC 3.2.1.1.] activity in the blood serum was determined by enzymatic method using the substrates of the Cormay Company and Cobas Mira Plus Company biochemical analyser. The activity of amylase was demonstrated in U/dl.

The spleens were defrosted in 0.9% solution of NaCl at +4 °C. One gram of spleen tissue was taken. The samples were dissolved and placed into a 0.3 M sucrose solution at +4 °C in proportions of 1.0 g of gland tissue to 5.0 ml of sucrose and homogenised. The obtained homogenate was centrifuged for 10 minutes at 2200 × g at +4 °C. The supernatant was decanted and centrifuged for 20 minutes at 35,000 × g. to obtain sample containing the free fraction of enzyme [23]. Cathepsin activity was assayed by the spectrophotometric methods using substrates (Sigma Chemical Company, St. Louis, MO, USA) which form coloured complexes when they react with the proteases [4, 24].

Cathepsin B [EC 3.4.22.1.] decomposes the substrate, benzoilo-DL-arginino-naphtyloamide (BANA) which liberates free naphtyloamine, which forms coloured complex with diazone salt Fast Blue B (FBB). Addition of 4-chloromercuro-benzoesal acid (CMB) activates the enzymatic reaction and enables formation of the coloured complex. The following substances were used as activators: 32.3 mg anhydride cysteine dissolved in 100 ml of activating buffer; 40 mg BANA in 1 ml dimethylsulfoxide as a substrate; and 10 mM solution of CMB, in a pH of 6.0, mixed with the equivalent amount of the stabilised solution of FBB as a binding factor. One and a half ml of the buffered activator was incubated with 0.5 ml of spleen homogenate at +37 °C for 5 minutes. Fifty µl of BANA was incubated with this mixture for 30 minutes. After this time, 2 ml FBB was added. After 10 minutes, the absorption was measured at 520 nm (Spekol 221).

Cathepsin D [EC 3.4.23.5.] and L [EC 3.4.22.17] decompose denatured azocasein, at pH 5.0, in a solution of 3M urea, releasing free azopeptides and azidocasein. The azopeptides remain dissolved in 10% trichloroacetic acid (TCA) while azidocasein remains as a precipitate [28]. The azopeptides in the solution have an absorbancy maximum at 366 nm. The following solution was used for spectrophotometric analysis: 2% azocasein solution in 0.1M acetic acid buffer, pH 5.0, containing 6M urea and 0.1% Triton X-100, as a substrate; 50 mM acetic acid buffer, pH 5.0, containing 0.1% Triton X-100 and 5 mM L-Dithiotreitol, which serves as an incubating buffer: 4.0 µM pepstatine in 50 mM acetic acid buffer, pH 5.0, as an inhibitor of cathepsin D, and 10% TCA as a solvent. To measure the activity of cathepsins D and L, 50 µl of the salivary gland homogenate was pre-incubated with 50 µl of the incubating buffer for 10 minutes at room temperature. The solution of 100 µl of incubating buffer and 200 µl azocasein was then incubated for 24 hours at +37 °C. These solutions were then cooled in ice water, and 400 µ1 of 10% TCA was added. After 10 minutes, each solution was centrifuged for 5 minutes at 35,000 × g. The absorption was measured at 366 nm for the homogenate solution. To measure the activity of cathepsin L, 50 µl of homogenate was added to 50 µl of pepstatine and incubated for 10 minutes at room temperature. Pepstatine blocks -SH groups of

cathepsin D, inhibiting the activity of the enzyme while having no effect on cathepsin L. A control solution containing 100 µl of incubating buffer and 200 µl of azocasein was prepared. The absorbency for cathepsin L was measured using the above-described procedure. The level of protein had been carried out by Lowry's method [18]. The values were demonstrated in µg/l (as a miscalculate of 1 mg of protein/l hour of incubation).

Out of group B and group C 5 rats were used for the histological examinations (one out of each period of time). Samples of pancreas and spleen were taken and placed in 10%-neutralized formaline for 3 days. After dehydratation, the tissue fragments were embedded in paraffin, and sliced in 7 mm slices. After deparaffination, samples were dyed in review stain with hematoxylin and eozin according to Bagiński method and the photographic documentation was done in the Jenalumar luminous microscope Zeiss brand.

The statistical analysis was done using the SAS system v. 6.11 (SAS Institute Inc.; SAS Campus Drive, Carry, NC 27513, USA). Results are expressed as mean ±SD. Differences between groups were calculated by analysis of variance (ANOVA). If $p<0.05$, differences between the mean values were considered statistically significant. Five percent risk of an experimental error was accepted in this thesis. The mean values are illustrated by curves of changes between 2 and 48 hours. In these figures the mark "o" symbolizes a statistically significant comparison to group A. The mark "*" means a statistically significant difference with comparison between groups B and C.

Results and Discussion

During the experiment 16 animals died: in group B – 5 individuals (mortality rate 10% and in group C – 11 individuals (mortality rate 22%). The detailed experimental data are given in Table I.

In our experiment the severity of AP was monitored by measuring the amylase activity in blood. The initial concentration was measured to have an average value of 293 U/dl (Fig. 1). Twelve hours after induction of AP the activity, increased to nearly 800 U/dl in both experimental groups. After 48 h the peak level of 950 U/dl in group C was observed. The analysis of variance showed that there were highly significant differences between the mean values of amylase activity of intact (A) and in the animals belonging to groups B and C ($p<0.01$), but there were significant differences between the mean values of groups B and C after 2 and 6 h of experiment ($p<0.05$).

Table I

Mean activity of cathepsins and amulase in the spleen of intact (A), injected with physiological saline (B) and experimental (C) rats injected by sodium taurocholate by retrograde way into the pancreas, in the different periods of acute pacreatitis (in μg/l)

Enzyme	A	B (n=45)					C (n=39)				
	(n=15)	2 h (n=8)	6 h (n=9)	12 h (n=9)	24 h (n=10)	48 h (n=9)	2 h (n=7)	6 h (n=10)	12 h (n=8)	24 h (n=7)	48 h (n=7)
Cathepsin B	2397.12 (423.07)	1762.56 (1116.0)	2140.71 (797.11)	2206.73 (805.14)	1531.59 (481.70)	2331.12 (461.13)	2509.50 (1261.0)	2652.88 (1211.0)	3334.32 (954.90)	2117.74 (954.90)	1735.52 (447.62)
Cathepsin D	165.921 (53.167)	166.226 (38.236)	161.335 (50.704)	173.232 (51.914)	215.203 (28.010)	130.907 (30.183)	181.359 (41.857)	142.222 (24.757)	36.157 (14.300)	183.176 (47.916)	206.680 (68.982)
Cathepsin L	123.976 (38.066)	158.636 (77.252)	151.309 (45.331)	160.859 (40.023)	220.785 (133.26)	204.014 (53.425)	131.776 (85.923)	195.687 (98.280)	212.607 (86.199)	122.689 (53.580)	100.477 (42.325)
Amylase	293.1 (73.4)	385.4 (79.7)	516.4 (61.5)	784.3 (171.3)	808.6 (134.7)	814.2 (176.9)	486.2 (125.3)	721.7 (285.3)	769.3 (289.1)	880.7 (231.1)	950.9 (261.8)

The values of standard deviation are given in parentheses. Statistical significances are given on the Figures 1–4.

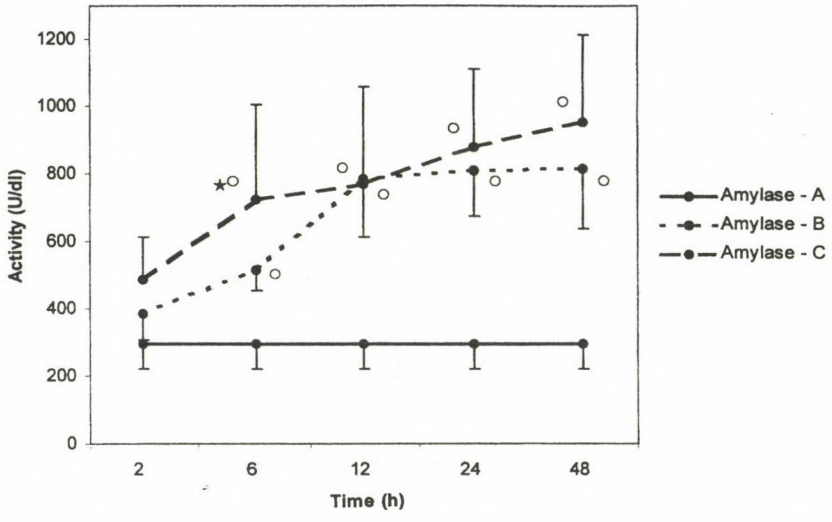

Fig. 1. The curve of changes of amylase activity in blood during AP (mean values)

