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In vivo dose-response characteristics of endothelin-1: Studies on rat saphenous vein

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Microangiometric studies were made *in vivo* on rat saphenous vein to obtain dose-response contraction curves with endothelin-1. During superfusion of the drug, threshold concentrations were found to be in the range of 4×10^{-11}–4×10^{-10} moles/lit, maximal concentrations were about 4×10^{-8} moles/lit. These values are close to published *in vitro* threshold values. During intravascular administration of the drug contraction could be observed in response to a dose as small as 4.37 femtomoles/s. The data presented show that characteristic dose response curves resembling those found *in vitro* can be recorded *in vivo* with endothelin on appropriate vascular preparata. The rat *in situ* saphenous vein microangiometric technic can be used for delicate pharmacologic examination of venoactive substances *in vivo*.

Keywords: rat, saphenous vein, endothelin-1

Endothelin-1 is a very effective vasoconstrictor drug for both arteries and veins [1–3, 5–10, 12–17, 19, 20–29]. Dose response contraction curves of endothelin for the venous smooth muscle *in vitro* have been published earlier [1, 3, 5, 7, 10, 14–16, 19, 22–24, 27, 28]. Because of several reasons, however, *in vivo* dose response curves have not yet been published in the literature with one exception [20], where, for methodical reasons endothelin doses directly acting on the venous system could not be estimated. Difficulties to obtain such curves with endothelin have been stressed by some authors [13, 21]. Slow development of the contraction, practical impossibility of the washout of the drug, large avidity of the substance to its receptor [10] are listed as factors that impede the registration of *in vivo* dose response curves. Knowledge of

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these curves would be very important as it makes possible the evaluation of physiological or pathological significance of substance concentrations measured *in vivo* in tissue fluids [4]. In the present study the rat in situ saphenous vein preparation [18] was applied to obtain *in vivo* dose response curves for endothelin-1.

Methods and Materials

The experiments were carried out on Sprague-Dawley rats of both sexes, weighing 250–350 grams. The animals were anaesthetized with pentobarbital (Nembutal, CEVA) 40–50 mg/kg body weight intraperitoneally. Third of this dose was repeated when needed, intravenously. After tracheal cannulation the right femoral artery and right femoral vein were cannulated, for arterial pressure measurement and for the introduction of repeated doses of the anaesthetic, respectively. Then the left saphenous vein was prepared under the dissection microscope at the level of the knee-joint for *in vivo* microangiometric measurement of the vessel diameter as described earlier [18]. In some animals, a side branch of the saphenous vein with a diameter of 160–220 micrometer was also cannulated for local intraluminal administration of endothelin-1. Superfusion studies were performed on five animals, infusion studies were attempted on six animals but could be successfully performed only on two animals.

Outer diameter of the saphenous vein was continuously measured with the aid of an *in vivo* microangiometer setup developed in our Institute. The animals were fixed onto a warm water jacketed stage. The site of the measurement was illuminated with a fiber-optic lamp (Volpi Intralux 6000). The exact site of the diameter measurement was indicated with a video-marker. Continuous microscopic video-angiometric records were made using a dissection microscope (Wild M3Z type-S, Heerbrugg, Switzerland), a solid state camera (Philips) and a monitor (Philips). A microcomputer developed in our institute analysed the signal of the monitor. Magnification at the screen was 143-fold. During measurement two light spots (video-markers) appeared on the screen, whose position could be changed with the aid of two turnknobs. The markers were positioned at the two contours of the outer diameter of the vessel. The distance between the markers was automatically measured and transmitted for evaluation to an IBM 386 AT personal microcomputer. Calibrations were made with the aid of a microetalon. Arterial blood pressure was measured with a Gould pressure head.

Stock solutions of synthetic endothelin-1 (Sigma) were made with distilled water (10 microgram/ml) and kept at −18 °C. Solutions for superfusion were freshly made with saline in concentrations of 0.1, 1.0, 10, 100 and 1000 nanogram/ml which corresponds to 4×10^{-11}, 4×10^{-10}, 4×10^{-9}, 4×10^{-8} and 4×10^{-7} moles/lit, respectively. Superfusion was performed just over the region of diameter measurement of the saphenous vein with the aid of a polyethylene microcannula and a Harvard pump. The rate of superfusion was 65.4 microliters/min. In the infusion studies endothelin was intraluminally applied through the side-branch of the saphenous vein. Endothelin-1 was infused in a concentration of 1 microgram/ml with the aid of a Harvard pump, at rates of 0.65–65.4 microliters/m, which corresponds to 4.37–437 femtomoles/s, respectively.

Data representation, storage and evaluation were made on an IBM PC using a specifically developed software system ("Microangiometry"). Arterial blood pressure and saphenous vein diameter values were continuously recorded. Selected parts of the recordings were stored on the computer hard-disc together with typed-in notes. During data retrieval values corresponding to marked time-points could be read digitally on the screen.

Statistical analysis was done with paired *t*-test.

Results

In some superfusion experiments saphenous vein contraction could be observed at endothelin-1 concentrations as low as of 4×10^{-11} moles/lit. At 4×10^{-10} moles/lit the contraction was statistically significant (external diameter: 721 ± 50 micrometers from 788 ± 46 micrometers). Close to maximal contractions were reached with 4.0×10^{-8} moles/lit concentration of endothelin-1. The extent of average maximal contraction was 64.2% of original outer diameter and on the monitor it could be observed that hardly any lumen was left. The dose response curve seemed to be biphasic (Fig. 1). The contractions usually began in less than one minute after the onset of the superfusion and fully developed stabilized contractions could be observed within ten-fifteen minutes. Attempts to wash out the drug turned out to be impossible thus separate animals were used for each dose-response curve.

During the intraluminal application of endothelin-1 in many experiments a problem arised from the extreme effectivity of the drug: even at very low infusion rates the vessel totally contracted just above the level of the infusion microcanulla which made further infusion of the drug impossible. Out of six attempts only two were successful. One representative intraluminal *in vivo* dose response curve can be seen on Fig. 2. That curve shows a threshold dose of 10.9 picogram/s (4.37×10^{-15} moles/s) and a maximal dose of 109 picogram/s (43.7×10^{-15} moles/s). The intraluminal dose-response curve also appears to be biphasic.

Mean arterial blood pressure values were over 80 mm Hg throughout the experiments. The applied endothelin doses did not induce systemic blood pressure rises.

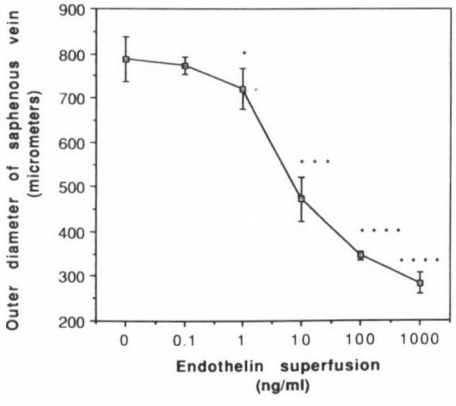

Fig. 1. *In vivo* contraction dose response curve of the rat saphenous vein. Superfusion of endothelin-1. n = 5 Mean±SEM. Significance levels of paired *t*-tests are marked, *p<0.05, **p<0.02, ***p<0.01, ****p<0.001

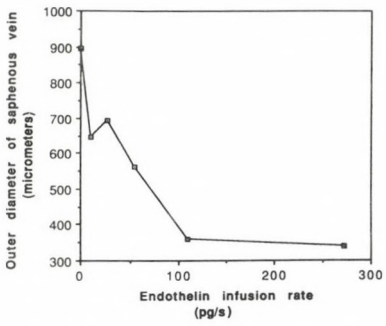

Fig. 2. *In vivo* dose response contraction curve of the rat saphenous vein. Infusion of endothelin-1. Individual experiment

Discussion

The experiments carried out in the present study show that with the use of the rat spahenous vein microangiometric technic [18] *in vivo* endothelin-1 dose response curves could be obtained. It was thoroughly shown that endothelin-1 induces a very effective contraction not only in arteries but also in veins *in vitro* [1–3, 5–10, 14–17, 19, 22–24, 27, 28] and *in vivo* [11–13, 20, 26]. Endothelin-1 dose-response contraction curves were observed on several venous preparata *in vitro*. *In vivo* dose response curves were difficult to obtain [13, 21] because the binding of the drug to its receptor was effective [10], the contraction was delayed in its onset, slowly developing etc. The only *in vivo* dose-response curve was published by Pernow [20]. He infused the endothelin-1 into the brachial artery while the venous capacitance was measured. As metabolism of the drug could be expected, in these experiments it was impossible to estimate the doses or concentrations directly acting on the venous wall. Dose-response curves taken *in vitro* usually showed threshold concentrations of about 10^{-11}–10^{-10} moles/lit and maximal concentrations of about 10^{-7}–10^{-9} moles/lit [1, 3, 5, 7, 10, 14–16, 19, 22–24, 27, 28]. These values were close to the *in vivo* values we obtained in the superfusion experiments (Fig. 1). Our data confirm an earlier view that the basal plasma levels of endothelin-1 cannot be very effective in maintaining venous tone. Specific measurements of immunoreactive ET-1 in plasma were in the range of 1–5 pg/ml (around 10^{-12} moles/lit, [4]). We observed that the dose response curve was biphasic (Fig. 1). This is in good accordance with observations reporting that endothelin-1 contraction of veins *in vitro* affects two populations of endothelin receptors with different binding constants, most probably ET_A and ET_B receptors [3, 5, 10, 15, 19, 23, 24, 27, 28].

The very low amounts we found to induce vascular contraction *in vivo* with local intraluminal application show the extreme effectivity of the drug (Fig. 2). The observed threshold dose, 4.4 femtomoles/s, taking into consideration the rat saphenous vein blood flow (around 1 ml/m Nádasy, unpublished data) corresponds to intraluminal endothelin-1 concentrations of about 2.6×10^{-10} moles/lit of blood, which is similar to what has been found in *in vitro* experiments in the literature (see above, and *in vivo* by us (Fig. 1).

In conclusion, we have succeeded in obtaining *in vivo* dose-response curves with endothelin-1 on a vein. Our studies confirm earlier *in vitro* studies that venous tissue is very sensitive to the drug. Still, the published *in vivo* endothelin-1 plasma concentrations do not seem to be sufficient to ensure an important role for this substance in maintaining basal tone. The rat in situ saphenous vein microangiometric technic which has originally been developed for *in vivo* electrophysiology studies [18], can also effectively be applied for delicate *in vivo* pharmacologic evaluations of venoactive substances.

Acknowledgements

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Comparison of the vasodilatory effects of bradykinin in isolated dog renal arteries and in buffer-perfused dog kidneys

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This study was undertaken to investigate the role of nitric oxide (NO), cyclooxygenase products and bradykinin (Bk) receptors in the Bk evoked responses of canine renal arteries and perfused kidneys.

Rings of isolated canine renal arteries were mounted in organ chambers for measurement of isometric force. The isolated canine kidneys were perfused with Krebs-solution (constant flow) and the perfusion pressure was continuously recorded. The influence of the cyclooxygenase inhibitor indomethacin and the nitric oxide (NO) synthase inhibitor Nω-nitro-L-arginin (L-NOARG) on the vasocontractile responses to phenylephrine (PE) were examined in both preparations. Furthermore, the effects of Bk on the tone of canine isolated renal arteries and on the vasopressor responses of isolated buffer-perfused kidneys of dogs were tested in the absence and presence of enzyme inhibitors and the B_2 kinin receptor antagonist HOE-140.

It was found that indomethacin enhanced the contractile responses of large renal arteries to PE by 77±10%. In intact artery rings L-NOARG (0.1 mM) caused an additional potentiation of the PE-induced contractions in the presence of indomethacin (from 11.5±1.2 mN to 21.6±1.7 mN). However, L-NOARG failed to affect contractile responses to PE in endothelium-denuded rings. Bk produced a concentration-dependent relaxation of the precontracted endothelium-intact renal arteries. The IC_{50} value for Bk was 11.2±3.7 nM. The relaxant activity of the peptide in renal artery rings was not affected by indomethacin (3 μM). However, in the presence of L-NOARG a significantly higher concentration (IC_{50}=860±300 nM) of Bk was required to relax renal arteries. The Bk receptor antagonist HOE-140 (10 nM for 40 min) attenuated the relaxant effect of Bk in renal artery rings (from an IC_{50} of 14.2±2.5 nM to 216±37 nM).

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Indomethacin (3 μM for 20 min) did not significantly alter the arteriolar vasoconstriction (from 45±4 mm Hg to 48±5 mm Hg, n=5) evoked by PE. By contrast, L-NOARG (0.1 mM) potentiated (from 56±7 mm Hg to 94±11 mm Hg) the PE-induced vasopressor responses in perfused kidneys. Bk reduced the size of the pressor responses at relatively low concentrations (2–60 nM) but the dose-response curve was flat and the maximum inhibitory effect hardly exceeded 50 percent. Indomethacin (3 μM) did not modify the inhibitory effect of Bk in perfused kidney. In the presence of L-NOARG, Bk depressed the PE induced vasopressor effects with a maximum of 18±20%. Preincubation of the kidney preparations with the Bk antagonist HOE 140 (10 nM for 40 min) almost completely abolished the inhibitory effect of Bk on the PE induced vasopressor responses.

The results suggest that the endothelial NO plays a fundamental role in the relaxant effect of Bk and considerably modulates vascular reactivity to PE in canine renal vasculature. Furthermore, significant difference exists between conduit and resistance vessels of dog's kidney in the effect of indomethacin on the adrenergic contractions.

Keywords: isolated dog renal artery, isolated dog kidney, bradykinin, indomethacin

Bradykinin (Bk) relaxes dog renal arteries *in vitro* [1] and increases renal blood flow in anaesthetized dog *in vivo* [2, 6]. The relaxant effect of Bk on renal arteries and renal vasculature has been shown to be due to various mechanisms and various mediators [18, 25]. Bk is thought to act mainly through the release of endothelium-derived relaxing factor and cyclooxygenase products. Inhibition of prostaglandin biosynthesis by indomethacin had no effect on vasodepressor responses to Bk in dogs [12], but inhibited the relaxation evoked by the activation of muscular kinin receptors (B_1 and B_2) in isolated renal arteries [18]. It was demonstrated that the selective B_2 receptor antagonists (e.g. HOE-140) produced a marked inhibition of the endothelium-dependent response to Bk in dog renal arteries [18] and blocked the vasodepressor effect of Bk *in vivo* [12]. The Bk induced endothelium (NO)-dependent relaxation responses of dog carotid, coronary and renal arteries are due to the activation of endothelial B_2 receptors [16, 18]. These studies suggest the involvement of NO-dependent mechanisms in the vasodilator responses of renal vessels. However, the involvement of NO-dependent mechanisms in the Bk induced vasorelaxations of isolated buffer-perfused dog kidney has not been demonstrated. The present study was undertaken to establish and to compare the underlying mechanisms of action of Bk on isolated canine renal artery and perfused kidney. In the current experiments we demonstrate that the contribution of nitric-oxid and cyclooxygenase products to the regulation of vascular tone in large and resistance vessels may differ.

Methods

Mongrel dogs of either sex, weighing 8–12 kg, were anaesthetized with pentobarbitone sodium (30 mg/kg, i.v.). After administration of heparin (500 IU/kg) the animals were killed by bleeding from the common carotid arteries, and both kidneys were rapidly removed. The experiments were performed on isolated dog kidneys and renal artery rings.

Isolated renal artery experiments

The renal arteries (1.5–2.0 mm in diameter) were cut into 3 mm long rings and suspended in an organ bath (5 ml) containing Krebs-solution at 37 °C. The composition of the bathing fluid was (mM): NaCl 113, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.5, Na_2EDTA 0.03. The solution was continuously aerated with a mixture of 95% O_2 and 5% CO_2. The contractile and relaxant responses of the arterial ring were measured isometrically (Ugo Basile, Type DY 1) and recorded on potentiometric recorder (Radelkis).