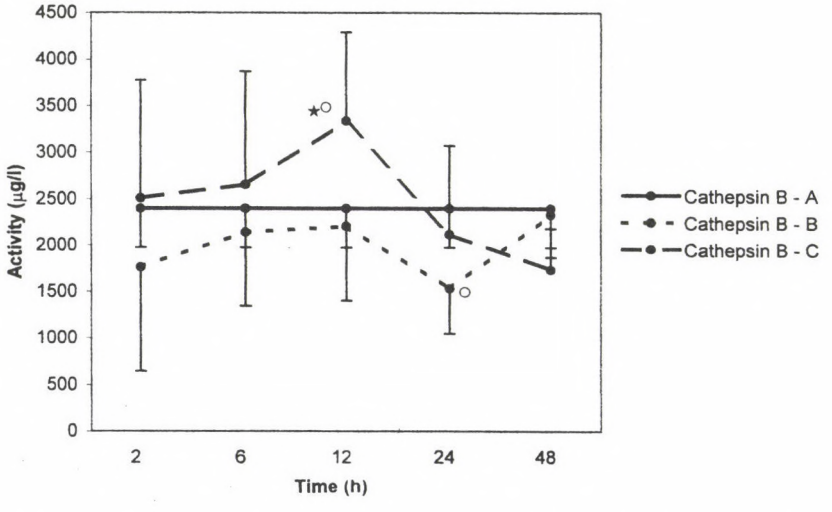

Fig. 2. The curve of changes of cathepsin B activity in spleen during AP (mean values)

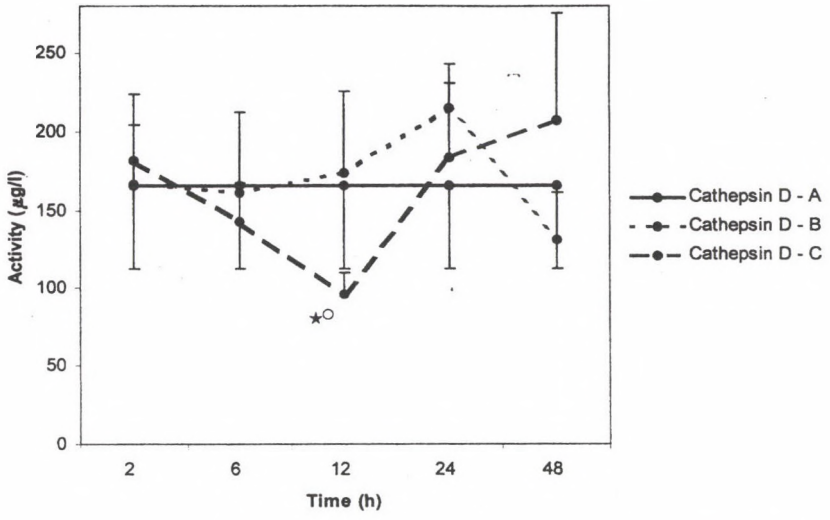

Fig. 3. The curve of changes of cathepsin D activity in spleen during AP (mean values)

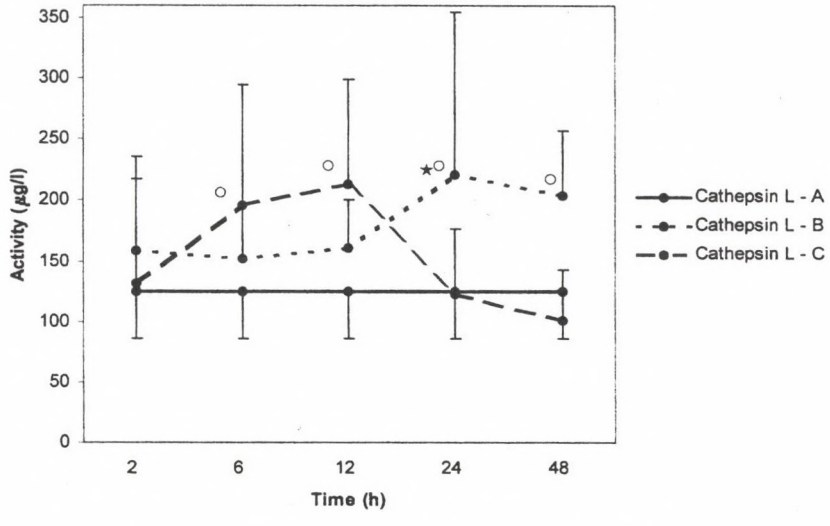

Fig. 4. The curve of changes of cathepsin L activity in spleen during AP (mean values)

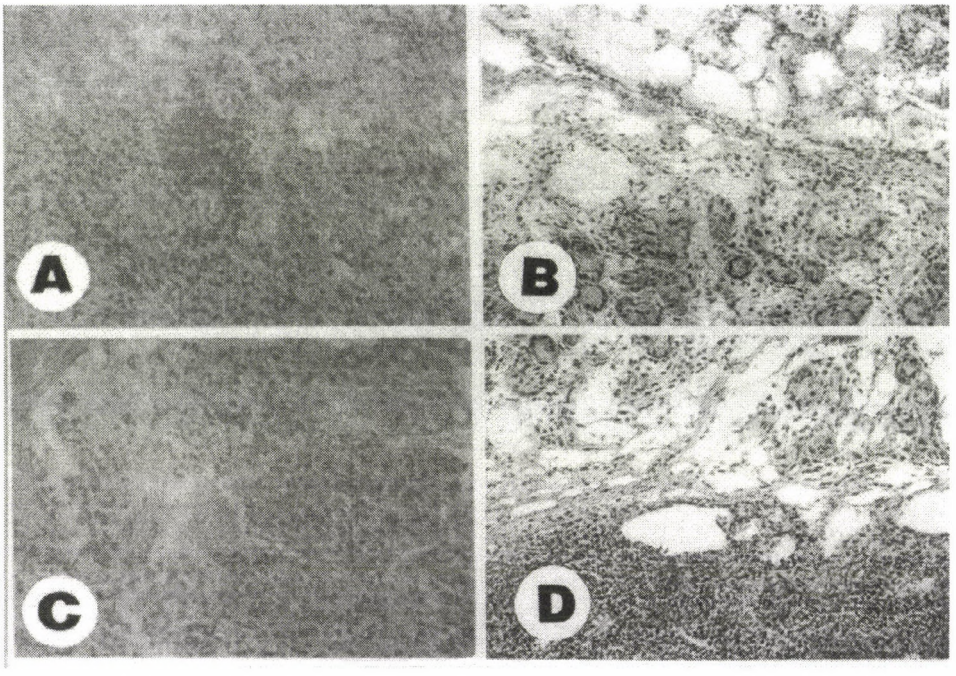

Fig. 5. Histological (H+E, x157) observations of spleen (A and C) and pancreas (B and D) in rats after 48 hours of experimental pancreatitis

The activities of amylase and cathepsins B, D and L in the spleen in the different periods of AP are presented in Table I.

The activity of cathepsin B differed between the groups B and C (Fig. 2). This value decreased below levels of intact animals after 24 h of experiment. The mean value activity of cathepsin D is shown in Fig. 3. Only the inconsiderable differences between the curve of changes of groups B and C were seen. The activity of the free fraction of cathepsin L (Fig. 4) was 124 μg/l in the intact rats.

After 24 h this value had approximately doubled in group B. There were no significant differences between the mean values of groups A, B and C and between groups B and C in different periods of experiment.

After 48 hours of experiment in group B the features of oedema and inflammation were dominant in the spleen and pancreas. Increase of fibrous elements of connective tissue with parenchyma and fat necrosis were observed too (Figs 5A, 5B). In group C oedema, parenchyma necrosis, interlobular space dilatation and lymphocyte inflammation were visible at that time in both organs (Figs 5C, 5D).

There are many mediators of the systemic complications associated with AP. Cathepsins seem to play an important roles in inflammatory, degenerative and neoplasmatic diseases among them [2]. The scientific research has proven that lysosomal cathepsins are very important during initiation of pancreatitis and multiorgan complication occurrence [6, 11, 15, 17, 29, 30]. We have found only a few papers devoted to the issue of splenic complications of pancreatitis being published during last few years [12, 13, 20, 21, 22, 26].

Norman et al. have established that cytokine production is correlated with disease severity and occurs within the pancreas first and subsequently within distant organs including spleen [22]. Banks et al. and Hughes with co-workers concluded that severe AP in the rat model is associated with significant upregulation of TNF alpha mRNA in splenic mononuclear cells [3, 13]. However, we have found no previous reports of dysregulation of cathepsins production in the spleen during pancreatitis. It seems that impairment in enzymes activity is dependent not only on the inducing substance, but as well as on the increase of duct pressure. This pathomechanism, which resembles biliary acute pancreatitis in humans, is also stressed in works by Luthen et al [19].

The present data provide evidence, that the acute pancreatitis can induce dysregulation of cathepsins at a distant organ, the spleen, but it seems that these changes are non crucial.

Conclusions

1.) The activity of the free fraction of cathepsin B, D and L taken from spleen changed non-specifically over the course of experimentally induced acute pancreatitis in rats.

2.) No statistically significant difference was found in the enzyme activity of the saline-induced and the taurocholate-induced pancreatic tissue damage.

3.) There was no correlation between cathepsins activity in the spleen and the intensity of pancreatitis established by serum amylase level.

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Influence of the acute intraperitoneal administration of tetrazepam on blood glucose level and serum lipids in normoglycemic and normolipidemic rats

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The acute ip administration of tetrazepam (5, 7.5, 15 mg/kg) in normoglycemic and normolipidemic rats induced an increase in blood glucose level, a delay of fibrinolysis (when administered at the first two doses) and variable changes of serum lipids. These results are different from those obtained in hyperlipidemic rats treated with tetrazepam.

Keywords: tetrazepam, normoglycemic and normolipidemic rats, glucose, lipids

In the previous paper [8], it was shown that tetrazepam, a 1,4-BZD, given to rats which were rendered hyperlipidemic by the ip administration of Triton WR-1339, evoked significant reductions of serum lipids and blood glucose level. Similar results (with regard to serum lipids) were obtained by the use of other BZDs [5]. On the other hand, when BZDs were given to normoglycemic and normolipidemic rats, the results were inhomogeneous [6, 7].

In the present paper, we investigated the effects of tetrazepam on blood glucose level and serum lipids in normoglycemic and normolipidemic rats.

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Materials and Methods

The experiments were carried out in male Wistar-Bratislava rats, weighing 150–200 g. The animals were fed a common rat chow, with water *ad libitum*. They were maintained at constant temperature (20–22 °C) and humidity. Twenty hours prior to the blood sampling, they received ip an injection of the following drugs: fenofibrate (200 mg/kg) or tetrazepam at the doses of 5, 7.5, 15 mg/kg. All the compounds were suspended in a mixture of 0.2% methylcellulose +1% glycerin. The control rats were administered only the mixture. Two hours later, the food was withdrawn, but the water was allowed *ad libitum*. In this way, the conditions of the experiments carried out in hyperlipidemic rats were reproduced (with the exception, of course, of the administration of Triton WR-1339, the hyperlipidemic agent, see 5.) Twenty hours after drugs administration, blood was sampled by the punction of the retroorbital sinus. The following determinations were performed: blood glucose level, by the enzymatic method (Merck's test), total lipids by the technique of Zöllner and Kirsch [9], total cholesterol by the enzymatic procedure (Reanal's test), and triglicerides by the enzymatic procedure (Merck's test). In addition, the fibrinolysis was estimated by the dilute blood clot lysis time (method of Gallimore et al. [3]). The biochemical results were expressed as mmol/ml, with the exception of TL level, which was expressed as mg%. The clot lysis time was expressed in minutes.

The results obtained for each experimental group were compared with the control (normoglycemic and normolipidemic rats [p]) and with the effect of the reference compound (fenofibrate [p]). The statistical evaluation of the differences was performed using the unpaired *t*-test [10].

Results

Fenofibrate and the first two doses of tetrazepam raised blood glucose level, while the last dose was ineffective (Table I).

Total lipid levels were not affected by fenofibrate, but they diminished by the doses of 5 and 15 mg/kg of tetrazepam, while the dose of 7.5 mg/kg was devoid of any influence.

Total cholesterol concentration was not changed by fenofibrate, however tetrazepam administered at the lowest dose of 5 mg/kg increased its content while the highest dose decreased it. The dose of 7.5 mg/kg was ineffective.

Table I

Effects of tetrazepam on blood glucose level and serum lipids in rats

Group/dose	Clot lysis time (minutes)	Blood glucose glucose (mmol/l)	Total lipids (mg%)	Total cholesterol (mmol/l)	Triglycerides (mmol/l)
Control (normolipidemic and normoglycemic)	43.18 ± 1.02	3.04 ± 0.12	395.3 ± 11.5	1.80 ± 0.09	0.76 ± 0.12
Fenofibrate (200 mg/kg)	40.2 ± 1.1 p n.s.	4.12 ± 0.105 $p<0.001\uparrow$	362.4 ± 12.7 p n.s.	1.58 ± 0.072 p n.s.	0.595 ± 0.037 p n.s.
Tetrazepam (5.0 mg/kg)	61.55 ± 4.4 $p<0.001\uparrow$ $p_1<0.001\uparrow$	5.7 ± 0.193 $p<0.001\uparrow$ $p_1<0.001\uparrow$	297.67 ± 14.9 $p<0.001\downarrow$ $p_1<0.001\downarrow$	2.18 ± 0.07 $p<0.001\uparrow$ $p_1<0.001\uparrow$	0.3424 ± 0.0274 $p<0.001\downarrow$ $p_1<0.001\downarrow$
(7.5 mg/kg)	69.44 ± 4.57 $p<0.001\uparrow$ $p_1<0.001\uparrow$	3.54 ± 0.146 $p<0.001\uparrow$ $p_1<0.001\uparrow$	390 ± 10.32 p n.s. p_1 n.s.	1.58 ± 0.055 p n.s. p_1 n.s.	0.77 ± 0.032 p n.s. $p_1<0.001\uparrow$
(15.0 mg/kg)	45.3 ± 4.65 p n.s. p_1 n.s.	3.89 ± 0.195 $p<0.001\uparrow$ p_1 n.s.	264.32 ± 12.45 $p<0.001\downarrow$ $p_1<0.001\downarrow$	1.44 ± 0.058 $p<0.001\downarrow$ p_1 n.s.	0.37 ± 0.031 $p<0.001\downarrow$ $p_1<0.001\downarrow$

All groups consisted of 10 rats, with the exception of the control group, which comprised 20 rats.
p – compared with control normolipidemic rats; p_1 – compared with fenofibrate; n.s. – not significant; ↓ – decrease; ↑ – increase

Triglyceride levels were not modified by fenofibrate but they were reduced by the lowest and highest doses of tetrazepam (5 and 15 mg/kg) while the dose of 7.5 mg/kg did not evoke any changes.

Fenofibrate failed to affect the blood clot lysis time. The same was valid for the dose of 15 mg/kg of tetrazepam. However, other doses of this drug evoked highly significant prolongation of it, i.e. they had antifibrinolytic activity.

Discussion

Comparison of the results obtained in normoglycemic and normolipidemic rats with those characteristic of the hyperlipidemic, Triton WR-1339, treated rats [8], showed the following differences:

– In normoglycemic and normolipidemic rats the blood glucose level was raised, while in hyperlipidemic animals it was variably affected by different doses of

tetrazepam, mostly diminished. This difference could be explained by the fact that the control blood level was different, although in both situations it might be considered as being normal.

– In fact, in Triton-administered rats, the blood glucose level was higher than in normoglycemic and normolipidemic rats. The difference was observed also in other similar experiments [1]. It is conceivable that the differences between the control blood glucose would direct the changes in this parameter evoked by tetrazepam in opposite directions. However, it must be emphasized that the changes were never large enough to create a pathological state.

Serum lipid level, with the exception of TG, which were reduced by the lowest and the highest doses of tetrazepam, were inhomogeneously modified by tetrazepam in normoglycemic and normolipidemic rats. However, the highest dose induced highly significant decreases in all the components of serum lipids. In contrast, in hyperlipidemic rats, the dose-response curve of serum lipids had a bell-shape, the maximum being reached at the dose of 10 mg/kg [5].

The action of tetrazepam on fibrinolysis was investigated only in normoglycemic and normolipidemic rats, since, as it was previously demonstrated by our investigations [2], Triton induced by itself an obvious acceleration of fibrinolysis.

Fenofibrate, which in hyperlipidemic rats induced very significant decreases in blood glucose level and in serum lipids [8], was completely inactive in normoglycemic and normolipidemic rats. These results confirm the importance of the initial levels of blood glucose and serum lipids to the response to the antihyperlipidemic drugs.

When the results obtained by the treatment of normoglycemic and normolipidemic rats with tetrazepam were compared with the findings acquired in similar conditions with other BZDs, the following features could be observed: dipotassium chlorazepate, chlordiazepoxide, diazepam and medazepam induced small, but significant, elevations in blood glucose levels [7], whereas oxazepam and lorazepam dipotassium salt reduced them [6, 7]. Serum lipid levels were significantly decreased by diazepam, partly by lorazepam [7] but chlorazepate dipotassium salt and chlordiazepoxide failed to affect TL content, increased TChol, but decreased TG concentration. Oxazepam augmented TL and TG, but failed to affect TChol. Medazepam increased all contents of all studied lipids [6].