Before the start of measurements, the renal artery rings were equilibrated for 60 min, and during this time the resting tension was continually adjusted to 15 mN. The arterial rings were used with intact endothelial layer or the endothelium was removed by gentle rubbing of the intimal surface with a toothpick. The presence or absence of endothelium was confirmed in each ring by the ability of acetylcholine (ACh, 1 μM) to induce at least 80% reduction of the sustained contraction. The arterial preparations were partially precontracted with 0.1–0.2 μM PE, the contractions being in a range between 15–25% of the maximum contraction induced by 0.1 mM PE. Concentration response curves for Bk were obtained by cumulative addition of the drugs to the organ bath. The effect of Bk was tested in the presence of indomethacin (3 μM), in the presence of indomethacin plus Nω-nitro-L-arginin (L-NOARG, 0.1 μM) or indomethacin plus HOE-140 (10 nM for 40 min).

Perfused kidney experiments

The kidney was cleaned of adherent connective tissue, the main artery exposed, cannulated with a polyethylene tube and flushed with heparinized (50 U/ml) saline. In dogs, the main renal artery usually divides into two (or three) side branches (15–30 mm in length) before reaching the kidney. To exclude the participation of extrarenal arterial segments in the vasopressor responses of perfused kidney, the side branches were cannulated and perfused separately from each other by the same way (5 ml/min). The cannulas were advanced into the side branches of the renal arteries as near to the kidney as possible. The drugs were injected or infused into the cannula in which the perfusion pressure was continuously measured. The preparation was placed into a thermostated kidney holder (37 °C) and perfused with Krebs-solution containing 1% dextrane. The perfusion fluid was maintained at a temperature of 37 °C and aerated with a mixture of 95% O_2 and 5% CO_2. The perfusion flow was kept constant at a rate of 5 ml/min (Kutesz, Peripump "D"). The perfusion pressure was continuously measured by a pressure transducer (Bentley Trantec Model 800) connected to the cannula. The pressure changes were recorded on a potentiometric recorder (Radelkis, OH-814). Before cannulation the pressure in the cannula was 5–10 mm Hg at the flow rate of 5 ml/min. After cannulation the pressure amount to 20–40 mm Hg and did not change significantly for at least 90 min. Following the equilibration period (30 min), the renal vasculature was made to constrict by bolus injections of phenylephrine (PE) into the cannula at 6 min intervals. The injected PE (7–10 nmol) produced a transient rise in perfusion pressure of 45–60 mm Hg. The injection volume was less than 20 μl. After steady responses to the injected PE had been obtained, the measurements were started. The vasodilatory activity of increasing concentrations of Bk was tested on the PE induced pressor responses. Bk infusion into the cannula (Infumat, Kutesz) was begun 2 min before and discontinued 2 min after the administration of PE. The effects of Bk were also measured in the presence of indomethacin (3 μM), in the presence of indomethacin plus Nω-nitro-L-arginin (L-NOARG, 0.1 μM) or indomethacin (3 μM) plus HOE-140 (10 nM for 40 min). These substances were added to the perfusion fluid.

Drugs

The following drugs were used: L-Phenylephrine hydrochloride (Sigma), Acetylcholine iodide (Sigma), Nω-nitro-L-arginine (Sigma), Indomethacin (Chinoin). D-Arg(Hyp^3,Thi^5,D-Tic^7,Oic^8)Bk (HOE-140) was kindly provided by Hoechst (Frankfurt/Main, Germany).

Data were analyzed using Student's *t*-test. A p value of less than 0.05 was considered significant.

Results

The effects of indomethacin and L-NOARG on the vasocontractile responses of isolated dog renal arteries

In renal artery rings the PE-evoked contractions were significantly increased (from 5.8±0.4 mN to 10.2±1.3 mN) by indomethacin. The contraction potentiating effect of indomethacin did not change significantly after rubbing the intimal surface (Table I). In artery rings L-NOARG caused an additional potentiation of the PE-induced contractions in the presence of indomethacin (from 11.5±1.2 mN to 21.6±1.7 mN). However, in rubbed preparations L-NOARG failed to affect vasocontractile responses. Removal of vascular endothelium increased slightly, but not significantly, the size of vasocontractile responses to PE (Table I).

Table I

The effect of indomethacin and Nω-nitro-L-arginine on the vasocontractile responses to phenylephrine in isolated canine renal arteries.
(Values are means of 5 experiments)

	Contractile responses to 0.1 μM PE (mN±S.E.M.)	
	Intact rings	Denuded rings
Control	5.8±0.4	7.6±0.6
+Indo	10.2±1.3*	13.7±1.6*
Control$^{\Phi}$	11.5±1.1	12.4±2.2
+L-NOARG	21.6±1.7*	13.4±1.8

Indo = indomethacin (3 μM), L-NOARG = Nω-nitro-L-arginine (0.1 mM), $^{\Phi}$ = control responses in the presence of 3 μM indomethacin, $p<0.05$

Effects of indomethacin, L-NOARG and the Bk receptor antagonist HOE-140 on the relaxant effect of Bk in isolated dog renal arteries

In the presence of intact endothelium, addition of Bk to preconstricted (sustained tone was 10.2±2.3 mN; evoked by 0.2 μM PE) renal arteries produced concentration-dependent, and at the highest concentration almost complete, relaxation. The IC_{50} value for Bk was 11.2±3.7 nM ($n=6$). In the presence of indomethacin the concentration of PE was reduced by 50–60% to get a level of vasoconstriction comparable (9.3±2.4 mN) to that seen in control experiments. Relaxation responses of Bk in intact rings were not significantly affected by indomethacin (IC_{50} value for Bk in the presence of the cyclooxygenase inhibitor was 11.7±1.8 nM; $n=6$). The

concentration–relaxation curves for Bk were shifted to the right by L-NOARG in endothelium-intact renal artery rings (the IC_{50} value for Bk was 860±300 nM in the presence of indomethacin plus L-NOARG). The maximal relaxation produced by Bk (at 10 μM) in the presence of L-NOARG was 62±9%. The effect of bradykinin-2 (B_2) receptor antagonist HOE-140 was also tested on the Bk evoked endothelium-dependent responses in renal artery rings. HOE-140 (10 nM for 30 min) caused a rightward shift of the concentration-relaxation curve for Bk. The IC_{50} values of Bk measured in the presence of HOE-140 were markedly increased (from 14.2±2.5 nM to 216±37 nM, $n=5$).

The effects of indomethacin and L-NOARG on the vasopressor responses of isolated buffer-perfused dog kidney

In perfused kidneys the basal pressure, at a constant flow rate of 5 ml/min, was 25±10 mm Hg. PE (7–10 nmol, bolus) injected into the cannula, produced an increase in the perfusion pressure of 45–60 mm Hg. The pressor responses were transient and disappeared within 3 min after PE administration. The cyclooxygenase inhibitor indomethacin (3 μM for 20 min) slightly, but not significantly augmented the pressor responses (from 45±4 mm Hg, to 49±5 mm Hg, n=5) to PE (Fig. 1). The effect of NO-synthase inhibitor L-NOARG (0.1 mM) on the vasopressor responses were tested in the presence of indomethacin. L-NAORG potentiated the PE-induced vasopressor responses (from 56±7 mm Hg to 94±11 mm Hg).

Effects of indomethacin, L-NOARG and the Bk receptor antagonist HOE-140 on the inhibitory effect of Bk in perfused dog kidney

In perfused dog kidneys Bk reduced the pressor responses at relatively low concentrations (2–60 nM) but the dose-response curve was flat and the maximum inhibitory effect hardly exceeded 50 percent (Figs 1 and 2). A further increase in the concentration of Bk was not possible due to the contractile activity of high concentrations of this compound. For the above reasons, the IC_{50} values for Bk could not be determined correctly in perfused dog kidneys. Preincubation of the preparation with indomethacin (3 μM) did not modify the inhibitory effect of Bk. The effects of L-NOARG on the inhibitory action of Bk were investigated in the presence of indomethacin. The administration of L-NOARG to the renal vasculature reversed (4 preparations) or significantly attenuated (2 preparations) the inhibitory effect of Bk (Fig. 3). The maximum inhibition produced by Bk was 18±20% in the presence of L-NOARG. In another series of experiments we tested the action of B_2 receptor antagonist HOE 140 on the vasodepressor effect of Bk in perfused kidneys. Preincubation of the preparations with HOE 140 (10 nM for 40 min) almost completely abolished the effect of Bk on the PE induced vasopressor responses. In the presence of HOE 140 the maximal inhibitory effect of Bk (60 nM) was 8±4% ($n=4$).

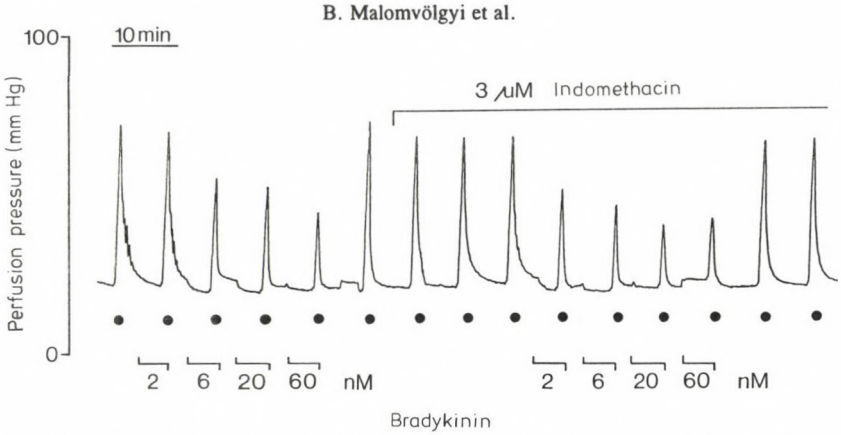

Fig. 1. The inhibitory effect of bradykinin (Bk) on the phenylephrine (• = 7 nmol bolus injection) induced vasopressor responses in perfused dog kidney, in the absence and presence of indomethacin

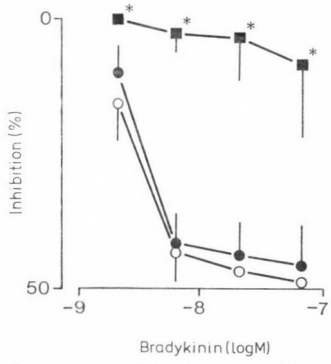

Fig. 2. The effect of bradykinin on the vasoconstrictions produced by bolus injections of phenylephrine in buffer-perfused dog kidneys in the absence (o) and presence (•) of indomethacin (3 μM) and in the presence of indomethacin + 0.1 mM Nω-nitro-L-arginin (▪)

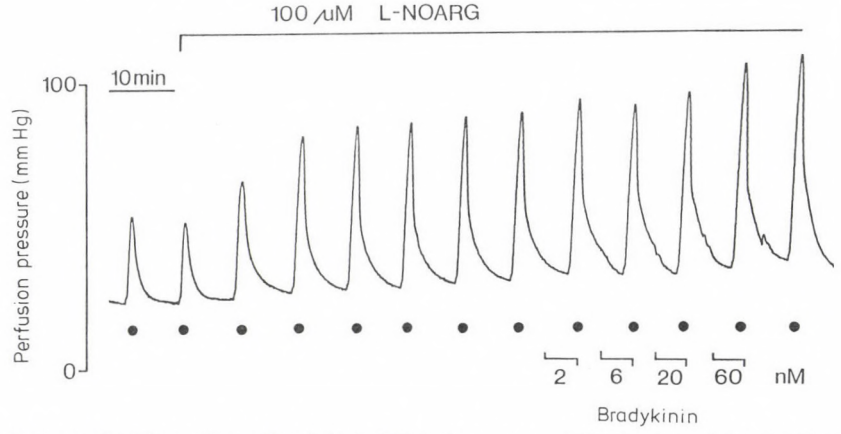

Fig. 3. The lack of inhibitory effect of bradykinin (Bk) in the presence of Nω-nitro-L-arginine (L-NOARG) on the phenylephrine induced (• = 7 nmol bolus injection) vasopressor responses in buffer-perfused dog kidney

Discussion

In our present study the involvement of NO-dependent mechanisms in the vasorelaxant effect of Bk on vascular tone of isolated dog renal arteries and perfused kidneys was investigated. Our results demonstrate that Bk which may modulate vascular tone by different ways act as an NO-dependent relaxant in both conduit and resistance renal arteries of dog. Our results also indicate that the role of endothelial factors (prostaglandins, NO) in the regulation of renal vascular contractility may differ from each other in large and resistance vessels.

The isolated kidney preparations perfused with Krebs-solutions are often used for the study of endothelium-dependent mechanisms and renal functions [3]. The buffer-perfused dog kidney is an unusual preparation for the study of endothelial-functions in renal microvessels. The Krebs-perfused dog kidney allows us to exclude the complicating effects of blood components (e.g. erythrocytes) which may modulate the vascular tone and EDRF level in renal microcirculation [9]. The isolated dog kidney preparations can be preserved for a long time without change in the endothelium-dependent responses and renal functions [19].

In the present study, different methods were used to study renal microvessels (perfused kidney) and large vessels (vascular ring). The results are mostly comparable qualitatively, but they may not be strictly comparable quantitatively.

Indomethacin did not alter the vasopressor responses of the perfused kidney preparations, whilst it significantly augmented the vasoconstriction of the isolated renal arteries. Our results show that the effect of indomethacin depends on the size of the blood vessels. Cyclooxygenase inhibitors variably affect renal hemodynamics *in vivo*. Several investigators have demonstrated that indomethacin produces a significant decrease in renal blood flow in anaesthetized dogs [4, 15] and enhances the vasoconstrictor response to angiotensin II [8]. By contrast, indomethacin did not alter renal blood flow significantly in conscious [22] or in anaesthetized dogs [23]. Although *in vitro* experiments cannot be adapted directly to *in vivo* situations, our results support the view that cyclooxygenase products do not contribute to the modification of adrenergic agonist-induced vasoconstriction in dog kidney.

In order to investigate the role of NO-dependent mechanisms in renal vessels, the effect of NO-synthase inhibitor L-NOARG was tested on the vasocontractile responses. In the presence of indomethacin, L-NOARG enhanced the PE-evoked responses in both isolated renal arteries and perfused kidneys. These observations suggest that the NO-dependent mechanisms may influence the vasocontractile responses in the whole renal vasculature and indicate the stimulated release of NO from the renal vascular tree. Removal of endothelium did not result in significant changes in vascular reactivity to PE in arterial rings. However, L-NOARG enhanced vasocontractile responses in endothelium-intact arterial segments (Table I). These results demonstrate the selective action of L-NOARG on endothelial function, which differs from the effect of mechanical denudation of renal artery rings.

As reported previously by others, Bk exhibited vasorelaxant activity in renal arteries [1, 18], in blood-perfused kidneys [7], and several studies showed that intrarenal infusion of Bk produces marked increases in renal blood flow [6]. In our experiments, Bk relaxed precontracted dog renal arteries almost completely. In contrast, the PE-induced vasopressor responses in the kidney were only partly inhibited by Bk. The partial inhibition produced by Bk in perfused kidney can be due to the presence of contractile Bk-receptors in the renal vascular bed.

There are at least two ways by which Bk may produce relaxation: releasing cyclooxygenase products (e.g. prostacyclin) and EDRF/NO from the vascular endothelium. We found that indomethacin failed to affect the relaxant or the inhibitory effect of Bk both in renal arteries and perfused kidneys. This finding indicates that cyclooxygenase products do not mediate the relaxant effect of Bk in dog renal vasculature. Our results are in accordance with the findings that cyclooxygenase inhibition had no effect on the vasodepressor responses to Bk in dog [12, 16] and in isolated renal arteries [1, 25]. Similar results were obtained by Feigen et al. [5] as well as by Strand and Gilmore [21] who found that the Bk induced renal vasodilation was not affected by cyclooxygenase inhibition in the dog. In contrast, the Bk induced relaxation in canine intrarenal arteries [24] and in blood perfused canine kidney [7] were slightly attenuated by indomethacin. The reason for this discrepancy may be the use of renal arteries of different size or animals of different ages.

In order to examine the possible involvement of the NO pathway in Bk-induced relaxations, we tested the effect of L-NOARG. In renal arteries the concentration-response curve for Bk was shifted to the right by L-NOARG, indicating the NO-dependent component of the responses. It should be noted that L-NOARG did not produce complete inhibition of the relaxation responses in arterial rings. The inhibitory effect of Bk on the vasopressor responses was significantly attenuated by the NO-synthase inhibitor in dog kidney. In contrast to the renal artery rings, the effect of Bk was partly reversed by L-NOARG in perfused kidneys. The relaxations produced by Bk were completely abolished in rabbit carotid arteries [17], but they were only partially inhibited by L-NOARG in bovine coronary arteries [13]. These observations show that there may be a significant tissue dependence of the inhibitory effect of L-NOARG on the endothelium-dependent relaxation. In dogs, the renal vasodilation caused by ACh [11], endothelin [25] and the Bk induced suppression of renal arterial vasoconstriction [14] were attenuated or reversed by NO-synthase inhibitor L-arginine analogs.