In conclusion, the changes in serum lipid levels brought about by various BZDs in normoglycemic and normolipidemic rats are heterogeneous. This is in sharp contrast to the antihyperlipidemic activity of all 1,4-BZDs observed in hyperlipidemic rats [5]. The causes of this discrepancy are not understood. It is noteworthy that in many conditions, the effects of various drugs are larger and more homogeneous in pathological conditions than in normal conditions. An illustrative example of this phenomenon are antipyretics.

The effects of various BZDs on fibrinolysis are also heterogeneous. So, besides tetrazepam, a delay in the fibrinolysis was induced by lorazepam and dipotassium chlorazepate, whereas diazepam brought about an acceleration [2]. Chlordiazepoxide was ineffective [2]. It is noteworthy that all these studies were performed in normoglycemic and normolipidemic rats. There is no relationship between the changes in the serum lipids and in fibrinolysis, since in hyperlipidemia induced by margarine, diazepam elicited both reductions of serum lipids and an acceleration of the fibrinolysis, whereas in normolipidemic rats diazepam evoked only an acceleration of the fibrinolysis [4].

In conclusion, tetrazepam, administered acutely ip in normoglycemic and normolipidemic rats, inhibited fibrinolysis, induced an increase in blood glucose level and caused heterogeneous changes in the serum lipids.

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Effects of acute intraperitoneal administration of tetrazepam on blood glucose level and serum lipids in hyperlipidemic albino rats

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In rats rendered hyperlipidemic by the i.p. injection of Triton WR-1339, the i.p. administration of tetrazepam, a benzodiazepine (BZD) used mainly as a central myorelaxant, evoked significant reductions of serum lipids and blood glucose level. The dose-response curve was bell-shaped for serum lipids changes, whereas no clear dose-response relationship for blood glucose level modifications could be established. Tetrazepam was less active on serum lipids than other BZDs as diazepam or midazolam.

Keyword: tetrazepam, hyperlipidemic rats, glucose, lipids

Our previous investigations reviewed in [3] have shown that BZDs induced marked diminution of serum lipids in hyperlipidemic animals and humans. The most sensitive parameter was the reduction of TG. In normolipidemic animals and humans, BZDs were less active or completely ineffective. Moreover, in some instances, they brought about even an increase of serum lipids [1, 18]. With the exception of the streptozotocin-induced diabetes in rats in which diazepam and midazolam elicited very significant reductions of the increase of the blood glucose level [2], BZDs induced variable changes of the blood glucose level [5, 10, 11].

BZDs act upon two different receptors: a central one, coupled to GABA, and a peripheral type receptor, which is independent of GABA. This receptor is located on the outer mitochondrial membrane [12].

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Our studies have shown that very probably the lipid lowering activity of BZD was due to the stimulation of the peripheral type receptors, since PK 11195, a selective antagonist of these receptors [14], prevented to a great extent the decrease of the serum lipids, elicited by diazepam, clonazepam, midazolam [2, 13]. At the same time, flumazenil (Ro15-1788, Anexate$^{(R)}$), an imidazobenzodiazepine with selective antagonistic properties of the central BZD receptors [9], failed to antagonise the effects of BZDs. Moreover, it mimicked the lipid-lowering activity of BZD. Since this action could be antagonized by PK-11195, it resulted in that, at least in certain doses, flumazenil could stimulate the peripheral type BZD receptors [13].

This view on the mechanism of action of BZD on serum lipids was strengthened by the fact that a selective agonist of the peripheral type BZD receptors, 4'-chlorodiazepam (Ro5-4864) induced the same changes of the serum lipids as did the common BZDs (diazepam, chlordiazepoxide, lorazepam, clonazepam, flunitrazepam, midazolam) [4]. Moreover, the effects of 4'-chlorodiazepam could be prevented by PK-11195, meanwhile flumazenil enhanced them [6].

In the experiments reported here, we studied the influence of tetrazepam, another BZD on blood glucose level and serum lipids in hyperlipidemic rats. Tetrazepam has, besides the "classical" therapeutic uses of BZD (anxiolysis, sedation-hypnosis, anticonvulsant activity) a more expressed myorelaxant effect than other BZD. It acts on the central mechanism which regulate muscular tone. It is used in the treatment of some muscular spasms. The normal muscular tone is little affected [15, 17].

Materials and Methods

The experiments were carried out in male Wistar rats, weighing 150–200 g. They were kept at a constant temperature (20–22 °C) and humidity and fed a common rat chow. Water was allowed *ad libitum*. Tetrazepam and fenofibrate, which was used as a reference compound, were suspended in a mixture of 0.2% methylcellulose +1% glycerin and administered i.p., two hours prior to the i.p. injection of Triton WR-1339 (200 mg/kg) which was used as a hyperlipidemic agent. After the injection of Triton, the food was withdrawn, but the water was allowed *ad libitum*. Eighteen hours after Triton-WR 1339 administration, blood was sampled from the retroorbital sinus. Blood glucose level was determined using the enzymatic method (Merck's test). From the serum, the following measurements were carried out: total lipids (TL) by the technique of Zöllner and Kirsch [19], total cholesterol (Tchol) with the enzymatic procedure (REANAL test), triglycerides (TG) with the enzymatic method (Merck's test).

The results were expressed as millimoles, with the exception of TL, which were expressed as mg%. The comparisons were performed between the groups which

received tetrazepam or fenofibrate and the control hyperlipidemic group (p) which received also the suspending mixture. Other comparisons were carried out between tetrazepam and fenofibrate (p_1).

The statistical evaluation was performed by means of the unpaired "t" test [18]. The differences were considered to be significant at $p<0.05$. The results are presented in Table I.

Results

I. The reference compound – fenofibrate – administered in a standard dose of 200 mg/kg, induced a very significant decrease of blood glucose level as well as of TL, TChol and TG.

II. Tetrazepam, given in doses of 2.5–5.0–7.5–10–15 and 20 mg/kg, induced the following changes, when compared to the control: the first two doses, and the dose of 10 mg/kg, elicited very significant reductions of the blood glucose level, whereas other parameters were not affected. The doses of 7.5–10 and 15 mg, induced very significant diminution of TL, TChol and TG. The highest dose (20 mg) was completely ineffective. The most active dose was 10 mg.

III. Comparing the results obtained by various doses of tetrazepam with those obtained fenofibrate, it resulted in that tetrazepam was equally active as fenofibrate (i.e. no statistical difference could be detected) or was less effective (see Table I).

IV. Tetrazepam induced a slight hypomotility and a diminution of the interest of animals on the environment. The muscular tone was not reduced. These changes were not quantified, since our previous studies have shown that no parallelism could be established between the behavioural changes induced by anxiolytics and the metabolic modifications [3]. Indeed, BZDs having predominantly anxiolytic or sedative-hypnotic properties as well BZDs acting preferentially upon the peripheral type BZD receptors are effective hypolipidemic agents [3]. Moreover, other anxiolytics as benactyzine and meprobamate are practically devoid of antihyperlipidemic actions [3].

Discussion

Our findings have shown that like other BZD, tetrazepam is an active hypolipidemic drug. As other BZDs, the dose-response curve was bell-shaped. However, it seems that tetrazepam is less active than most BZD, mainly diazepam and midazolam. Indeed, diazepam had a maximal activity at 5 mg, midazolam at 4 mg, whereas the optimal dose of tetrazepam was 10 mg. It is true that the molecular weights

of these compounds were different. However, even by reporting the doses of tetrazepam to those of diazepam (see column 1 of Table I) the differences persist.

Table I

Effects of tetrazepam on blood glucose level and serum lipids (means±standard error)

Group/Dose	Blood glucose level (mmol/l)	TL (mg%)	TChol (mmol/l)	TG (mmol/l)
Control*	5.18 ± 0.11	2706.16 ± 148	7.49 ± 0.20	20.22 ± 1.11
Reference (Fenofibrate 200 mg/kg i.p.)	4.2 ± 0.06 p<0.001 ↓	630.2 ± 22.3 p<0.001 ↓	2.3 ± 0.11 p<0.001 ↓	1.8 ± 0.18 p<0.001 ↓
Tetrazepam 2.5 mg/kg (equivalent to 1.67 mg diazepam)	4.54 ± 0.01 p<0.001 ↓ p_1 n.s.	2569.8 ± 137.5 p n.s. p_1<0.001 ↑	8.0 ± 0.27 p n.s. p_1<0.001 ↑	17.58 ± 1.25 p n.s. p_1<0.001 ↑
5.0 mg/kg (equivalent to 3.34 mg diazepam)	3.68 ± 0.11 p<0.001 ↓ p_1<0.001 ↓	2638.4 ± 221.8 p n.s. p_1<0.001 ↑	6.7 ± 0.35 p n.s. p_1<0.001 ↑	17.02 ± 1.51 p n.s. p_1<0.001 ↑
7.5 mg/kg (equivalent to 5.0 mg diazepam)	5.26 ± 0.1 p n.s. p_1<0.001 ↑	989.2 ± 182.4 p<0.001 ↓ p_1 n.s.	3.18 ± 0.43 p<0.001 ↓ p_1 n.s.	1.76 ± 0.54 p<0.001 ↓ p_1 n.s.
10 mg/kg (equivalent to 6.68 mg diazepam)	3.37 ± 0.11 p<0.001 ↓ p_1<0.001 ↓	533.3 ± 55.8 p<0.001 ↓ p_1 n.s.	2.7 ± 0.14 p<0.001 ↓ p_1 n.s.	1.16 ± 0.3 p<0.001 ↓ p_1 n.s.
15 mg/kg (equivalent to 10.0 mg diazepam)	5.12 ± 0.13 p n.s. p_1<0.01 ↑	1101.9 ± 180.9 p<0.01 ↓ p_1<0.001 ↑	4.63 ± 0.3 p<0.001 ↓ p_1<0.001 ↑	2.94 ± 0.5 p<0.001 ↓ p_1 n.s.
20 mg/kg (equivalent to 13.36 mg)	5.02 ± 0.085 p n.s. p_1<0.001 ↑	2450 ± 141.3 p n.s. p_1<0.001 ↑	6.78 ± 0.21 p n.s. p_1<0.001 ↑	18.36 ± 1.5 p n.s. p_1<0.001 ↑

All groups consisted of 10 rats, with the exception of the control group, containing 20 rats.

p: compared with control hyperlipidemic rats; p_1: compared with reference (fenofibrate) treated rats; n.s. = non significant; * Control group contains rats rendered hyperlipidemic (hyperlipidemia with Triton WR-1339, 200 mg/kg i.p.)

↓ – decrease; ↑ – increase

Our experiments have also shown that tetrazepam pertains to the BZDs which decrease the blood glucose level [5]. Since other BZD, despite being lipid-lowering drugs, have increasing blood glucose level or are ineffective [5], it results in that no correlation can be established between the hypolipidemic activity and the action on blood glucose level. It is noteworthy that no clear dose-response relationship could be established for blood glucose modifications. These peculiarities are difficult to explain. It is remarkable that in sharp contrast to the antihyperlipemic activity of all BZDs hitherto investigated, all effects of these drugs on blood glucose level are very variable. Some of them raise the blood glucose level, other diminish it, whereas diazepam is inactive [5]. These observations are valid for experiments carried out in Triton WR-1339 administered rats. In experimentally induced diabetes diazepam [2] and midazolam [8] induced very significant reductions of this parameter. This heterogeneity of responses can be considered due, to the fact that the effects on blood glucose are not mediated through receptors, but they are due to other, probably direct metabolic actions. On the contrary, the lipidic responses had a bell-shaped character, which enabled us to establish some certain structure-activity relationship [7]. The bell shaped aspect of the dose-response curve for serum lipids have been seen with all BZDs investigated so far. The cause of this phenomenon is not clear. It is possible that this is a consequence of the saturation of the peripheral mitochondrial receptors by the agonists, followed by an autoinhibition.

Concluding, it may be said that tetrazepam is a lipid lowering BZD when administered to hyperlipidemic rats. Most doses induced a reduction of the blood glucose level.

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ACE-inhibitors and defence reflexes of the airways

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The group of angiotensin-converting enzyme (ACE) inhibitors is one of the drugs of choice for the treatment of hypertension and congestive heart disease. However, it has been reported that in some of patients ACE-inhibitors induce hyperreactivity of the airways with occurrence of a persistent dry cough, dyspnoe and wheezing. We supposed that the mechanism of these hyperreactivity is connected to accumulation of bradykinin, tachykinins and other inflammatory mediators in the airways. Increased local concentration of inflammatory neuropeptides stimulates bronchial C fibres and rapidly adapting receptors and provoke the cough reflex. Inflammatory processes in the airways could be followed by contraction of airway smooth muscle.

In this study, our aim was to measure the changes of the number and intensity of mechanical induced cough in cats, which were treated for days with enalapril (5 mg/kg b.w.). After 15 days of treatment the reactivity of the lung and tracheal smooth muscles to the bronchoconstrictor mediator histamine was estimated.

As to our finding 15 days of administration of enalapril results in significant increase of cough parameters measured with a more significant sensitivity of the laryngopharyngeal part. In the experimental animals we observed increased reactivity of bronchial smooth muscle to histamine after 15 days of enalapril treatment. The reactivity of the lung smooth muscle to the histamine was not significantly changed.

These results confirmed the increased cough sensitivity and increased bronchial reactivity after enalapril treatment. These experimental animal model may be useful for the investigation of the pharmacological minimization of respiratory adverse effect of ACE-inhibitors.

Keywords: ACE-inhibitors, enalapril, experimental cough, hyperreactivity of the airways

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Angiotensin-converting enzyme (ACE) inhibitors are the drugs of first choice in the treatment of hypertension and congestive heart failure [1]. Their use results in fewer side effects than other antihypertensive medications. ACE-inhibitors lower the blood pressure without adverse effects on lipid and glucose metabolism [2]. However, it has been reported that in some patients ACE-inhibitors induce dry non-productive cough with frequency of occurrence between 0.2–33% [3, 4]. Other airway reactions following ACE-inhibitor therapy are increased airway reactivity with occurrence of dyspnoe and wheezing [5].

ACE is a dipeptidyl carboxypeptidase that plays a pivotal role in regulation of renin-angiotensin and kinin-kallikreine system. The mechanism of the respiratory adverse effects associated with ACE-inhibitor treatment is unrelated to inhibition of the renin angiotensin system because treatment with angiotensin receptor blocker losartan has not caused similar problems [6]. The negative effects of ACE-inhibitors on defence reflexes of the airways are linked to bradykinin and tachykinins accumulation in the airways. These inflammatory mediators stimulate the vagal afferents and nonmyelinated C fibres that potentiate the cough reflex [7]. Consequently the different inflammatory actions of bradykinin [8] and SP [9] in the airways could result in the increased contraction reactivity of airway smooth muscle.

The aim of the present study was to investigate the effect of ACE-inhibitor-enalapril administration on the mechanical stimulated cough in non-anaesthetized cats. Furthermore we investigated the effect of long-lasting administration of enalapril on the reactivity of the smooth muscle of the airways to bronchoconstrictor mediator histamine.

Materials and Methods

Adult non-anaesthetized cats of both sexes weighing 1500–2500 g were used for the experiments. After several days of quarantine a tracheal cannula was surgically implanted into the animals, which served for the mechanical stimulation of the airways with nylon fiber, 0.35 mm in diameter, as well as for recording of the side tracheal pressure. The cough parameters, i.e. the number of cough efforts /NE/, intensity of the cough attacks in expirium /IA^+/ and in inspirium /IA^-/ were evaluated on the basis of the pressure values recorded during the experiment from both laryngopharyngeal (LPh) and tracheobronchial (TB) part of the airways (Nosál'ová et al. [10]). The values of cough parameters obtained before administration of enalapril represented the control (C). Enalapril was administrated perorally as a water solution in a dose of 5 mg/kg b.m. during 15 days. The effect of enalapril on the cough parameters was monitored in the intervals 3, 5, 8, 10, 12, 15 days. The results of these experiment were evaluated by Wilcoxon and Wilcox method [11].

After 15 days of administration of enalapril the reactivity of the smooth muscles of the airways to bronchoconstrictor mediator histamine was investigated by the in vitro method. The preparation of cat tracheal and lung strips were placed in Krebs – Henselheit solution, aerated with mixture 95% O_2 and 5% CO_2. After a 20-minute loading phase (tension of 4 g), and a 30-minute adaptation phase (tension of 2 g) the cumulative doses of histamine (10^{-8}–10^{-3} mol·l^{-1}) were applied into the organ bath. The amplitude of contraction (mN) was used for evaluation of smooth muscle reactivity [12]. For statistical analysis Student's *t*-test for unpaired data was used.

Results

During the 15-days perorally administration of enalapril (5 mg/kg b.m.) the cough was induced by the method of the mechanical stimulation of the airways. A statistically significant increase in number of cough efforts – NE (Fig. 1) was observed during 3, 5, 8, 10, 12, 15 days of enalapril administration. The intensity of cough attack during expiration (IA^+) and inspiration (IA^-), both from laryngopharyngeal and tracheobronchial mucuos area of the airways were statistically significant increased, too (Fig. 2).