Both endothelium-dependent mechanisms and Bk are involved in the control of vascular tone and renal functions. Bk is known to induce diuresis, natriuresis and increased renal blood flow in dogs. B_1 and B_2 receptors are present in the vascular tissues of dog renal arteries [10, 18]. The infusion of the B_2 receptor antagonist D-Arg^0,[Hyp^3,D-Phe^7]-Bk selectively inhibited the maximal diuresis and rise in renal blood flow observed when Bk was infused alone [10]. The relaxant effects of Bk were probably mediated by the activation of endothelial B_2 receptors in our experiments, because the effects of Bk both in renal arteries and perfused kidneys were attenuated by

the selective B_2 kinin antagonist HOE-140. In accordance with our observations the effect of Bk was blocked by B_2 receptor antagonists in isolated dog renal arteries [18]. Bk concentration-dependently suppressed the endothelin-1-induced vasoconstriction of isolated dog renal arteries through the activation of endothelial B_2 receptors [14].

The results of our present study provide further evidence for the involvement of endothelial B_2 receptors and NO-dependent mechanisms in the relaxant effect of Bk in isolated dog renal arteries and perfused kidneys. They also indicate a difference in the vasomotor regulation of conduit and resistance vessel in dog's kidney.

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Effect of perinatal triiodothyronine (T3) treatment on thymic glucocorticoid and estrogen receptors and uterus estrogen receptors. Receptor selectivity during hormonal imprinting

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Triiodothyronine (T3) treatment at 1, 3 and 5 days after birth significantly increased the binding capacity (density) of the thymus glucocorticoid receptor of male and female rats in adult age. There was no change in the binding capacity of uterine and thymic estrogen receptors. The experiments point to the sensitivity of developing hormone receptors and call the attention to the possibility of overlap between the members of the steroid receptor superfamily at the perinatal critical period. This overlap is not dependent on the structure of the hormone, however it is dependent on the structure of receptors and acceptors. The difference in the reaction of glucocorticoid and estrogen receptors shows the differences in the sensitivity of receptors to false imprinting.

Keywords: triiodothyronine, thymus, glucocorticoid receptor, rat, estrogen receptor, hormonal imprinting

Cytosol receptors represent a superfamily including receptors of steroid hormones, thyroid hormone, D3 vitamine and retinol (retinoic acid) receptors [13]. PPAR (peroxisoma proliferator activated receptors –PPAR) has been classed among them recently. These receptors have similar structure. After bound to the hormone and separated from the heat-shock protein the receptor-hormone complex enters into the nucleus and looks for the acceptor site [2]. In spite of their similarities, the receptors specifically bind their ligands.

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Receptor maturation takes place perinatally in the presence of the adequate hormones. The primary interaction between the hormones and their receptors (hormonal imprinting) sets the receptor's binding capacity for life [4–6]. In this critical period the absence of the adequate hormone leads to receptor immaturation [9]. Related molecules capable to bind to an immature receptor can result in false imprinting [6–8].

It was found that perinatal treatment with synthetic steroids (diethylstilbestrol, allylestrenol) reduced the binding capacity of the uterus estrogen receptors to less than half in adulthood [10]. Overlap has been manifested between hormones act on different steroid receptors; synthetic sexual steroid (allylestrenol) treatment reduced the binding capacity not only of the uterine estrogen but thymic glucocorticoid receptor's binding capacity, too [15]. Aromatic hydrocarbons – environmental pollutants – after neonatal treatment reduced the binding capacity of both estrogen and glucocorticoid receptors [7, 8].

Various steroid hormones and aromatic hydrocarbons have similar structure and this is why the latter ones can influence steroid receptors in the state of maturation. However there are receptors in the steroid receptor superfamily which bind ligands considerably different from steroid structure. So, it seemed to be reasonable to examine whether triiodothyronine (an amino acid type hormone having a thyronine nucleus) can influence steroid receptor maturation by false imprinting.

Materials and Methods

Forty-four male and 52 female Wistar rats were treated with 1 μg triiodothyronine (T3, Fluka Buchs, Switzerland) freshly dissolved in 0.005 N NaOH in 0.9% NaCl, buffered and diluted to the final concentration with physiological saline on 1st, 3rd an 5th day after birth. 48 male and 56 female control animals received 10 μl solvent only. Cytosolic receptor fractions were prepared from thymi of males at the age of six weeks and at the age of 12 weeks from thymi and uteri of females 9 days after ovariectomy for receptor assays.

Preparation of cytosol fraction

Procedures were performed at ice/water temperature. Tissues examined were homogenized in 10 mM TRIS-HCl buffer containing 1.5 mM EDTA, pH 7.4 (freshly supplemented with 20 mM molybdate and 2 mM dithiotreitol) with a motor driven glass-teflon Potter homogenizer 1,5 ml/g wet wt. Homogenates were centrifuged at 100 000 g for 60 minutes at 4 °C and the supernatants were used for receptor assays. Protein content was estimated by the Coomassie-blue method.

Glucocorticoid receptor assay on thymus cytosol

Saturation analysis with 3H-dexamethasone: 500 μg protein was incubated with 0.625–40 nM ^{3}H-dexamethasone (Amersham, England, spec. act.: TBq/mmole) in duplicates in a total volume of 100 μl at 0 °C for 18 hours. Another set of tubes contained 1 μM unlabelled dexamethasone at each ^{3}H-dexamethasone concentration for non-specific binding.

Competition analysis with dexamethasone acetate: 500 μg protein was incubated with 10 nM ^{3}H-dexamethasone and increasing concentrations of dexamethasone acetate (0–1000 nM, Fluka Buchs Switzerland) at the same temperature and incubation time as in case of saturation analysis. Radioactivity measured in the presence of 1000 nM dexamethasone acetate was regarded as non-specific binding. Bound glucocorticoid was separated by the charcoal method.

Estrogen receptor assay on uterus and thymus cytosol

Saturation analysis with estradiol: 250 μg cytosolic protein of uterus or 500 μg cytosolic protein of thymus were incubated with 0.3–20 nM 2,4,6,7 ^{3}H-estradiol (Izinta, Budapest, Hungary, 3.4 TBq/mmole spec. act.) in duplicates in a total volume of 100 μl at 0 °C for 18 hours and another set of tubes containing 1000 nM unlabelled estradiol (Organon Oss, Holland) at each ^{3}H-estradiol concentration for non-specific binding. Termination of the reaction was identical that of receptor assay on thymus cytosol. Bound radioactivity was counted in Optiphase Hisafe cocktail (Pharmacia) with 47% efficiency in a Beckman counter.

Analysis of results

Analysis of the results were carried out by the computer program EBDA and LIGAND written by McPherson [18, 19]. EBDA was used the process of the raw data. Ligand (non-linear curve fitting program) was used to obtain final parameter estimates. Statistical analysis of the final parameters was calculated by the computer program DATAANALYSIS V.1.0. Statistical and Design Services.

Results and Discussion

The results of our present experiment show that repeated perinatal T3 treatment increased the glucocorticoid receptor's binding capacity in adulthood both in males and females according to saturation (Fig. 1) and competition (Fig. 2) binding studies. It means that the hormone (T3), which has a target belonging to the steroid receptor superfamily, can alter the binding character of the glucocorticoid receptors as it was monitored earlier in the case of structurally different steroid ligands [5, 6].

The alteration owing to false imprinting, however, seems to contrast with that caused by steroids structurally similar to the glucocorticoids [7, 14]. T3 treatment perinatally resulted in increased binding capacity in contrast with the treatment with synthetic steroid like molecules, which resulted in decreased binding capacity in adulthood. Similar observation was demonstrated when the binding capacity of glucocorticoid receptors increased by T3 stimulation in adult Xenopus [16].

No difference was found in the binding capacity of uterus (control Kd: 4.130; Bmax: 1.965; treated Kd: 3.884; Bmax: 1.870 M^{-10}) and thymus estrogen receptors between the control and T3 treated groups of animals. Considering that the glucocorticoid receptors responded to the perinatal T3 treatment (whilst estrogen receptors have not), a difference in the sensitivity of the superfamily's members can be suggested. The results are strengthened by the similar negative behaviour of estrogen receptors independent of their origin.

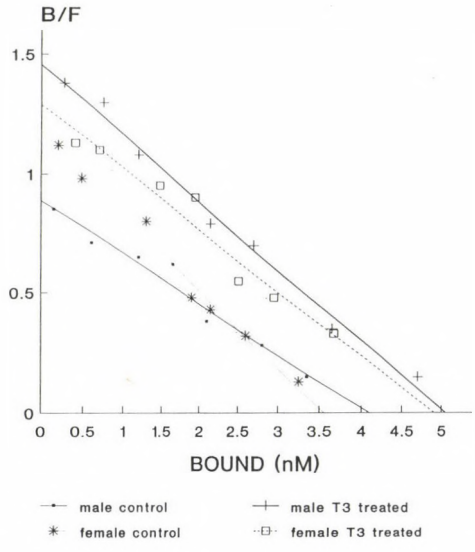

Fig. 1. SCATCHARD PLOTS transformed from the data of displacement study on thymus cytosol with ^{3}H-dexametasone-dexamethasone acetate. Kd and Bmax values are the mean of 4 and 5 independent measurements on control and T3 treated male groups, 5 and 7 independent measurements on control and T3 treated female groups of animals, respectively, calculated from the row data by the program LIGAND (see Mat. and Meth.). MALE: Kdcont.=3.88±0.51 nM; Kdt3=3.29±-0.87 nM. Bmax cont=0.385±-0.067 nM; BmaxT3=0.496±-0.059 nM ($p<0.05$). FEMALE: Kdcont.=2.92±-0.85 nM KdT3=3.17±-0.66 nM. Bmax cont.=0.357±-0.025 nM; BmaxT3=0.456±-0.081 nM ($p<0.05$)

It is worth mentioning that the differences in the effect of T3 on glucocorticoid and estrogen receptors has been demonstrated at the level of acceptors, too. There is a synergism between these receptors at the level of the acceptor, and the cross-talk is expressed in a way, that no response to glucocorticoids in the absence of T3 and T3 receptors [17] occurs. On the contrary, though T3 receptor binds to the estrogen responsive element with a high affinity, it inhibits the estrogen dependent activation [12].

The results of our experiment inspire that the ability of a ligand to bind to a receptor of the superfamily is more important in the development of imprinting, than the structural similarity. This can explain why the T3 could significantly influence the development of the glucocorticoid receptor, however its chemical structure is very different. The ligand specificity of the receptor and maturity in the perinatal critical period seems to be also very important. The lack of effect of T3 on the estrogen receptor can also be explained by the finding that between the T3 and glucocorticoid

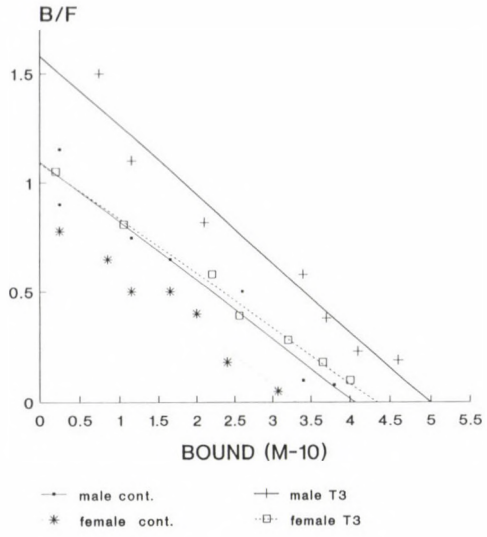

Fig. 2. SCATCHARD PLOTS of the EBDA data of saturation analysis with 3H-dexamethasone on cytosolic fraction of thymus. Kd and Bmax values are the mean of 4 and 5 independent binding assays performed on cytosolic fractions obtained from control and T3 treated male groups of animals, 5 and 7 independent binding assays of cytosolic fractions of female groups of animals, respectively. MALE: Kdcont. $=0.393\pm-0.12$ nM; KdT3 $=0.307\pm0.091$ nM. Bmaxcont. $=0.432\pm-0.042$ nM; BmaxT3 $=0.506\pm-0.061$ nM. ($p<0.05$) FEMALE: kdcont. $=0.346\pm-0.084$ nM; KdT3 $=0.354\pm-0.047$ nM. Bmaxcont. $=0.311\pm-0.073$; BmaxT3 $=0.415\pm-0.049$ nM. ($p<0.05$)

receptors a higher structural similarity exists than between T3 and estrogen receptors, moreover by the possible increased maturity (stability) of estrogen receptors at the time of treatment, in opposite to T3 receptors, the quantity of which increases 40-fold during this hormone sensitive developmental period [1]. However the acceptor level synergism of glucocorticoid and T3 receptors [3, 11] also have to be considered.

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Effect of neonatal treatment with monoclonal antibody to thyrotropin (TSH) receptor on the thyroxin (T_4) level and certain parameters of the internal genital organs of adult rats

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Treatment of newborn rats with a single dose of monoclonal antibody to TSH receptor, caused permanent changes in the body weight, T_4 level, and different sexual parameters. There are considerable sex and organ dependent differences in the sensitivity. Body weight and physical condition of males deteriorates, while the condition of the females is constant. However, T_4 level of females significantly increases while that of the males does not change. The weight of seminal vesicle increases, while ovarian weight decreases. Effect of hCG treatment in adult age is inhibited by neonatal antibody (to TSH receptor) treatment. The experiment calls the attention to the imprinter effect of antibodies in the neonatal critical period, which effect could be manifested on the antibody's own (provoked) or foreign (related) receptor.

Keywords: TSH receptor, gonads, hormonal imprinting, thyroxin, monoclonal antibody, neonatal critical period

Hormone receptors mature parallel with the cell differentiation [15]. For the maturation the presence of the adequate hormone is needed [4]. At the first encounter with the hormone (perinatally) develops the hormonal imprinting, which at the end of receptor development results in a binding capacity characteristic to the adult age [5, 6]. If in the perinatal critical period molecules – different from the adequate hormone but able to bind to the developing receptor – are present in excess, the receptor

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development is biased from the normal one [7, 8]. This used to be manifested in the change (first of all reduction) of the maximal evocable receptor number. However, the effect is measurable in other parameters, too, as altered hormone levels, deviations in behavior etc.

Thyrotropin (TSH) and gonadotropins belong to the same hormone family. Their alpha subunits are identical and the specificity is provided by the difference of some amino acids of the beta chain [2, 19]. Similarly, their receptors are also related [14, 17, 19, 21, 22]. As it was cleared in earlier experiments, neonatal treatment with gonadotropin alters the later thyroxin response to TSH, for life [11]. Neonatal thyrotropin treatment causes a change in the response of adult gonads to chorion gonadotropin (hCG) and an overlap between hCG and TSH develops [9, 10].

Monoclonal antibody to TSH receptor [18] is able to bind to the receptor, like the hormone. This could provoke the hyperfunction of the cell – as in the Graves disease [13] – or on the contrary, by covering the receptor, the failure of the binding of the target hormone by it. These well-known facts conveys the suggestion that the presence of specific antibody in the perinatal critical period can imprint the target cell or inhibit the imprinting (by blocking the receptor sites). Our experiment's aim was to clear the correctness of one or the other possibilities.

Materials and Methods

Eighty male and 80 female rats of our closed Wistar breed were used in the experiments. The animals received at birth (strictly before 24 h) monoclonal antibody to TSH [18] in a dose of 168 μg/newborn rats solved in 0.1 ml saline, subcutaneously under the skin of the neck. Controls got saline only.

Monoclonal antibodies were developed to porcine thyroid cell membrane TSH receptors according to the method of Islam et al. [16] in mice. The monoclonal antibody could bind to the non-reduced antigen 80 KD protein component, which was identical with the TSH receptor [21]. The specificity of the antibody was controlled by RIA, FACS and immunocytochemical methods, too [18].