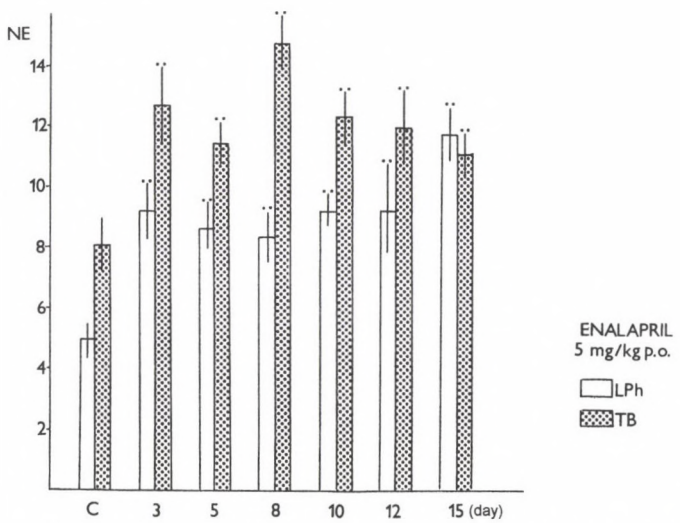

Fig. 1. The changes in the number of cough efforts (NE), during a 15-days peroral application of enalapril (dose 5 mg/kg b.m.). The open columns denote the cough values obtained from the laryngopharyngeal area (LPh), the stippled columns denote cough values obtained from the tracheobronchial area (TB). The control group (C) represents cough values recorded before enalapril therapy. Data are expressed as means ±S.E.M. (*$p<0.05$, **$p<0.01$)

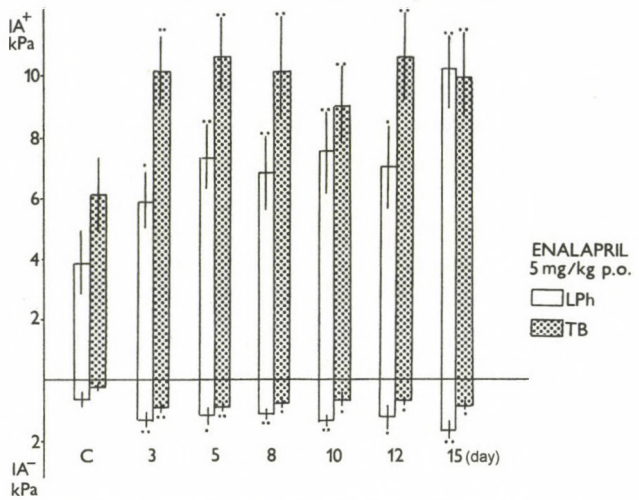

Fig. 2. The effect of enalapril on intensity of the cough efforts during expiration (IA^+) and inspiration (IA^-)

A comparison of the number of cough from laryngopharyngeal and tracheobronchial regions of the airways revealed, that the sensitivity of laryngopharyngeal area was more significant than that the tracheobronchial area (Fig. 3).

A 15-day administration of enalapril resulted in a statistically significant increase of reactivity of tracheal smooth muscle to the cumulative doses of histamine. This increase in the bronchoconstrictor activity compared with the control (C) was significant at the concentration range of histamine 10^{-8}–10^{-3} mol $\cdot l^{-1}$ (Fig. 4). Opposite to the effect of histamine on the tracheal smooth muscle, the reactivity of the lung smooth muscle was not effected significantly (Fig. 5).

Discussion

Cough accompanied by an increased sensitivity of the cough reflex is the most common symptom of inflammatory airway disease. This symptom is also frequently reported in patients receiving ACE-inhibitors. Cough as a defence reflex of the airways is elicitated by the stimulation of myelinated rapidly adapting receptors (RARs) and unmyelinated C fibres located throughout the larynx and tracheobronchial tree. The RARs are excited by some stimuli that evoke cough in man for example mechanical stimulation, distilled water, low chloride, PGE_2, bradykinin and capsaicin [13].

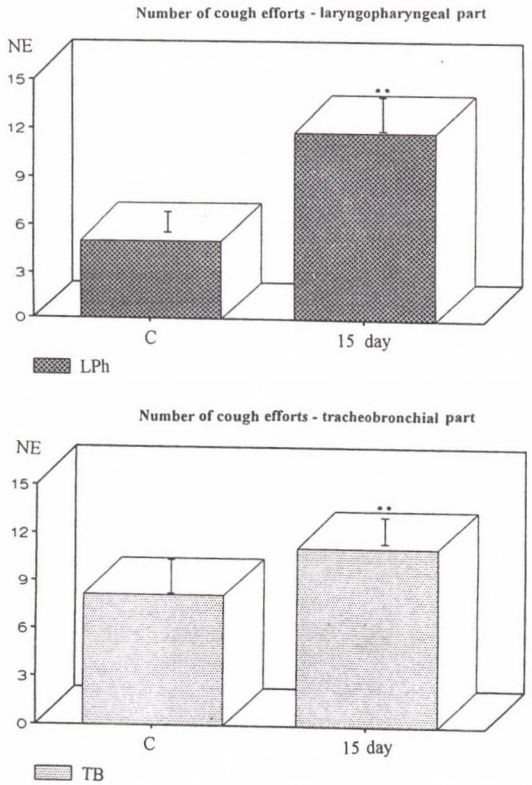

Fig. 3. Comparison in changes of the number of cough efforts (NE) from laryngopharyngeal (LPh) and tracheobronchial (TB) part of the airways before (C-control) and after a 15-day administration with 5 mg/kg b.w. enalapril

In our experimental conditions the animals treated for 15 days with enalapril (5 mg/kg b.m.) showed a statistically significant increased cough response to mechanical stimulus. The number of cough efforts and intensity of cough attacks were significantly increased both from the laryngopharyngeal and tracheobronchial regions of the airways. The effect from LPh region was more pronounced.

Increased cough incidence after enalapril treatment is linked with ACE inhibition and probably brought about bradykinin, SP and other neuropeptides accumulation in the airways. Fox et al. [14] suggest that the accumulation of bradykinin by ACE-inhibitors sensitize C fibres, thereby enhancing cough reflex. Furthermore, sensitized C fibres may release more SP in response to tussive stimuli via axon reflexes [15]. Ichinose et al. [16] have shown that bradykinin induced bronchoconstriction and cough were inhibited by tachykinin antagonists. Therefore, it is possible that increase in local

concentration of bradykinin by ACE-inhibition may stimulate C fibres and release of SP. In addition to bronchial C fibres, bradykinin stimulates the rapidly adapting receptors of the airways [17]. SP is also suggested to sensitize RARs by increases in microvascular leakage in rabbits [18]. In our study we found enhanced cough sensitivity after enalapril treatment to mechanical stimulation of the airways. Because the mechanical irritation of the airways is a relatively selective stimulant for the induction of cough by the rapidly adapting receptors [19], our results confirmed the participation of RARs in the production of the cough after enalapril treatment.

It is possible that increased local concentration of bradykinin and SP could facilitate vagal afferent activity undirectly through second messengers – e.g. histamine or prostaglandins [20]. Bucknall et al. [21] and Andersson [22] have demonstrated increased bronchial reactivity to histamine in subjects who cough after receiving ACE inhibitors. In our experimental in vitro conditions we observed the increased bronchoconstriction activity of the tracheal smooth muscle to the histamine. Bronchoconstriction as a defence reflex can trigger a cough reflex and may increase responsiveness of the cough receptors to the mechanical and chemical irritants [23].

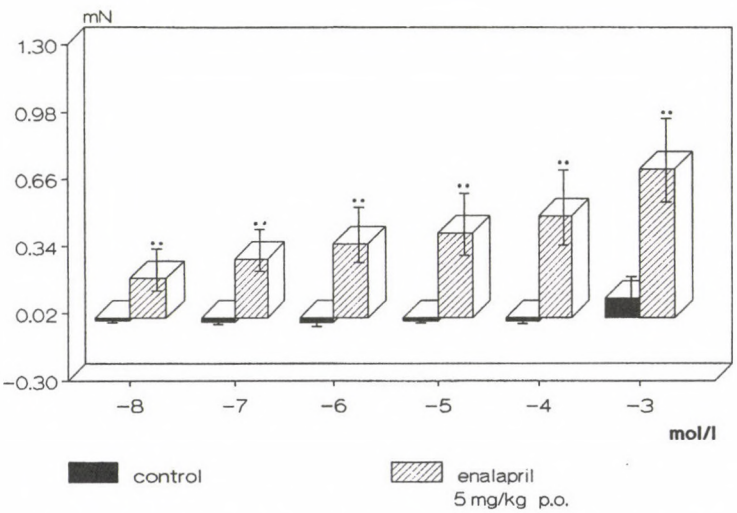

Fig. 4. Effect of the 15-day administration of enalapril (5 mg/kg b.w., per os) on the reactivity of tracheal strips of smooth muscles after histamine in cats. Black columns-control, stripped columns – enalapril. Data are expressed as means ±S.E.M. (*p<0.05, **p<0.01)

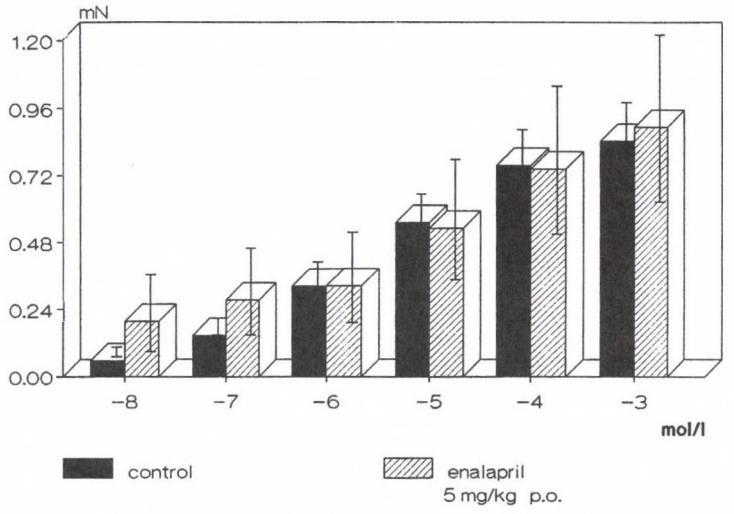

Fig. 5. Effect of the 15-day administration of enalapril (5 mg/kg b.w., per os) on the reactivity of lung strips of smooth muscles after histamine in cats. Black columns-control, stripped columns – enalapril. Data are expressed as means ±S.E.M. (*p<0.05, **p<0.01)

The reactivity of the lung smooth muscle to histamine was not affected significantly.