Groups of the two-month old neonatally treated or untreated animals (this age is the optimal for comparison with previous experiments and the indices are also well measurable in that time) received gonadotropin (hCG, Choriogonin, Richter, Budapest) treatment for three days (twice a day) in a dose of 2.5 IU, dissolved in 0.1 ml saline. Rupture of ovarian follicles (Ascheim–Zondek reaction), weight of uterus and ovary, number of follicles (by stereomicroscopy) and bleeding was studied in females. In males the weight of testes and seminal vesicles were measured. The weight of the animals were determined in both sexes.

In males and females alike thyroxin level of blood serum has been determined by T_4 RIA kit (Izinta, Budapest) without any further treatment and after treatment with 3 IU TSH (Ambinon, Organon, Oss).

Significance has been evaluated by Student's *t*-test.

Results and Discussion

The results of the experiments demonstrated the differences in many parameters caused by a single neonatal treatment with a monoclonal antibody to TSH receptor, related to the control animals. These differences were sex dependent.

The body weight of two-month old male animals decreased about 22%, significantly (Table I). On the contrary, there was no difference in the body weight of the females (Table I). Though this statement is subjective, the male animals seemed to be "looser" related to the controls, while there was no difference in the condition of the females. Considering that the antibody is bound by the TSH receptors of the thyroid gland, the effect could have been uniform in both sexes. Nevertheless it is known that the TSH and gonadotropin receptors are very similar [14, 17, 19, 21, 22] and there are overlaps at the hormone level, too [2, 9, 10]. Thus it cannot be excluded – and later this will be justified – that the TSH antibody exerted its effect also on the gonadotropin receptor. This means that the low body weight and looseness of males could be caused by the lower androgen level and this way – by failure of anabolic effect. There are other experiments which support the susceptibility of androgen level by imprinting [20].

The difference in the physical condition between males and females perhaps could be explained by the change in thyroxin level (differences in metabolism), too. However, there was no difference in males in the T_4 level of control and monoclonal antibody treated animals (Table II). At the same time a 40% significant increase in T_4 levels of females was recorded (Table II). Since this difference in T_4 levels is not manifested in the body weight (body weight of control and treated animals is similar), it seems not to be likely that this factor influenced the "loose" males. However the difference between the reaction of males and females is demonstrated by the T_4 levels, too.

Table I

Body weight values of control and IgG treated animals

	Body weight (g)	
	males	females
Control	290.0±12.65	216.3±4.6
IgG	226.5±12.63*	215.3±4.43

Significance:
* = $p<0.001$ to control

Table II

Organ weight and T_4 values of control and neonatally IgG treated animals, hCG treated or untreated in adult age

	con-con	con-hCG	IgG-con	IgG-hCG
	Weights (g)			
ovary	0.141±	0.169± b	0.110± b	0.177± be
	0.0070	0.0056	0.0073	0.0096
uterus	0.338±	0.298±	0.281±	0.264± a
	0.0298	0.0168	0.0273	0.0187
testis	1.617±	1.702±	1.767±	1.674±
	0.1680	0.1017	0.0856	0.1829
ves. seminalis	0.059±	0.296± b	0.103± a	0.216± cd
	0.0070	0.0549	0.0156	0.0257
	Relative weights (10^{-2})			
ovary/body	0.088±	0.111± b	0.080±	0.113± bd
	0.0039	0.0054	0.0042	0.0054
uterus/body	0.210±	0.197±	0.206±	0.168± af
	0.0173	0.0091	0.0224	0.0116
testis/body	0.961±	1.010±	0.994±	0.936±
	0.0787	0.0732	0.0251	0.0802
ves. sem./body	0.035±	0.183± b	0.0582± a	0.121± cd
	0.0046	0.0397	0.0090	0.0121

Significance:

a = $p<0.05$ to con-con
b = $p<0.01$ to con-con
c = $p<0.001$ to con-con
d = $p<0.01$ to IgG-con
e = $p<0.01$ to IgG-con
f = $p<0.05$ to con-hCG

	con-con	con-TSH	IgG-con	IgG-TSH
	T4 (μg/dl)			
(males)	3.84±	4.72± a	3.86± d	3.63± ae
	0.192	0.325	0.080	0.083
(females)	3.81±	4.21± a	5.24± cd	4.83± bdf
	0.099	0.264	0.360	0.340

Significance:

a = $p<0.05$ to con-con
b = $p<0.01$ to con-con
c = $p<0.001$ to con-con
d = $p<0.05$ to con-TSH
e = $p<0.001$ to con-TSH
f = $p<0.05$ to IgG-con

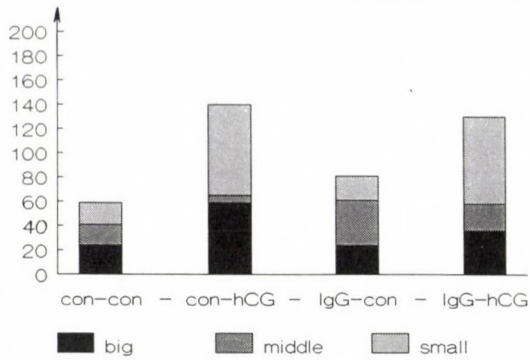

Fig. 1a. Number of follicles. con=control; IgG=anti-TSHR; hCG=chorion gonadotropin

It has to be mentioned that TSH treatment in adult age can elevate T_4 level in neonatally untreated animals, but can not do the same in neonatally antibody treated males. In females a significantly lower T_4 value is observed after TSH treatment in the antibody treated animals, than in the animals treated neonatally with antibody but not treated in adult age at all.

There was no difference in the testicular weight of control and treated animals (Table II), while the weight of seminal vesicle significantly increased in the treated males (Table II). This is not surprising, as the seminal vesicle is more sensitive to hormonal influences than the testis.

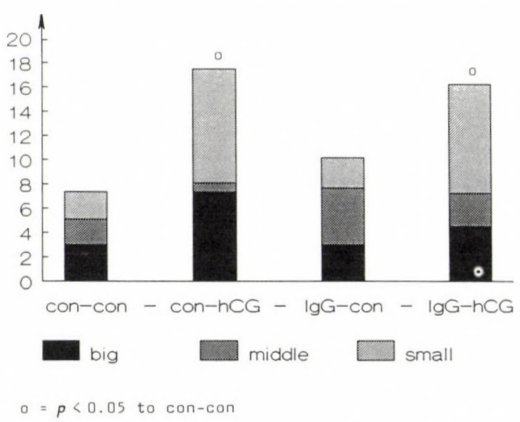

Fig. 1b. Number of follicles/animal. Abbreviations as in Fig. 1a

There was no difference in the weight of the uterus between the control and treated females (Table II). Nevertheless, the weight of the ovaries decreased in the treated group (Table II). However, this observation has to be handled cautiously, as the ovarian weight decrease related to the body weight is not significant.

The hCG treatment in adult age was done for studying the change in receptor sensitivity due to the neonatal treatment. It is remarkable that the weight of the seminal vesicle – which enormously increased in animals which were neonatally untreated but treated in adulthood – is lower in the neonatally antibody treated, hCG treated when adult group (Table II). This seems to be justified even if we related to the body weight. There was also a decrease in the uterine weight of rats which received antibody in neonatal and hCG in adult age compared to the untreated controls or to those treated with hCG in adult age (Table II).

There is an increase in the number of follicles in the group not treated in neonatal age whereas receiving hCG in adulthood (Figs 1a and b). This was hardly inhibited by the neonatal antibody treatment. However in this latter case there is a lower number of large follicles present. There were no heavily bled follicles – in contrast to the control – in the neonatally antibody treated group, but this appeared after (adult) hCG treatment (Fig. 2).

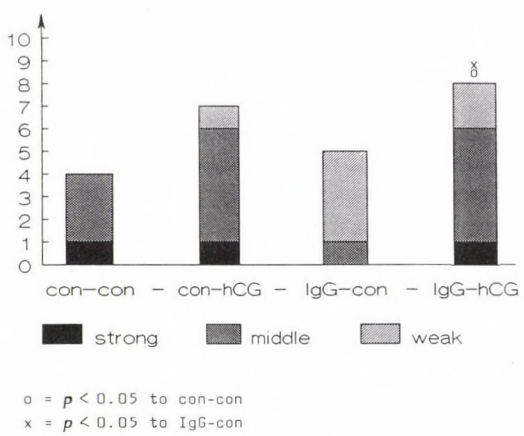

Fig. 2. Follicle bleeding. Abbreviations as in Fig. 1a

Based on our present findings it can be stated:

1. life-long changes are provoked in certain parameters by a single neonatal antibody to TSH receptor treatment. From this point of view the antibody treatment is very similar to the effect of hormones (molecules) causing false imprinting.
2. There is a sex-dependent difference in the reaction to the antibody to TSH receptor. The males' body weight decreases and their physical condition deteriorates, while there are no signs of similar problems in females. The T_4 level is constant in males and significantly higher than control in females.
3. Neonatal treatment with antibody to TSH receptor causes long lasting changes in sexual parameters. This means that the antibody is overlapping on the receptors like the hormones of the same family.

4. Considering that the tendency of the changes is different in different sexes and sexual organs, on the basis of the experiments there is no possibility to establish whether the antibody functioned similarly to hormones or just the opposite: it inhibited the binding of hormones neonatally.

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Alterations in skeletal muscle microcirculation of head-down tilted rats**

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The head-down tilted whole body suspended (HDT/WBS) rat is recognized as a model that reproduces many of the responses seen during exposure to microgravity including an increase in systemic blood pressure. Functional alterations of microscopic blood vessels (arterioles) in skeletal muscle (cremaster muscle) were assessed for their role in the observed elevations of blood pressure associated with HDT/WBS. Arteriolar baseline diameters, vasoconstrictor responses to norepinephrine and vasodilation to nitroprusside were assessed in control rats, rats suspended for 7 or 14 days, and rats allowed to recover for 1 day after 7 days of HDT/WBS. Using *in vivo* videomicroscopy, neither baseline diameters nor ability to dilate were altered by HDT/WBS. Maximum vasoconstriction to norepinephrine was significantly greater in arterioles of hypertensive 14 day HDT/WBS rats. This study of the intact microvasculature of skeletal muscle reveals an elevated contractility of arterioles to norepinephrine in suspended rats, and suggests that an elevated peripheral resistance in skeletal muscle may contribute to the increase in blood pressures among animals subjected to HDT/WBS.

Keywords: weightlessness, head-down tilt, hypertension, rats, cremaster muscle

The head-down tilted whole body suspended (HDT/WBS) rat is widely accepted as a model for responses seen during or following exposure to weightlessness. Many studies using this earth-side model have dealt with muscle and bone responses and have corroborated space flight results. In contrast, cardiovascular adjustments to weightlessness have not received much attention. The few recent studies have often lacked consensus [17, 22, 23, 24, 27].

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Previous work with the HDT/WBS rat disclosed a moderate hypertension, defined as a statistically significant elevation in blood pressure [17, 22]. The suspension induced hypertension could result from increases in cardiac output and/or total peripheral resistance. There has been little systematic investigation of changes in cardiac output or total peripheral resistance to indicate which factor may be more contributory to the development of hypertension. In the suspended rat, Deavers reported a negative water balance [4] which may be responsible for observed reductions in plasma volume [2]. This would be expected to minimize rather than elevate the cardiac output. Measurements of cardiac output have not been performed in suspended rats which developed an elevation in blood pressure. Thus, the possible contribution of elevated cardiac output is not clear.

Data from several studies support the contention that vascular changes occur in the suspended rat, particularly in the hindquarter, which is composed mostly of skeletal muscle. An increase in iliac artery resistance has been observed at 24 h [30] and 72 h [11] of suspension. After 9 days of suspension [22], iliac artery blood flow velocity decreased more among suspended rats than in control rats during submaximal exercise suggesting that the hindquarter vasculature of suspended rats was more constricted by a physiological condition associated with an increase in sympathetic drive. These observations raise the possibility that an increase in skeletal muscle vascular resistance may contribute to an increase in mean arterial blood pressure. An increase in vascular resistance may be due functionally to an increase in vascular responsiveness or due structurally to vascular hypertrophy, or a decline in the number of perfused vessels, e.g. rarefaction. Structural alterations have been observed in the vasculature of the hypertensive rat. Vascular hypertrophy, reflecting a greater vascular smooth muscle content, would be expected to contribute to a greater contractility. Since circulating norepinephrine levels have been shown to be elevated in the suspended rat [29, 31], a functional change in the vascular smooth muscle may be evident in the suspended rat.

However, this is in contrast to conclusions drawn by others [15, 16] that the vascular responsiveness to sympathetic stimulation is reduced in the suspended rat. Our study was designed to investigate functional changes in the small resistance arterioles of skeletal muscle among rats exposed to simulated weightlessness. We used the well-established cremaster muscle preparation for microvascular observations. Previously, functional alterations in early stages of various forms of experimental hypertension have been observed using this preparation [9, 10, 21]. Atrophy of load bearing muscles has been a well studied phenomenon in suspended rats. The cremaster muscle is not a load bearing muscle and is not expected to atrophy with the reduced ambulatory state of suspension. However, functional changes evident in the vasculature may be indicative of generalized changes in the skeletal muscle vascular bed.

In this study, comparisons were made of the basal diameters of resistance arterioles in the cremaster muscle of control (non-suspended) rats and rats which had been suspended for seven days, fourteen days or had been allowed to recover for one day after seven days of suspension. Vivarian caged rats were used as "non-

weightlessness" exposed control rats. Arteriolar reactivity to exogenous norepinephrine was assessed since this vasoconstrictor is known to play an important role in the regulation of blood pressure and skeletal muscle blood flow. Finally, the dilator responses to nitroprusside were assessed as an indicator of the degree of basal tone of the arterioles.

Materials and Methods

Procedures described have been evaluated and approved by the University of Louisville institutional animal care and use committee. Male Sprague-Dawley rats (Charles River, Wilmington MA) weighing 150–170 g were allowed to acclimate to laboratory conditions for at least five days. Systolic blood pressure was estimated by the tail cuff method while the rats were conscious. They were assigned to one of 4 experimental groups: control, seven, or fourteen days HDT/WBS and one day recovery from seven days of suspension.

Whole-body Suspension Procedure

The suspension method has been previously described in detail [20]. Each rat was lightly anaesthetized with pentobarbital (25 mg/kg i.p.), then fitted with a custom tailored whole-body cloth suit and suspended at a head-down tilt of approximately 20° from the horizontal. Free movement of fore- and hindlimbs was possible, but only the forelimbs could be used for body position adjustments. The animal movements included some side to side movement to access water and rat chow *ad libitum* and for purposes of grooming. After seven or fourteen days, each rat was removed from suspension. One day recovery rats were removed from the suspension apparatus after seven days of suspension and returned to a cage for normal ambulatory movement for a period of one day before experimentation. Tail artery pressures were measured immediately after the rats were removed from the suspension apparatus and just prior to the start of the microvasculature experiments. The rats were then anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and placed on a heating pad to maintain rectal temperature at 36.0–37.5 °C. Tracheal cannulation (to maintain airway) and carotid artery cannulation (to monitor blood pressure) were performed.

Microvascular Experiments

The right cremaster muscle was prepared as described previously [9]. The scrotum was opened and the cremaster muscle was incised longitudinally and kept moist during handling with physiological salt solution (PSS) superfusion as needed. The muscle was stretched over a cover slip in a 50 ml plexiglass bath using sutures. The bath was filled with a physiological salt solution (PSS) containing: NaCl, 113 mM; $NaHCO_3$, 25 mM; dextrose, 11.6 mM; KCl, 4.7 mM; $CaCl_2 \cdot 2H_2O$, 2.5 mM; $MgSO_4 \cdot 7H_2O$, 1.2 mM; KH_2PO_4, 1.2 mM; (osmolality: 285 mOSM/kg H_2O). The temperature of the bath PSS was maintained at 34.5 ± 0.5 °C by a feedback system connected to an indwelling heating coil. The bath pH was maintained at 7.40 ± 0.05 by aerating the solution with carbon dioxide.

The rat was then positioned on a microscope stage to visualize the microvessels of the cremaster muscle with transilluminated light. A closed-circuit television system displayed the microvessel images on a video monitor, and a video cassette recorder stored the images for later analysis. The magnification of the image was determined by displaying a stage micrometer scale on the monitor screen. Vessel diameters were then calculated from the measured image using the magnification factor. The experimental protocol was begun after a one hour equilibration period.

Based on branching order and size, the first- (A1), a second- (A2; 60–90 μ), and a third-order arteriole (A3; 10–20 μ) were identified and selected for observation. Only A3 arterioles which subsequently branched and formed capillaries were included.

Experimental Protocol

The procedures, briefly described, are commonly used and details are found in the literature [9, 10]. The average baseline diameter of the selected vessels was obtained, then nitroprusside (10^{-5} M bath concentration) was added to the bath to cause dilation. The arteriole diameters were measured after 10 minutes of exposure. The solution was drained from the bath and the muscle washed several times with drug-free PSS to remove the nitroprusside.

After a thirty minute equilibration period, new baseline diameters were measured, then norepinephrine was added to the bath. The diameters, when the arterioles were maximally constricted, were determined from responses to multiple bath concentrations ranging between 10^{-7} M and 3×10^{-5} M. The bath solution was drained, and the muscle was washed several times with drug-free PSS.

Drug Preparation

Drugs were freshly prepared for use on a daily basis. Norepinephrine (Sigma) was prepared in a saline solution containing ascorbic acid solution (1 mg/mg) to retard oxidative degradation. Nitroprusside (Malinkrodt) was prepared in 0.9% saline.

Statistical Analysis

Analysis of variance (ANOVA) was used to compare baseline diameters, minimum and maximum diameters after nitroprusside and norepinephrine, respectively, between the four experimental groups. When statistical significance was noted, Student's *t*-tests were employed. With all tests, a probability of less than 5% ($p < 0.05$) was considered statistically significant. Data are presented as mean $\pm$ standard error of the mean (SEM), and statistically significant differences are indicated with an asterisk.

Results

Table I shows mean blood pressure measurements for each experimental group. Significant increases in tail artery blood pressure were observed among the 14 day suspended and 1 day recovery groups (post vs presuspension, and suspended vs control). Mean arterial pressures measured by direct arterial cannulation following pentobarbital anaesthesia were also elevated among the 14 day and 1 day recovery animals.

The resting baseline diameters of the A1, A2 and A3 arterioles of the 3 experimental groups did not differ significantly from those of the control (non-suspended) group (Fig. 1). Norepinephrine significantly constricted the A1, A2, and A3 arterioles of all 4 experimental groups ($p < 0.05$). The A1 arterioles of the 14 day suspended rats contracted to a significantly ($p < 0.05$) smaller diameter than those of control non-suspended rats in response to norepinephrine. No differences in A2 and A3 arteriole constriction to norepinephrine were noted between the groups. Nitroprusside dilated the A3 arterioles from the non-suspended control; seven day and fourteen day suspended animals to comparable values, but did not dilate those A3 arterioles of the one day recovery rat group (NS). Nitroprusside did not significantly dilate any A1 and A2 arterioles (Fig. 2).

Table I

Rat body weights and blood pressures

	Weight (g)	Tail artery B.P. (mm Hg)		
		MAP	pre-HDT	post HDT
Control	166±4 (12)	103±4 (11)	-	89±4 (7)
7d HDT/WBS	156±3 (9)	116±8 (8)	94±6 (4)	108±7* (3)
14d HDT/WBS	165±6 (9)	134±4* (9)	95±6 (8)	113±5$^{\alpha}$* (8)
1d Recovery	157±3 (9)	129±9* (9)	92±5 (6)	112±6$^{\alpha}$* (6)

Mean arterial blood pressure (MAP) of anaesthetized rats at time of microvascular measurements, and tail artery blood pressure (B.P.) among conscious rats, before and after whole body suspension. Data are expressed as mean ± SEM. (*) denotes statistically significant ($p<0.05$) differences from corresponding values from the control group, (α) denotes statistically significant ($p<0.05$) differences from presuspension measurements

Discussion

Alterations in body fluid and/or cardiovascular variables have been reported in experimental models of weightlessness [2, 17, 22, 30, 31]. Musacchia and colleagues reported an elevated mean arterial blood pressure in conscious HDT/WBS rats as early as 3 days, which remained significantly elevated throughout seven days [17, 19]. Similarly, a modest but significantly elevated mean arterial blood pressure in conscious rats 9 days after suspension has been reported [22]. In contrast, no change in mean arterial blood pressure was noted among conscious rats [24] or anaesthetized rats [27] after seven days of suspension. Likewise, others [2] using a different suspension model (the tail suspended rat), have reported no change in mean arterial blood pressure after seven days of suspension.

We considered the possibility that the development of hypertension among suspended animals was time-dependent, requiring more than seven days for consistent manifestation. In this study the suspension period among one group of rats was extended. While conscious, a significant elevation of tail artery blood pressures was observed after seven days of suspension. Following anaesthetization, only a trend for increased mean arterial blood pressure (direct cannulation) was found among these same rats. It is possible that anaesthesia normalized blood pressure among the suspended rats as has been shown for other hypertensive rats [28]. After 14 days of suspension, both conscious tail artery pressure and anaesthetized mean arterial blood

pressure were significantly elevated indicating that this period of suspension evoked overt cardiovascular changes evident after removal from suspension, which were not masked by induction of anaesthesia.

Our direct observations of the intact cremaster muscle microvasculature revealed that the resting diameters of the A1 and A2 arterioles did not significantly differ across groups. Conclusions cannot be drawn regarding A3 diameters since A3 arterioles of comparable resting diameters were selected from each muscle to assess vasoconstrictor-dilator responses. Unchanged [12] but also reduced [10, 21] baseline arteriolar diameters have been previously reported in hypertensive rat cremaster microcirculation. Thus, reduced baseline arteriolar diameters in the skeletal muscle microvasculature is not a feature of every form of hypertension studied. The etiology and duration of the hypertension may determine whether changes in resting arteriolar diameter are observed. With modest hypertension, such as that which we have detected in the whole body suspended rats, changes in resting arteriolar tone may have been masked by the dilatory effect of anaesthesia [13]. Alternatively, since resting blood pressure was elevated despite unchanged basal diameters in suspended rats, the hypertension observed in the suspended animals may be due to an elevation of resistance in vascular beds other than skeletal muscle. It is also appropriate to consider that although the diameters are not different under anaesthetized resting conditions, the microvessels of suspended rats may exhibit a greater constriction than control animals under sympathetic stimulation resulting in a more elevated blood pressure among the suspended rats. This concept was suggested by previous data [22]. Measurements of hindquarter blood flow velocity during exercise [23] suggest that hindquarter blood vessels of suspended rats constrict more than those in non-suspended rats. Thus, we reasoned it appropriate to assess arteriolar reactivity to an endogenous vasoconstrictor.

Since significantly elevated basal plasma norepinephrine levels have been reported after seven [31, 32] and fourteen days of suspension [32], an enhanced arteriolar contractility (i.e. maximal vasoconstriction) to norepinephrine could then lead to an increased vascular resistance and systemic hypertension. Furthermore, since skeletal muscle blood flow and cremaster muscle arterioles are particularly sensitive to norepinephrine, we assessed arteriolar responses to this endogenous agonist.

In seven day suspended rats, which showed only a tendency towards increased mean arterial blood pressure, the A2 arteriole showed only a tendency towards a heightened contractility to norepinephrine (Fig. 1). While in the fourteen day suspended rats, which were significantly hypertensive, the A1 arteriole contractility was significantly elevated. Similarly, enhanced norepinephrine contractile responses have been demonstrated in the cheek pouch of renovascular hypertensive hamsters [3] and in the cremaster A3 of one-kidney one-clip hypertensive rats [9, 10]. Enhanced contractility to norepinephrine appears to be common to an early developmental stage of suspension-induced hypertension as has been observed in other forms of experimental hypertension.

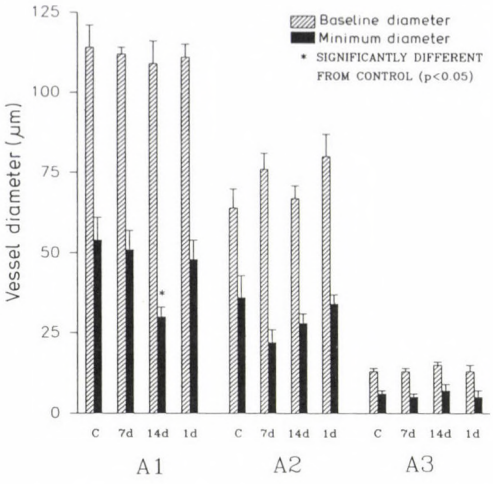

Fig. 1. Resting baseline and minimum (maximum norepinephrine response) diameters of first (A1), second (A2) and third order (A3) arterioles in control (C), 7 day HDT/WBS (7d), 14 day HDT/WBS (14d) and 1 day recovery from 7 days HDT/WBS (1d). Data are expressed as mean ± SEM. Statistically significance ($p<0.05$) is denoted by (*)

The mechanism of this enhanced arteriolar contractility was not addressed in this work. There has been substantial work using various forms of experimental hypertension in rats that has yielded observations suggestive of contributing mechanisms. These tend to be either functional or structural in type. Functional

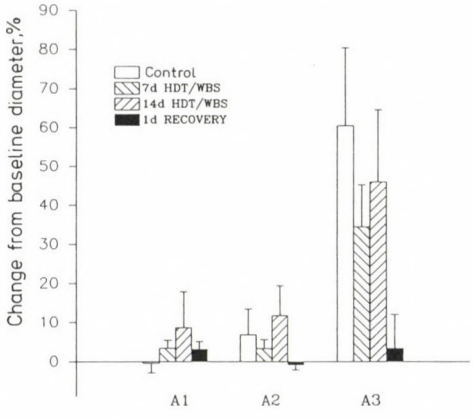

Fig. 2. The dilation of first (A1), second (A2) and third order (A3) arterioles to topical nitroprusside (10^{-5} M). The data are expressed as percent change in diameter accomplished by topical nitroprusside stimulation (Mean ± SEM)

changes of the vascular smooth muscle generally include receptor and post-receptor mechanisms. Observations have included heightened production and release of the vasoconstricting neurotransmitter [7], increased vasoconstrictor sensitivity [33] and alterations in intracellular second messengers [1], increased receptor stimulated calcium influx [14] and increased calcium sensitivity of the vascular smooth muscle [26]. Structural alterations that have been observed in hypertensive rats have included vascular hypertrophy [25] and vessel rarefaction [12]. Recently, cremaster microvessel rarefaction has been reported after three days of suspension [6]. This may provide some insight into increases in resistance and rather than to observations of hyperresponsiveness. Thus there are a number of possible mechanisms that may be contributing to the observation of enhanced arteriolar contractility. In addition, the observation of adrenal hypertrophy in the suspended rat [18] suggests that stress may also be a contributing component to responses observed in this model.

A3 arteriolar dilation to nitroprusside (Fig. 2) revealed comparable maximum diameters for A3 arterioles among all groups. These data indicate that the degree of basal tone (difference between maximum diameter and resting diameter) was not different between groups. Furthermore, A3 dilation dispelled any notion that structural changes had occurred in the walls of the arterioles to limit the dilator and/or constrictor capacities. Such structural changes leading to impaired small arteriolar dilation have been reported to occur within one week following the development of diabetes [8] but, is usually not manifested until much later in the development of hypertension [21].

In conclusion, head-down tilted whole-body suspension induces a modest elevation of arterial blood pressure that becomes significant after fourteen days. At this time, the large arterioles of the cremaster microcirculation exhibit an enhanced contractility to exogenous norepinephrine. It is unknown whether similar changes take place throughout all skeletal muscle vasculature or in other vascular beds. But our observations suggest the possibility that total peripheral resistance may be increased during episodes of sympathetic stimulation and lead to an increase in mean arterial blood pressure among suspended rats.

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Autologous connective tissue chamber as a tool for introducing active substances into the CNS

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A new method of introducing active substances into the CNS is described. The autologous connective tissue chambers were obtained by implantation of a silicone tube under the back skin of rats. Subsequently they were filled with fibrine and additionally with NGF or submicrosomal fractions from nonpredegenerated and predegenerated peripheral nerves. Filled chambers were implanted stereotaxically into the injured hippocampus. The neurite outgrowth was examined by means of FITC-HRP and acetylcholinesterase-method. Implanted connective tissue chambers are very useful in getting active substances into the CNS. This method allows to avoid inflammatory processes and does not hinder the histological procedures.

Keywords: autologous connective tissue chambers, hippocampus, regeneration, submicrosomal fractions

The inability of axons to grow across damaged central nervous system (CNS) tissue is a well-known consequence of injury to the brain and spinal cord of adult mammals. The cause of axonal regeneration failure in the mammalian CNS is probably multifactorial. Recently it was made clear that some adult CNS neurons may regenerate when appropriate environmental conditions are created. It is, for instance, possible to induce regrowth of injured axons into grafts prepared from peripheral nerves [3].

A variety of chemicals have been tested in order to recognize factors enhancing the regrowth of central axons. Among these substances are hormones [6, 23, 26, 30, 31], extracellular matrix components [8, 10, 33], different tissue extracts [7] and neurotrophic factors [16, 22]. The effects of growth factors on neurite outgrowth and their ability to prevent neuronal death after mechanical injury suggest that they may be useful in promoting regeneration [34]. The same applies to different tissue extracts containing known or undefined neurotrophic substances. One of the main problems in

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using NGF and other growth factors is that they do not pass the blood-brain barrier, and so they have to be delivered in the neighbourhood of the target cells in the CNS. For this reason practical difficulties of and limitations on the administration and delivery of such molecules must be overcome.

The best so far characterized neurotrophic molecule NGF, is often administered intraventricularly by means of miniosmotic pumps [4, 11, 36, 39, 41]. Recently, other methods of its administration were developed, such as releasing from polymer matrix [13] or hollow polymer fibres [35], placement into the wound cavity NGF-soaked gel foam pieces [27] and implantation of genetically modified cells secreting NGF [9]. A more complicated task is to find an adequate delivery system for intracerebral administration of tissue extracts. Earlier they were administered intraarterially [21] or injected directly into the wound [14, 32]. Concentrated protein solutions were also incorporated into polyacrylamide gels, sodium alginate gels or gelatin, and placed in different CNS regions [7, 28].

Regeneration in the peripheral nervous system was also examined and analysed by means of different types of synthetic guidance channels e.g. silicon permeable [15] and inpermeable chambers [2, 29, 38, 40], semipermeable polyvinylchloride acrylic copolymer tubes [1, 19, 37]. Similar materials were used in experiments concerning the role played by different factors in the promotion of the regeneration of injured central axons [17, 20].

In 1979 Lundborg and Hanson introduced the use of preformed autologous connective tissue chambers for the study of peripheral nerve regeneration [25]. These tube-shaped chambers were called pseudosynovial or mesothelial chambers [24]. On the basis of the mentioned papers we tried to elaborate a method for preparation of autologous connective tissue chambers as well as a way of implanting them into the CNS. The purpose of this experiments was to ascertain whether this technique could be suitable for the long-term administration of tissue extracts or growth factors into the CNS.

Materials and Methods

Preparation of autologous connective tissue chambers

Experiments were carried out on 40 male adult Wistar rats. Animals were anaesthetized intraperitoneally with chloral hydrate (420 mg/kg b.w.).

Silicone tubes of 10 mm length and 2 mm in diameter were implanted for 4 weeks subcutaneously on the back of the animals (Fig. 1). During this period a connective tissue layer grows around the tube, forming a tube-shaped connective tissue chamber. After 4 weeks the animals were reanaesthetized, the silicone tubes were dissected together with the surrounding connective tissue (Fig. 2a) and placed in sterile cold Ringer's solution for mammals. Subsequently one end of the chamber was cut and the connective tissue was pulled down the silicone tube. In this way the chamber was prepared for the filling (Fig. 2b–c).

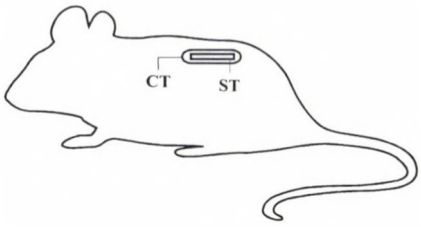

Fig. 1. Schematic diagram illustrating the silicone tube implantation. CT – connective tissue, ST – silicone tube

Filling of the chamber

Williams et al. [40] have revealed that the presence within the silicone chamber of acellular fibrin matrices prepared from dialysed plasma is essential for successful regeneration in peripheral nerves. Knoops et al. [18] had demonstrated that the same applies to the adult rat CNS. They obtained a fibrine-fibronectine-containing matrix by linking the dissected sciatic nerve ends with a piece of semipermeable polysulfone tube. After the lapse of 7 days this tube was filled with fibrine-fibronectine-containing matrix exudated from the nerve stumps. We filled our chambers with fibrine derived from rat blood. For purification of fibrinogen we employed the method described by Blombäck and Blombäck [5]. This method allows to obtain plasma extracts containing 80% of fibrinogen.