In conclusion, our experimental data provide consistent evidence of respiratory negative effects of ACE-inhibitors accompanied with increased incidence of the mechanically induced cough and increased reactivity of the tracheal smooth muscle to the bronchoconstrictor mediator histamine.

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Effect of dietary supplementation of vitamin E in partial inhibition of Russell's viper venom phospholipase A_2 induced hepatocellular and microsomal membrane damage in rats

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The current investigation furnishes a good correlation between the α-tocopherol content of the liver and microsomes and corresponding inhibition of Russell's viper venom phospholipase A_2 inflicted damage to them. Dietary supplementation of dl-α-tocopherol at a concentration of 100 mg and 200 mg per kg of diet displayed less damage caused by venom phospholipase A_2 in sharp contrast to the vitamin E deficient rats. α-tocopherol due presumably to the formation of complexes with the phospholipid hydrolysis products of the membranes, plays a significant role in membrane stabilization.

Keywords: membrane stabilization, microsomal membrane, alpha-tocopherol, Russell's viper, phospholipase A_2

Russell's viper envenomation clinically manifests a variety of toxic effects [1] including disorder of vital organs [2, 3]. Viper bite often brings about malfunctioning of the liver [4] and also affects the lipid profile of the liver microsomal fraction [5]. The latter, besides possessing enzymes for steroid and lipid biosynthesis, contains membrane bound cytochromes, involved in the detoxification of many drugs and toxins. Therefore, the viper venom generated disorder of liver, particularly its microsomal fraction must drastically affect the metabolic activities.

Our recent findings vouch for the paramount role played by phospholipase A_2, a major component of viper venom, in destabilizing the lysosomal membrane of the vitamin E deficient rats [6]. However, the vitamin E uptake and concentration have been documented to vary from tissue to tissue [7] and even among subcellular

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organelles in the same tissue [8]. Contemplating on the information the current experiment has been designed to explore whether the differences in the α-tocopherol content significantly contribute to differential degree of membrane stabilization in microsomes and liver as compared to lysosomal membrane.

Materials and Methods

Lypophilized *Vipera russelli* venom was supplied by Haffkine Institute, Mumbai. Sephadex G-50 and CM-Sephadex C-25 and were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. dl-alpha-tocopherol acetate was from E. Merck (India) Ltd., Mumbai. All other reagents used were of analytical grade and obtained from Sigma Chem. Co., St. Louis, MO.

All the experiments were done in accordance with the 'Ethical principles and guidelines for scientific experiments on animals' of the Swiss Academy of Medical Sciences. Laboratory inbred Wister strain albino rats (100±10 g), were divided into three groups. Group A received vitamin E deficient diet [6] for about four months. Groups B and C consumed identical diet, supplemented with 100 mg and 200 mg dl-α-tocopherol acetate respectively per kg of diet. Groups A, B and C will hereinafter be referred to as deficient, normal and high vitamin E content rats respectively. Deficiency of vitamin E was assessed according to Pappu et al. [10].

Viper venom phospholipase A_2 at a dose of LD_{50} was injected into individual rats of the three groups. Animals were sacrificed 5 h thereafter. Liver microsomal fraction was isolated conventionally [8] from both the injected as well as uninjected (control) rats. The supernatant following centrifugation at 105,000 g of liver homogenate was used for the assay of hepatocellular enzymes viz. alanine and aspartate aminotransaminase [11], alkaline phosphatase [12] and 5'-nucleotidase [13]. Glucose-6-phosphatase of microsomes was assayed according to Recknagel and Lombardi [14] respectively. Protein content was determined by the method of Lowry et al. [15]. The purification of venom phospholipase A_2 and lipid analysis of liver microsomal fraction were performed by procedures described earlier [6].

Results

Purity of microsomal fraction was judged on the basis of enrichment of marker enzyme glucose-6-phosphatase. Microsomes prepared from both vitamin E deficient and supplemented rats gave fairly same type of purity (data not shown). LD_{50} of the purified viper venom phospholipase was 3.5 μg/g body weight of rats. With an increase

in the dietary supplementation of vitamin E, there was a concomitant elevation in the level of α-tocopherol in liver and microsomes (Table I). Contrastingly enough, vitamin E deficiency never displayed any decrease in the lipid profile of liver and microsomes (Table I), as expected.

Although administration of viper venom phospholipase A_2 resulted in a significant elevation in the activities of all the hepatocellular enzymes tested, percent increase in the enzyme activity differed markedly among the groups of rats (Table II). Vitamin E dose dependently inhibited the release of liver enzymes after phospholipase A_2 treatment. Liver enzymes were significantly elevated in vitamin E deficient rats ($P<0.001$) and significantly reduced in high vitamin E content rats as compared to rats containing normal vitamin E, following phospholipase A_2 injection.

Table I

Vitamin E content and lipid profile of liver and liver microsomal fraction from rats fed different levels of vitamin E for 4 months

	Dietary Vitamin E (mg/kg of diet)		
	0	100	200
Vitamin E Status of rats:	Deficient	Normal	High
		α-tocopherol level	
Liver (μg/g tissue):	7.7 ± 0.08	165.0 ± 16.5*	234.0 ± 23.0$^+$
Microsome (μg/g tissue):	2.0 ± 0.48	45.5 ± 4.6*	58.9 ± 6.2$^+$
		Lipid Profile	
Liver			
Total lipid (mg/g tissue):	127.0 ± 3.9	130.0 ± 4.5	131.0 ± 5.0
Total phospholipid (mg/g tissue):	56.2 ± 3.8	59.2 ± 3.0	60.0 ± 4.5
Total cholesterol (mg/g tissue):	5.9 ± 1.0	6.0 ± 0.8	6.0 ± 1.0
Microsome			
Total lipid (μg/mg protein):	540 ± 39.0	590.0 ± 40.0	619 ± 54.0
Total phospholipid (μg/mg protein):	389.0 ± 30.0	436.0 ± 35.0	446.0 ± 37.0
Total cholesterol (μg/mg protein):	40.0 ± 4.0	44.0 ± 4.0	47.0 ± 5.0

Values are mean ±S.D. of four rats per group. Levels of significance when compared with rats fed no vitamin E with diet. *$P<0.01$, $^+P<0.001$

Table II

Release of liver enzymes from different groups of rats 5 h post viper venom phospholipase A_2 (PLA_2) administration

	Enzyme activity (unit/mg protein)			
	ALT[a]	AST[b]	ALP[c]	5'-NT[d]
		Group A		
Control:	11.0 ± 1.2	6.3 ± 0.6	12.1 ± 1.2	9.3 ± 0.8
After PLA_2 treatment:	25.0 ± 2.2^{+}	10.0 ± 0.4^{+}	19.2 ± 1.5^{+}	15.0 ± 1.5^{+}
% increase in enzyme: activity	227.3 ± 3.0^{x}	159.4 ± 7.0^{y}	158.7 ± 3.0^{y}	161.9 ± 2.0^{x}
		Group B		
Control:	9.2 ± 1.0	5.6 ± 0.5	10.3 ± 1.0	8.8 ± 1.0
After PLA_2 treatment:	15.0 ± 2.0**	7.9 ± 0.6**	15.0 ± 2.2**	13.0 ± 1.5**
% increase in enzyme: activity	173.3 ± 3.0	140.4 ± 2.0	145.7 ± 6.0	149.7 ± 3.0
		Group C		
Control:	10.0 ± 1.0	5.5 ± 0.5	11.8 ± 1.2	8.6 ± 0.8
After PLA_2 treatment:	13.7 ± 1.8*	6.3 ± 0.3*	13.8 ± 1.4	9.5 ± 0.6
% increase in enzyme: activity	137.3 ± 4.0^{x}	114.3 ± 4.0^{x}	117.3 ± 1.0^{x}	110.6 ± 3.0^{x}

$^{+}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$; when compared with respective control values $^{x}P<0.001$; $^{y}P<0.01$. Significantly differed compared to group B rats.