In our experiments we applied the following active substances: Purified extracts (submicrosomal fractions) from distal stumps of predegenerated and non-predegenerated rats' sciatic nerves and NGF. The connective tissue chambers were filled through the free end of the tube (Fig. 2d), with a mixture of 20 μl of nerve extract or 20 μl of NGF, 20 μl of fibrinogen solution and 5 μl of thrombine (Immuno AG, Wien) (Table I). To get the same volume of all chambers, 20 μl of buffer was added to chambers containing fibrin only. It was the same buffer with which the submicrosomal fractions were diluted. Few minutes after addition of thrombine the content changed its consistence from liquid to jelly, i.e. the matrix was formed. Subsequently this "sausage-like" formation was implanted into the CNS.

Table I

Contents of the implanted connective tissue chambers. PNE – purified nerve extract

	I Fibrin only	II Fibrin + NGF	III Fibrin + PNE
PNE or NGF	----	20 μl	20 μl
Fibrinogen	20 μl	20 μl	20 μl
Thrombine	5 μl	5 μl	5 μl

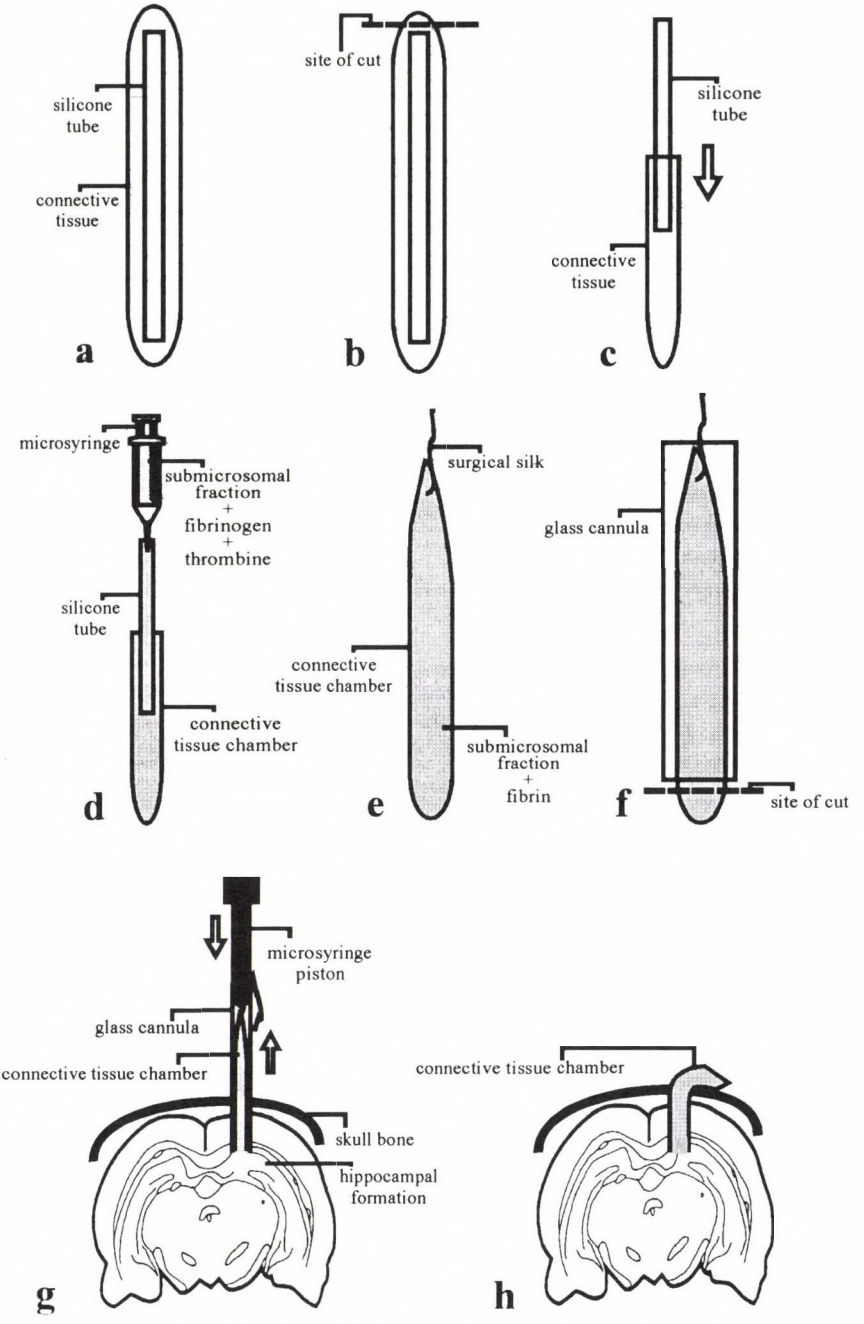

Fig. 2. Filling and implantation of connective tissue chambers. For details see text

Implantation of chamber into the hippocampus

For further experimentation the hippocampal formation was used and this for reasons as follows. First, it belongs to the structures of CNS which easily gives off fibres to implanted peripheral nerve grafts. Second, its characteristic structure allows a quick histological identification. And it is also important that the rodents' hippocampus is relatively big, what makes surgical procedure easier.

After dissection of chambers animals were placed in a stereotaxic apparatus. After opening the skull integument a hole was bored at a stereotaxically settled point (3 mm caudally from bregma, 3 mm laterally). Afterwards the brain tissue was injured stereotaxically by means of a knife for paracentesis (1.5 mm width, 10 mm length), 3 mm in depth (this is the distance necessary to reach the hippocampus). One end of the chamber was tied with surgical silk (Fig. 2e), and by pulling the thread the filled connective tissue chamber was drawn into a glass cannula (Fig. 2f). Its free end was cut to allow contact between the content of the chamber and the injured hippocampal tissue. The cannula was fastened in a stereotaxic apparatus and subsequently introduced into the trepanation foramen. When in place the chamber was thrust out by means of a microsyringe piston (Fig. 2g). Subsequently the glass cannula was carefully slipped off. While slipping of the cannula the chamber was held with the help of the mentioned piston to avoid its pushing out by the brain tissue pressure. The chamber's length was about 10 mm, and we fixed its protruding end to the skull bone with a two-component fibrine tissue glue Tissucol Kit (Immuno AG Wien) (Fig. 2h).

Histology

Six weeks following surgery the animals were perfused transcardially with 5% sucrose in phosphate buffer and subsequently with fixative solution in the same buffer. Whole grafted brains were dissected, 10 mm thick frontal frozen section were made (Cryotom 620, Anglia Scientific) and subjected to three different histological procedures. In order to verify the anatomical position of the graft, hematoxylin-eosin staining was used. To show the origin of central fibres growing into the chamber we decided to use the FITC-HRP (horseradish peroxidase conjugated with fluoresceine isothiocyanate) retrograde tracing method. Twenty-four hours before perfusion 10 μl of 0.4% FITC-HRP solution was injected into the extracranial end of the connective tissue chamber. These sections were examined in the fluorescence microscope (Labophot 2, Nikon) at 480 nm wavelength. The number of traced cells was considered as an exponent of the neurotrophic activity of the applied factors. In order to verify the presence of growing fibres inside the chamber the acetylcholinesterase staining method of Hedreen was used [12].

Results

Light-microscopic examination of H–E stained slices showed the proper anatomical position of the graft and its satisfactory fastening in the brain tissue (Fig. 3).

Fluorescence microscope examination revealed that FITC-HRP-positive cells were present in the hippocampal tissue of all examined brains. Traced cells were seen in all cases in the neighbourhood of the graft's tip. However, their number was different according to the content of the chambers filling. The lowest number of labelled cells was observed in slices derived from brains treated with chambers filled with fibrine alone (Fig. 4).

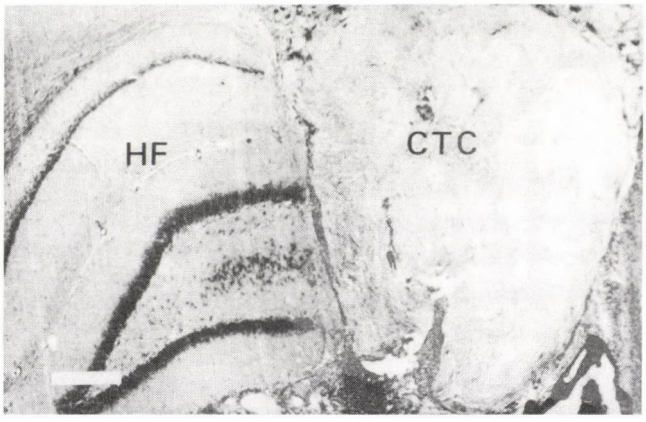

Fig. 3. Frontal section of the rat's brain from the site of implantation. CTC – connective tissue chamber, HF – hippocampal formation. H-E – staining. Bar=200 μm

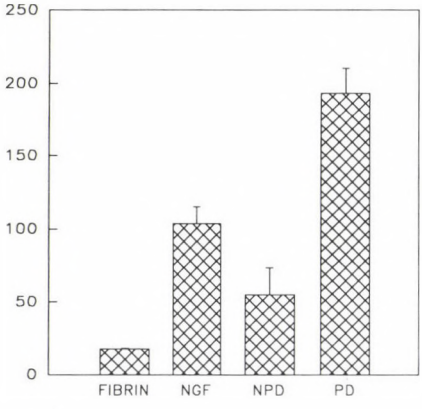

Fig. 4. Histogram showing the number of traced cells according to the implanted substance. FIBRIN – chambers filled with fibrin, NGF – chambers filled with nerve growth factor, NPD – chambers filled with fraction from nonpredegenerated nerves, PD – chambers filled with fractions from predegenerated nerves

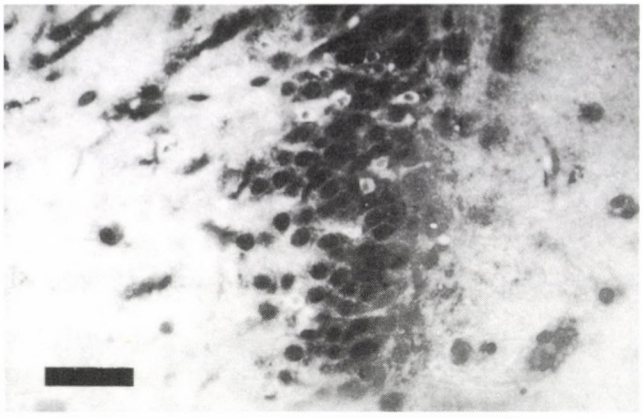

Fig. 5. FITC-HRP-positive cells in the hippocampus. Chamber filled with fraction from nonpredegenerated nerves. Bar=40 μm

In groups grafted with chambers containing NGF or purified peripheral nerve extracts, the number of traced cells was significantly higher (Figs 5, 6). In brains subjected to the method of Hedreen, cholinergic fibres were present in all implanted chambers (Fig. 7).

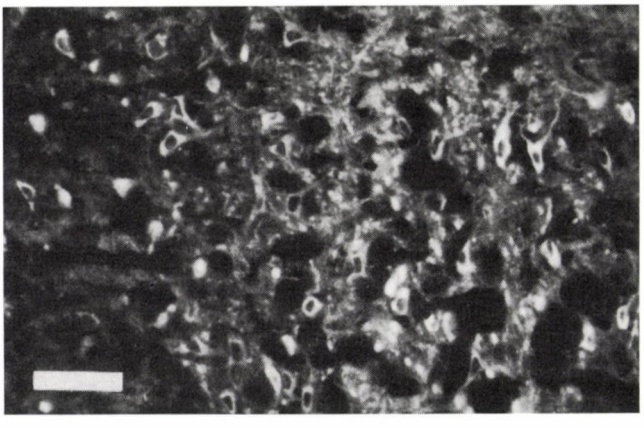

Fig. 6. FITC-HRP-positive cells in the hippocampus. Chamber filled with fraction from predegenerated nerves. Bar=40 μm

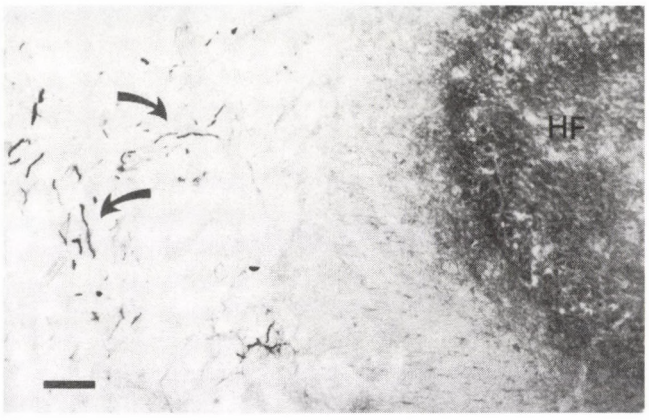

Fig. 7. AChE-positive fibres inside the connective tissue chamber (arrows). HF – hippocampal formation. Bar = 100 μm

Discussion

On the basis of the obtained results we can state, that the described method is a useful delivery system of long term application of active substances into the CNS. Autologous connective tissue chambers posses some advantages recommending their employment in the CNS. We did not notice any signs of an inflammation or unexpected traumatization of brain tissue. The employed connective tissue formation is very soft and elastic and that makes possible the use of relatively long chambers and their easy placement under the scalp. The soft consistence does not hinder histological procedures, so frozen as well as paraffin sections of whole grafted brains can be easily made. The way of obtaining as well as filling of these chambers is relatively simple, and the device for chamber implantation is not complicated and is feasible in most laboratories. However a few practical remarks should be added. After dissection of the tube together with the surrounding chamber, the excess of tissue should be removed very carefully to avoid the chamber's perforation. While filling the chamber, it is important to take care in order that the chamber does not slip down from the tube. The best protection is to hold the chamber on the tube by means of a pair of forceps. To avoid the pushing back of the chamber by the pressure of the brain tissue, the microsyringe piston should stay for a few minutes in the cannula after insertion of the chamber into the hippocampus. Experiments carried out by Lundborg (in PNS) and Knoops (in CNS) showed, that the presence of fibrin matrix is necessary for successful outgrowth of axons through the chamber [17, 25]. However they did not fill the chambers with fibrin before implantation. In their experiments the ends of the transected nerve were intubulated into the chamber and the fibrin matrix was formed from the endoneurial fluid exudating from the nerve ends into the chamber. The

described way of chamber filling before implantation creates new possibilities for the addition of various factors into the inner environment of the implant. In our experiment we used NGF and submicrosomal fractions, but other substances can be applied as well. Additional advantage of this method is the smaller traumatization of animals. Obtaining of fibrin in the previously mentioned way would require an additional transection of the peripheral nerve in the same animal that is donor and host of the autologous chamber.

In experiments concerning regeneration of CNS neurites a control group with chambers filled with fibrine only is required. The fibrin which formes a "path" for regrowing neurites, exerts neurotrophic activity that could intensify the activity of the applied substances [18]. The same applies to the collagen present in the connective tissue layer forming the chamber. Some results indicate the inductive role of collagen for the outgrowth of the neurits, especially that of types I, III and IV [37]. Using gel grains as reservoirs of diffusible proteins requires high concentrations of active substance in a little amount of gel [7]. The proposed method allows the use of relatively big amounts of a solution which has not to be of high concentration and the exposure of injured neurites to the applied substances.

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Solcoseryl^{R} improves learning and memory in rats

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Our previous experiments have shown that Solcoseryl (S), a protein-free extract of calves' blood stimulates locomotor activity and decreases haloperidol catalepsy in rats. In this study the influence of S on acquisition, consolidation, and recall of both, conditioned avoidance responses (CARs) and passive avoidance behaviour was tested. S at the intraperitoneal (i.p.) dose of 1.25 ml/kg significantly improved acquisition and at the dose of 1.0 ml/kg recall of CARs. In the passive avoidance situation the significant effect on acquisition and recall of information was observed after i.p. injection of 1.0 ml/kg of S, and on consolidation after 0.75 ml/kg. These data indicate that S may positively affect the CNS processes responsible for learning and memory.