Each value represents mean ±S.D. of four independent observations.

[a]ALT: Alanine aminotransferase; Unit-μ mole pyruvate/h

[b]AST: Aspartate aminotransferase; Unit-μ mole pyruvate/h

[c]ALP: Alkaline phosphatase; Unit-μg-p nitrophenol/h

[d]5'-NT: 5'-Nucleotidase; Unit-μ mole P_i/30 min.

Lipid composition of the microsomes was drastically changed following viper venom phospholipase A_2 administration (Table III). However this decrease was significantly less as compared to the liver lysosomal fraction of rats consuming identical diets in our previous experiments [6]. Besides, phospholipid/cholesterol ratio after venom injection was more markedly changed in group A rats than in the other two groups.

Table III

Changes in the lipid composition of the liver microsomes after viper venom phospholipase A_2 (PLA_2)

	Total lipid (μg/mg protein)	Total phospholipid (μg/mg protein)	Total cholesterol (μg/mg protein)	Phospholipid cholesterol
			Group A	
Control:	540 ± 39.0	389.0 ± 30.0	40.0 ± 4.0	9.7 ± 0.4
Treatment:	270.0 ± 3.0[a]	185.0 ± 29.0[a]	28.0 ± 3.0[b]	6.6 ± 0.2
% decrease:	49.0 ± 1.8[x]	51.4 ± 2.0[x]	31.2 ± 1.0[x]	32.0 ± 1.0
			Group B	
Control:	590.0 ± 40.0	436.0 ± 35.0	44.0 ± 4.0	9.9 ± 0.3
Treatment:	369.0 ± 35.0[a]	267.0 ± 31.0[a]	36.0 ± 4.0[c]	7.4 ± 0.2[#]
% decrease:	38.0 ± 1.5	39.2 ± 1.7	172.0 ± 1.1	25.0 ± 0.8
			Group C	
Control:	619.0 ± 54.0	446.0 ± 37.0	47.0 ± 5.0	9.5 ± 0.4
Treatment:	443.0 ± 36.0[a]	314.0 ± 31.0[b]	40.0 ± 4.0[c]	7.9 ± 0.5[+]
% decrease:	27.6 ± 1.1[x]	29.8 ± 1.3[x]	15.0 ± 0.8[y]	16.8 ± 0.7

[a]$P<0.001$; [b]$P<0.01$; [c]$P<0.05$. Significantly different compared to respective control values.

[x]$P<0.001$; [y]$P<0.05$. Significantly different compared to group B rats.

[#]$P<0.05$; [+]$P<0.01$. Significantly different compared to group A rats.

Microsomes were isolated 5 h post PLA_2 injection as described in the text. Value represents mean ± S.D. of four observations.

Discussion

Supplementation of vitamin E along with diet leads to an increase in the level of α-tocopherol in the plasma, liver and microsomes. This agrees well with the results reported earlier [6, 9]. Although vitamin E protects tissues against the lipid peroxidation and prevents the release of polyunsaturated fatty acids (PUFA) from the membranes, its deficiency does not influence the lipid profile of the microsomes significantly. This may be due to the presence of enzymes in the microsomes capable of synthesizing lipids even in vitamin E deficiency [8]. VRV-PL-VI, a major basic phospholipase A_2 of Russell's viper venom promotes cell membrane dysfunction by hydrolysing the membrane phospholipids [16]. Phospholipids of the biological membranes appear to be bound to lipoproteins and are suitable substrate for the action of venom phospholipases [17].

Damage of the hepatocytes and microsomes by viper venom phospholipase A_2 is well consistent with our previous investigation [6]. Hepatic injuries almost invariably

concur with changes in the activity of liver enzymes, which are very useful for diagnosis and monitoring liver disorder. Release of cytosolic (amino transferases) and membrane bound enzymes (alkaline phosphatase and 5-nucleotidase) after phospholipase A_2 treatment is a clear consequence of liver cell necrosis and membrane damage. Similarly decrease in the quantity of phospholipids and hence total lipid from the microsomes, along with the release of cholesterol and free fatty acids might be attributed to the phospholipid hydrolytic action of the injected phospholipase A_2 [6, 18].

The present investigation reinforces the correlation between α-tocopherol content of liver and microsomes and inhibition of viper venom phospholipase A_2 induced injury to them. Vitamin E dose dependently inhibits the liver cell necrosis and microsomal membrane hydrolysis by phospholipase A_2. This is further evident from the comparison of the data from the current and previously reported experiments [6]. Since the uptake rate of α-tocopherol may vary [8], therefore microsomes from all the three groups of rats contain much more vitamin E than lysosomes. As a result the lipid constituents of microsomes are less hydrolysed then those of lysosomes under identical experimental condition. Therefore the higher vitamin E contents of microsomes compared to lysosomes, may account for their differential response to injected phospholipased A_2. This argument also holds good to explain the better stabilization of hepatocellular membranes from group B and C rats compared to group A rats against the detrimental action of viper venom phospholipase A_2. Further phospholipid/cholesterol ratio significantly increases in groups B and C rats, compared to group A rats after phospholipase A_2 treatment and this phenomenon is due mainly to the protection of the microsomal membrane phospholipids by α-tocopherol in the former two groups of rats [6]. A high phospholipid/cholesterol ratio increases the membrane fluidity and thereby stability.

Despite its low concentration in the cell membrane, vitamin E, the nature's most effective lipid-soluble antioxidant, protects unsaturated fatty acids in the cell membranes that are important for membrane function and structure [19]. Vitamin E can stabilize the biological membranes against the damaging action of phospholipid hydrolysis products, but never modulates the activity of phospholipase A_2 [20]. Extensive hydrolysis of phospholipids results in a sharp increase in the free fatty acids and lysolecithin level in the microsomes and liver, which may provoke further membrane damage and cell necrosis, consequently facilitating phospholipase A_2 action. Since α-tocopherol forms complex with free fatty acids and lysolecithin, both in solution and membrane system [21], therefore in biological membranes, α-tocopherol will predominantly interact with phospholipid hydrolysis products [20]. Besides, elevation in the α-tocopherol level of microsomes results in increased complex formation with the arachidonyl moieties of the membrane phospholipids [22] which thus become less vulnerable to the action of phospholipase A_2.

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AKADÉMIAI KIADÓ, BUDAPEST

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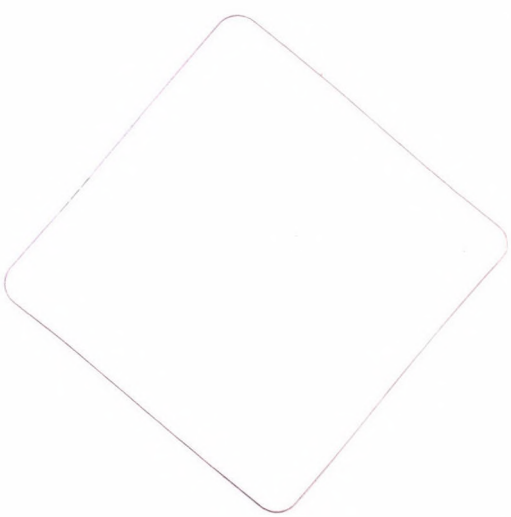

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Three complete copies of the manuscript including all tables and illustrations should be submitted. Manuscripts should be typed double-spaced with margins at least 3 cm wide. Pages should be numbered consecutively.

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An abstract of not more than 200 words should be supplied typed before the text of the paper. The abstract should be followed by (no more than) five key-words.

Abbreviations should be spelled out when first used in the text. Drugs should be referred to by their WHO code designation (Recommended International Nonproprietary Name): the use of proprietary names is unacceptable. The *International* System *of Units* (SI) should be used for all measurements.

References

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Examples:

Székely M., Szelényi, Z.: Endotoxin fever in the rat. Acta Physiol. Hung. **53**, 265-277 (1979).

Schmidt, R. F.: Fundamentals of Sensory Physiology. Springer Verlag, New York-Heidelberg-Berlin 1978.

Dettler J. C.: Biochemical variation. In: Textbook of Human Genetics, eds Fraser, O., Mayo. O., Blackwell Scientific Publications, Oxford 1975, p. 115.

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Figures (line drawings, diagrams, photographs). These should be numbered consecutively using Arabic numerals. One original copy and two additional copies should be sent. Please, indicate the figure number, the name of the first author and the top of the figure on the backside. Their approximate place should be indicated in the text. Captions should be provided on a separate page.

Prepare *line drowning and diagrams* in Indian ink at their expected final size. Good quality computer graphs produced on a laser printer are acceptable.

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