Keywords: Solcoseryl, learning, memory, rats

In a parallel study [4] we have shown that Solcoseryl (S), a protein-free extract of calves' blood containing low molecular weight peptides and nucleic acids derivatives [17] stimulates psychomotor activity of rats.

Considerable body of evidence indicates that this drug may possess the nootropic activity. According to Gabryel and Trzeciak [11] and Mondadori [26], the nootropic drug should favour optimal metabolism in brain cells, increase cerebral oxygen and glucose metabolism, and increase blood supply selectively to ischaemic areas. Clinical observations indicate that S markedly improves the mental capabilities of elderly [31], and retarded [20] patients. Lanner and Argyropoulos [21] have demonstrated that S facilitates recovery of patients after craniocerebral trauma and operations for benign brain tumors and for the restoration of the cerebral circulation. These positive effects are probably dependent on its influence on the uptake of oxygen and transport and utilisation of glucose in the central nervous system (CNS) [18, 21, 25, 36]. These

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observations suggest that S, just like other nootropic drugs [9, 13, 30, 35] may influence learning and memory.

The purpose of this study was to examine the effect of S on cognitive behaviour i.e. acquisition, consolidation, and retrieval of information in active and passive avoidance situations in rats.

Materials and methods

Animals

White male Wistar rats weighing 180–200 g were used. The animals were kept under standard laboratory conditions (light on from 7 a.m. to 7 p.m., commercial rodent food and tap water freely accessible).

Conditioned avoidance responses (CARs)

CARs were studied using shuttle box described in detail by Forster and Skinner [10]. Conditioned stimulus was 85 dB buzzer and unconditioned stimulus 1 sec, 1 mA scrambled footshock. The number of positive (+) conditioned responses was recorded every day and expressed as per cent of total number of trials. CARs acquisition training consisted of 7 consecutive daily 20 trial sessions. The extinction procedure was essentially the same: the animals were trained over 7 consecutive days and tested for extinction 7 days later in the same manner except for the unconditioned stimulus not being applied.

Passive avoidance performance

Passive avoidance performance was studied in a one trial, step-through passive avoidance situation [1]. The apparatus consisted of an illuminated platform attached to the large dark compartment. The subjects were placed on the platform and were allowed to enter the (naturally preferred) dark compartment. Two more trials were given on the following day. At the end of the second trial an inescapable electric footshock (0.5 mA for 3 sec) was delivered through the grid floor of the dark compartment. Retention of the passive avoidance was tested 24 h later by measuring the latency to re-enter the dark compartment up to a maximum of 300 sec.

Drugs

Solcoseryl (Solco, Basle, Switzerland) was given intraperitoneally (i.p.) as a water solution the osmolality of which was 300 mOsm i.e. equal to the osmolality of the physiological saline. The drug was given once in one of the 3 doses (0.75, 1.0, and 1.25 ml/kg) which had been established as centrally active in the previous study [4]. The saline was used to inject control animals. If one of the extreme doses was found to be effective (0.75 or 1.25 ml/kg) lower or higher doses of S were also tested, respectively. S was given 60 min before the learning session and testing the animals when acquisition and recall were examined, respectively, and immediately after learning trial for testing consolidation. The pre-trial administration of the drug was designed to affect the acquisition of information, while post-trial injection was to influence consolidation [23]. Administration of the drug before testing the retention was designed to affect recall.

Statistical analysis

Statistical comparisons were made by the Student's *t*-test.

Results

Effect of S on learning and memory of CARs

S at the dose of 1.25 ml/kg given i.p. once on Day 1, 60 min before the learning session, markedly enhanced acquisition of CARs (Fig. 1). The effect was significant from the third day of observation ($p < 0.05$ vs control) and persisted to the seventh day ($p < 0.02$ vs control). The higher dose of S (1.5 ml/kg) also stimulated acquisition of CARs, but this effect did not reach the level of statistical significance. The doses of 0.75 and 1.0 ml/kg of S were essentially ineffective. When the influence of S on the consolidation of CARs was examined, the animals were trained for 7 days, subsequently treated i.p. with 0.75, 1.0 or 1.25 ml/kg of S, and than tested 7 days after the drug administration. No effect of S on the consolidation of learned responses was observed (Fig. 2). Nevertheless S at the dose of 1.0 ml/kg given 7 days after the last learning session, 60 min before testing the animals, significantly improved recall of the avoidance responses, whereas the doses of 0.75 and 1.25 ml/kg of S were ineffective (Fig. 3). The animals injected with 1.25 mg/kg of S performed better than the controls, but the effect was no significant.

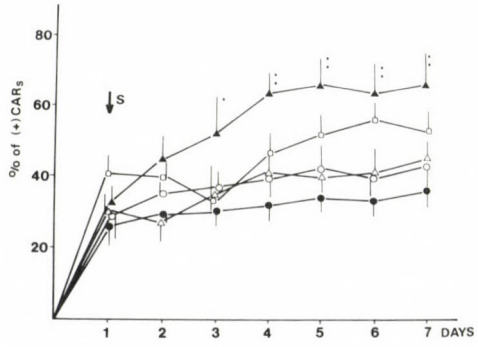

Fig. 1. Effect of Solcoseryl (S) 0.75 •, 1.0 Δ, 1.25 Δ, 1.5 □, ml/kg given i.p. 60 min before the first learning session on Day 1, on the acquisition of conditioned avoidance responses (+) (CARs) during the subsequent 7 days. Control (O) animals received 0.9% NaCl i.p. Points represent means ±SEM of the values obtained from 8 control, and 8, 9, 8, and 10 experimental subjects, respectively, *$p < 0.05$, **$p < 0.02$ vs control rats group (Student's *t* test)

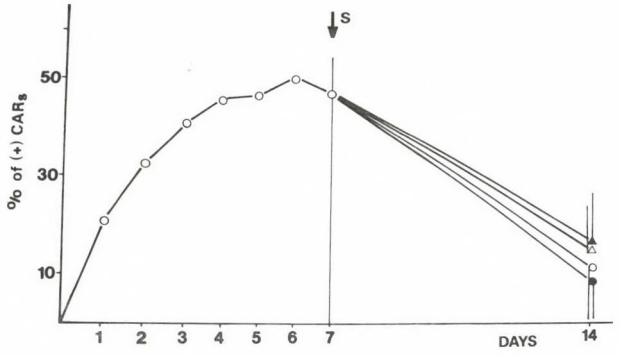

Fig. 2. Effect of Solcoseryl (S) 0.75 •, 1.0 Δ, 1.25 Δ ml/kg given i.p. immediately after the last learning session on Day 7, on the consolidation of conditioned avoidance responses (+) (CARs) tested 7 days later. Control (O) animals received i.p. 0.9% NaCl. Points represent means ±SEM of the values obtained from 8 control and 9, 8 and 10 experimental rats, respectively

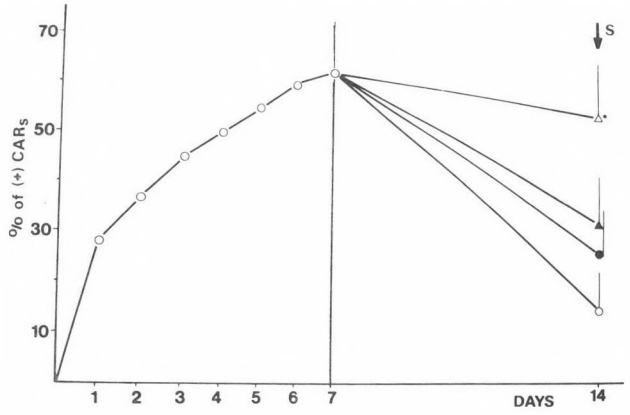

Fig. 3. Effect of Solcoseryl (S) 0.75 •, 1.0 Δ, 1.25 Δ ml/kg, given i.p. 60 min before testing the retrieval of conditioned avoidance responses (+) (CARs) 7 days after the last learning session. Control (O) animals received 0.9% NaCl i.p. Points represent means ±SEM of the values obtained from 8 control and 8, 9 and 10 experimental rats, respectively. *$p<0.05$ vs control group (Student's *t*-test)

Effect of S on learning and memory in passive avoidance situation

In the passive avoidance situation S at the dose of 1.0 ml/kg, given 60 min before learning trial (Fig. 4A), or 60 min before testing the latency to re-enter the dark compartment (Fig. 4C) significantly ($p<0.05$ and $p<0.01$ vs control, respectively) prolonged the time spent by rats on illuminated platform. In both cases the animals injected with 0.75 or 1.25 ml/kg of S stayed longer on the platform than control

animals, but these effects were insignificant. When S was given immediately after learning trial, injected animals also remembered better the unpleasant footshock experienced in the dark compartment, and stayed longer on the platform in comparison to the controls, but this effect was significant ($p<0.01$ vs control) only in animals injected with 0.75 ml/kg of S (Fig. 4B).

Discussion

Our experiments have shown that S similarly to the typical nootropic drugs [9, 13, 14, 29, 30, 33, 35] facilitates certain brain functions. In our study S facilitated learning and memory of both passive and active avoidance behaviours. To explain these effects a number of possibilities could be considered.

It is well established that S promotes oxidative metabolism of the cell. Krüger and Quadbeck [19] have shown in the laboratory animals and Scholing and Clausen [31] in patients with CNS disorders that S improves tolerance to hypoxia. The influence of S on the uptake of oxygen and transport and utilization of glucose was confirmed by the numerous experiments with isolated organs performed by Jaeger et al. [18], Lanner and Argyropoulus [21], and Sugiyama et al. [34]. Yamasaki et al. [36] studied the effect of S on local cerebral glucose utilization in spontaneously hypertensive Wistar rats with a tendency to stroke and observed a distinct improvement of the initially lowered glucose utilization. Gold et al. [15] and Messier and White [24] have demonstrated in passive avoidance responding, respectively, that post-trial, non-contingent injections of glucose solution could retroactively improve retention. The latter group have suggested that the effect may depend on the activation of a peripheral glucose transport mechanism. It is of interest that the effective doses of this sugar in both experiments fell within a narrow range. The existence of an optimal dose for the effect of glucose on memory is consistent with the effect of many other agents that improve retention when administered post-trial. These include electrical stimulation of the brain [3], and amphetamine [22]. In our experiments S also exerted its beneficial effect on learning and memory in one i.p. dose of 0.75, 1.0, or 1.25 ml/kg depending on the behavioural test used. The simplest interpretation of these results would be that these doses bring about an optimal, different for different behaviour level of arousal according to the arousal theory of cognition [16]. The theory says that consolidation of memory is facilitated by some optimal level of arousal and that levels of arousal too far above or below the optimum do not facilitate and may even disrupt consolidation.

In 1962 Brever [5] and later Schwabe [32] showed that S also renders cell more permeable to potassium. According to Gibbs and Ng [12] a short-term memory is dependent on an early phase of hyperpolarization induced by changes in the membrane permeability to potassium. This may add to the explanation of beneficial influence of the drug on the acquisition process.

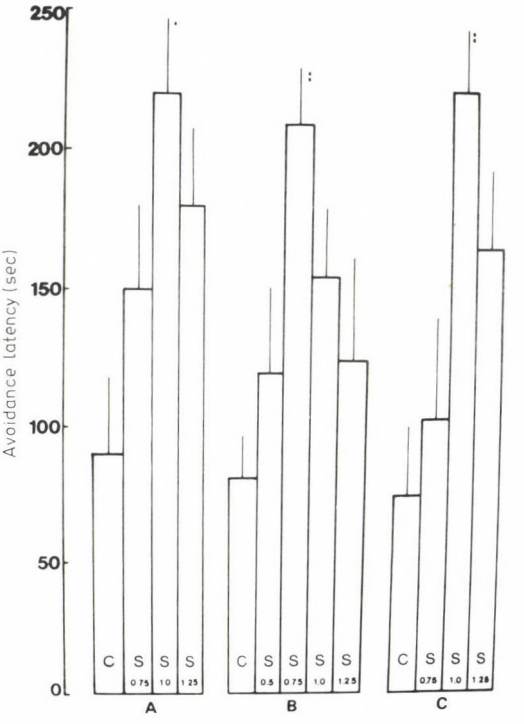

Fig. 4. Effect of Solcoseryl (S) given i.p. (at the doses indicated at the bottom of each column) 60 min before learning trial (A), immediately after it (B), and 60 min before the retention testing (C), on the re-entry latencies in a passive avoidance situation. Columns represent means ±SEM of the values obtained from 8 subjects. *$p<0.05$, **$p<0.01$ vs respective, injected with 0.9% NaCl control (c) groups (Student's *t*-test)

According to a widely accepted view memory involves synthesis of some specific proteins [7]. It has been demonstrated that nootropic piracetam stimulates protein synthesis and energy metabolism in the brain [28] and thus significantly improves the processes of learning and memory in both animals and normal humans [9, 11, 13]. Nakahara [27] has shown that also S induces endogenous kinases activation and polypeptide formation. In the parallel paper [4] we report that S has an anticataleptic and barbiturate sleep attenuating action possibly through the stimulation of dopamine and acetylcholine transmission, respectively. Both amines are known to be involved in cognitive processes in the CNS [2, 6, 8].

In addition to the results reported in the present study a number of experimental and clinical observations support the view that S meets the criteria of the nootropics [14, 29, 30]: i) S enhances learning and memory in animals, improves mental capabilities of elderly [31] and retarded [20] patients, ii) S protects brain against

harmful chemical and physical factors [21], iii) S alleviates amnesia in hypoxic and ECS treated rats [J. Boguszewicz, M. M. Winnicka and J. J. Braszko, unpublished results], iv) S enhances cerebral metabolism both in man and in animals [18, 21, 25, 36], v) since S is a deproteinized calves' blood it contains only substances of low molecular weight which should cross blood-brain barrier in most cases. Also, from the clinical point of view it is very important, that S has no side effects.

Although, at present the mechanism of action of S is unclear it is exciting from the practical point of view that the drug of natural origin devoid of side effects stimulates learning and memory and could be a new type of nootropic drug.

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Solcoseryl^{R} stimulates behavioural activity of rats

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The influence of Solcoseryl (S), a protein-free extract of calves' blood given intraperitoneally (i.p.) on the behavioural measures of activity of the central nervous system of male Wistar rats was examined. The drug (1.0 ml/kg i.p.) given 60 min before testing the animals in electromagnetic motimeter significantly enhanced overall and vertical motility of rats. S at the doses of 0.5, 1.0 and 2.0 ml/kg did not significantly influence the activity of rats in "open field". 1.0 ml/kg of S given 15, 45 and 60 min before thiopental (30 mg/kg i.p.) did not change the onset and time of sleep following the latter drug, except for the significant shortening of the time of sleep of animals injected with S 15 min before thiopental. S at the dose of 1.0 ml/kg did not change stereotypies produced by apomorphine (2.0 mg/kg i.p.) and amphetamine (6.5 mg/kg i.p.) but decreased intensity of haloperidol (1.0 mg/kg i.p.) catalepsy.

Keywords: Solcoseryl, psychomotor activity, stereotypy, catalepsy, rats

A great body of evidence showing the important role of several peptides and amino acids in the central nervous system (CNS) function has been published [2, 5, 7, 9, 10, 11, 14, 32, 35]. It has been shown that peptide hormones (ACTH, LPH) of their fragments resulting from enzymatic digestion have other than known biological activities, for instance they influence brain function [11, 14]. In our laboratory it was shown that the degradation products of many proteins (albumin, globulin, collagen, kininogen, angiotensinogen) influence the CNS [38, 39]. These peptides can modulate function of classic neurotransmitters such as norepinephrine, dopamine (DA), and serotonin [39].

Solcoseryl (S) is a chemically and biologically standarized, protein free, non-antigenic and non-pyrogenic haemodialysate of blood of healthy veal calves containing low molecular weight peptides and nucleic acids derivatives [19]. In experimental [16, 20, 27, 36, 40] and clinical [25] studies S has been shown to stimulate oxygen uptake,

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glucose transport and utilization, thus activating cell metabolism. In clinical studies [24, 25, 26], S increased brain tolerance to hypoxia and improved impaired mental performance. These observations indicate that S possesses similar activity as nootropic drugs [15, 28].

The purpose of this study was to examine effects of S on gross psychomotor activity as well as on the behavioural patterns largely dependent on acetylcholine (thiopental sleep [18]) and DA (stereotypy [33] and catalepsy [8]) in rats.

Materials and Methods

Animals

White male Wistar rats weighing 180–200 g were used. The animals were kept under standard laboratory conditions (lights on from 7 a.m. to 7 p.m., commercial rodent food and tap water freely accessible).

Overall and vertical motility

Individual rats were placed in a round transparent chamber covered by the ceiling with a plane electromagnetic coil glued to it [4]. All the vertical movements of an animal in the electromagnetic field generated by the cover coil produced impulses were counted and taken as a measure of its vertical motility. In addition, foot steps of the walking animal producing sounds which were detected by the sensitive microphones mounted under the membrane floor were counted giving the measure of the overall motility. Each rat was allowed a 1-min adaptation time, then it was tested for 10 min in the chamber.

Locomotor and exploratory activity

Locomotor and exploratory activity was measured in an open field apparatus as described earlier [5]. Its square (100 cm × 100 cm), white floor was divided by 8 lines into 25 equal squares and surrounded by a white wall, 47 cm high. Four plastic bars, 20 cm high, were located in 4 different line crossings in the central area of the floor. The single rat was placed in the apparatus for 1 min of adaptation. Subsequently, crossing, rearings and bar approaches were counted manually for 10 min.

Barbiturate sleep

The sleep was evoked by thiopental injected intraperitoneally (i.p.) at the dose of 30 mg/kg. S was given immediately (time 0 min), 15, 45 and 60 min before thiopental. Onset of the ensuing sleep was recorded as the time between thiopental injection and loss of the righting reflex. The barbiturate sleep was that between the loss and regaining the righting reflex time.

Stereotyped behaviour

Stereotypy was recorded according to a scale described by Kennedy and Zigmond [21]: –1, quiet or asleep; 0, normal activity; 1, occasional non-directed sniffing; 2, continuous sniffing; 3, continuous sniffing on a restricted area of the cage floor; 4, as 3 but with occasional licking; 5, continuous licking; 6, continuous licking with occasional biting; 7, continuous biting. Stereotyped behaviour was produced by an i.p. injection of 2.0 mg/kg of apomorphine and 6.5 mg/kg of amphetamine dissolved in 0.9% NaCl and administered in a volume of 1.0 ml/kg.

Haloperidol catalepsy

Catalepsy was evoked by haloperidol (1.0 mg/kg i.p.) – an antagonist of DA receptors [8]. The depth of catalepsy was scored according to Campbell et al. [8]. Six tests were applied in the following order: 3 cm high rod, parallel bars, four corks, crossings of both limbs on either side of the trunk and sitting on the hind limbs and tail with both forelimbs withdrawn from the floor. Each test was scored 0 or 1. In the tests 1–4 score one was given if the rat stayed in the abnormal position for at least 10 sec, and in the tests 6 and 7 for 3 sec. The scores of 6 tests were added so that the maximum score for one rat at the given time was 6.

Drugs

The following drugs were used: Apomorphine hydrochloride (Sandoz, Basel, Switzerland), d,l-amphetamine sulphate (Psychedrinum, Polfa, Warsaw, Poland), haloperidol (Polfa, Warsaw, Poland), Solcoseryl (Solco, Basel, Switzerland), thiopental sodium (Thiopental, Spofa, Praha, Czechoslovakia). All the drugs except for S were dissolved in saline before injection. S was given i.p. as a water solution the osmolality of which was 300 mOsm i.e. equal to the osmolality of the physiological saline. The drug was given at one of the three doses: 0.5, 1.0, and 2.0 ml/kg, 60 min before testing the overall and vertical motility of rats, and 30, 60 and 120 min before testing the animals in the "open field". In all the other experiments the most active dose 1.0 ml/kg of S was administered. S at that dose was given 0, 15, 45 and 60 min before the thiopental injection, immediately before apomorphine and amphetamine, and 90 min after haloperidol. Normal saline (0.9% NaCl solution) was used to inject control animals.

Statistical analysis

Statistical comparison were made by the Student's *t*-test.

Results

S at the dose of 1.0 ml/kg significantly ($p < 0.001$ vs control) enhanced overall (Fig. 1) and vertical (Fig. 2) motility of rats estimated in electromagnetic motimeter. In the "open field" test S given at the doses 0.5, 1.0 and 2.0 ml/kg, 30, 60 and 120 min before 10 min period of observation did not significantly change any of the recorded behaviours i.e. forward locomotion, number of rearings and bar approaches. The most pronounced changes in number of rearings were observed after injection of 1.0 ml/kg of S (Fig. 3). From these results it appeared that the dose of 1.0 ml/kg of S is the most effective behaviourally and therefore it was selected for the other experiments.

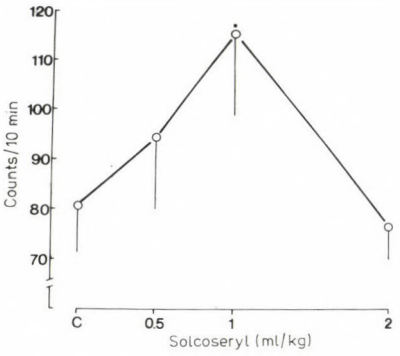

Fig. 1. Effect of S (0.5, 1.0 and 2.0 ml/kg), given i.p. 60 min before testing, on the overall motility of rats in magnetic field motimeter. The points represent means ± SEM of 8, 10 and 9 subjects, respectively, *$p < 0.001$ vs control (C) group ($n = 8$) (Student's *t*-test)

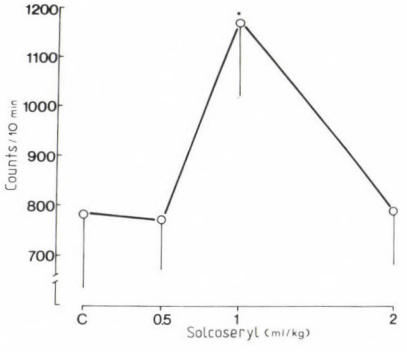

Fig. 2. Effect of S (0.5, 1.0 and 2.0 ml/kg), given i.p. 60 min before testing, on the vertical motility of rats estimated in magnetic field motimeter. The points represent means ± SEM of 8, 10 and 9 subjects, respectively, *$p < 0.001$ vs control (C) group ($n = 8$) (Student's *t*-test)

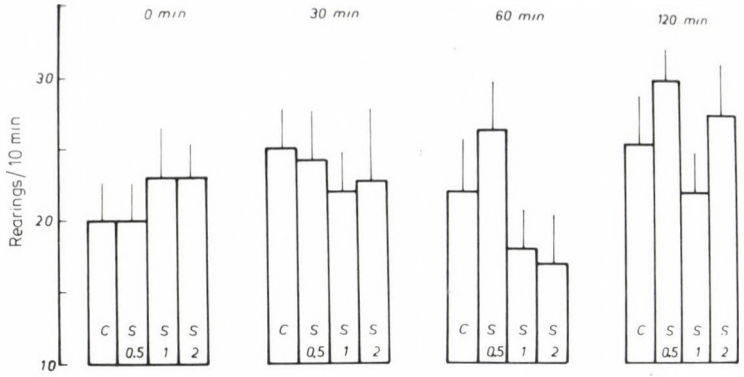

Fig. 3. Effect of S (0.5, 1.0 and 2.0 ml/kg i.p.) on the number of rearings, counted immediately (0 min), 30, 60 and 120 min after the injection, in the "open field" test. The columns represent means ± SEM of 8 control (C), and 8, 9 and 10 experimental rats, respectively

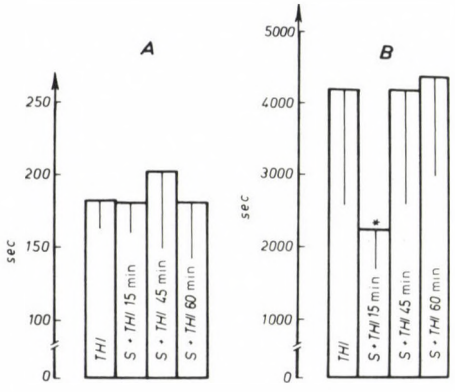

Fig. 4. Effect of S (1.0 ml/kg i.p.) given 15, 45 and 60 min before thiopental (THI, 30 mg/kg i.p.) on the onset (A) and time (B) of barbiturate sleep. The columns represent means ± SEM of 8 control (THI), and 10 experimental rats, *$p < 0.01$ vs THI group (Student's *t*-test)

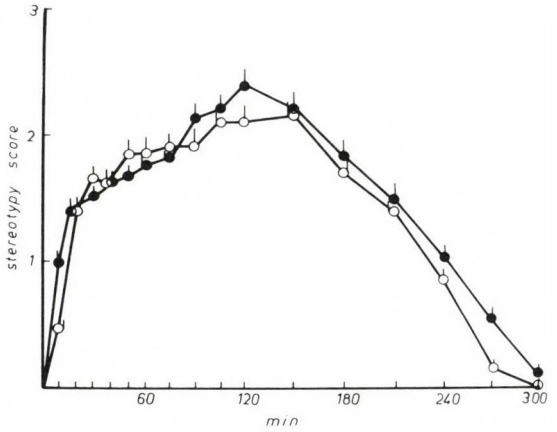

Fig. 5. Effect of 1.0 ml/kg of S (•) given i.p. immediately before apomorphine (2.0 mg/kg i.p.) on the apomorphine induced stereotypy. The points represent means ± SEM of 8 control (o), and 10 experimental rats

1.0 ml/kg of S did not change the onset of the sleep evoked by thiopental given 15, 45 and 60 min earlier (Fig. 4). Also the time of the sleep was not changed by S given at any time before thiopental except for 15 min, at which the drug significantly shortened the time of sleep ($p<0.01$ vs control). S at the dose of 1.0 ml/kg did not change the intensity of stereotypy evoked either by apomorphine (Fig. 5) or by amphetamine (Fig. 6). Nevertheless S at the same dose significantly ($p<0.05$ vs control) decreased intensity of haloperidol catalepsy (Fig. 7). This effect became evident immediately after S injection and persisted till the end of the observation period (300 min).

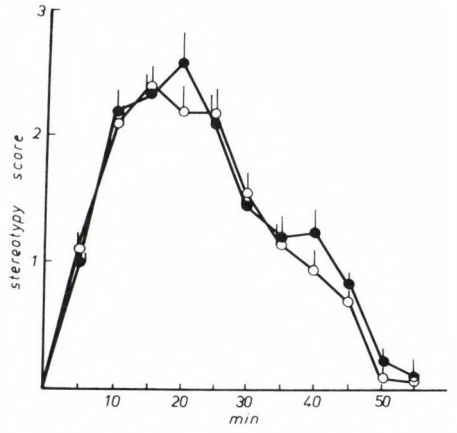

Fig. 6. Effect of 1.0 ml/kg of S (•) given i.p. immediately before amphetamine (6.5 mg/kg i.p.) on the amphetamine induced stereotypy. The points represent means ± SEM of 8 control (o), and 10 experimental rats

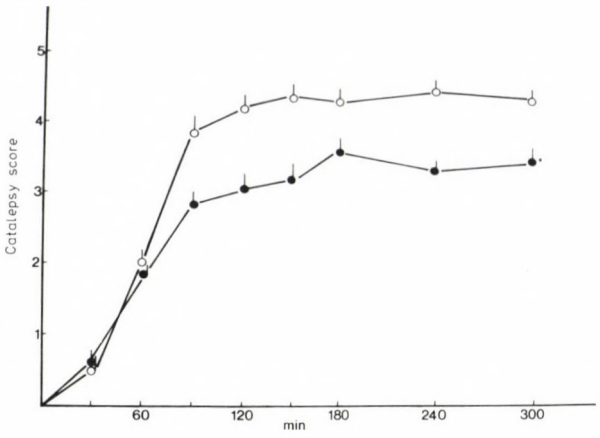

Fig. 7. Effect of 1.0 ml/kg of S (•) given i.p. 90 min after haloperidol (1.0 mg/kg i.p.) on haloperidol induced catalepsy. The points represent means ± SEM of 8 control (o), and 10 experimental rats. Overall effect of S was significant (*$p<0.05$, Student's *t*-test) vs control group

Discussion

The present results leave little doubt that S stimulates CNS activity. In the experiments assessing psychomotor activity of animals (overall and vertical motility) it was found that the active dose is 1.0 ml/kg of S given 60 min before testing. Although behavioural changes seen after 1.0 ml/kg of S in the "open field" were insignificant, this dose tended to be effective in changing number of rearings after its administration. Also, only at the dose of 1.0 ml/kg, S significantly shortened the time of sleep after barbiturate and decreased the intensity of haloperidol catalepsy. The above experiments indicate that S stimulates activity of CNS, but its effect is not dose-dependent. Of the three doses of S (0.5, 1.0 and 2.0 ml/kg) only 1.0 ml/kg was consistently active throughout 3 behavioural tests.

Because DA is the main neurotransmitter controlling, besides locomotor [1, 30], stereotyped [33] and cataleptic [8] behaviour, apomorphine and amphetamine stereotypy and haloperidol catalepsy were used to evaluate possible influence of S on central DA systems. While S significantly enhanced motility of rats and diminished depth of haloperidol catalepsy, it apparently had no influence on the stereotypies evoked by both apomorphine and amphetamine. Neuronal mechanisms controlling catalepsy involve, besides DA, opioid [12], and cholinergic [22] systems. Since in our experiments stereotypy controlled probably exclusively by DA [33] was not changed by S, while catalepsy was diminished, the influence of S on the activity of opioid and cholinergic systems may be assumed. The shortening the time of barbiturate sleep by S is in accordance with this assumption since barbiturates exert much of their sedative action through the cholinergic mechanism [37]. Besides, barbiturates decrease

consumption of oxygen in the brain tissue [18], while S increases it [20], the action that helps to explain the diminution of the thiopental sleep by S. S is a mixture of low molecular weight peptides, amino acids and nucleic acids derivatives [19]. Some of these compounds may have regulatory influence on the CNS function. Especially peptides like angiotensin and its fragments [3–6], neurotensin [29, 34], vasopressin and oxytocin [2, 23], cholecystokinin [13], substance P [31], neuropeptide Y [17], as well as many unknown substances possibly present in S may account for the behavioural effects of S seen in our study.

In conclusion, S has an obvious stimulatory influence on CNS function which involves increased oxygen consumption [20], but also probably a myriad of transmitter modulations responsible for the eventual net behavioural effect. The isolation and evaluation of the active ingredients of S seems to be the only way to fully understand its action in the brain.

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Assessment of a neuropathic factor in HIV-1 associated impotence: penile electrodiagnosis

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The value of bulbocavernosus reflex latency verses nerve conduction velocity of the dorsal nerve of penis and penile brachial index was evaluated in 50 impotent individuals infected with the human immunodeficiency virus, type 1 (both symptomatic and asymptomatic) and in 50 aged matched HIV-1 sero-negative impotent men serving as controls. All the subjects were neurologically asymptomatic and non demented. Both HIV-1 infected asymptomatic and symptomatic impotent patients exhibited a significant decrease ($P<0.0005$) in the nerve conduction velocity of the dorsal nerve of penis as well as penile branchial index from the controls of the same age group. The latency of bulbocavernosus showed no significant difference between the groups and was within the normal limits. A non-significant association in the study parameters between HIV-1 infected asymptomatic and AIDS positive impotent men was also observed. These findings suggest that impotence and altered erectile electrodiagnostic responses are likely to be associated with an increased frequency to neuropathy in these patients irrespective of their disease state.

Keywords: HIV-1, AIDS, impotence, neuropathy, penile electrodiagnosis

HIV-1 infection is commonly associated with neurological complications, which can be attributable to different etologies; infections, neoplastic and vascular. Neurological complications can occur in a simple way due to a direct HIV infection of central (CNS) or peripheral (PNS) nervous system [1, 21] or secondary to immunodeficiency [20]. Although peripheral neuropathy is often observed in HIV infection [4] a direct action on the central nervous system by HIV has also been described recently [9], however the precise mode of attack and progression of the disease from HIV in the CNS has not yet been clarified. According to some authors,

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CNS can be attacked early in the course of the disease, and as the infection proceeds very slowly, the clinically asymptomatic period can last long.

Despite the extensive body of research including the characterization, localization and pathology of HIV involvement of the CNS and to a lesser extent, the PNS [5], there are scant data about the involvement of the autonomic nervous system (ANS) by HIV [23]. In a single series of AIDS patients 4 of 5 were found to have abnormalities on ANS testing [12]. In a recently published study eleven of 17 HIV infected individuals (64.7%) have been reported to have symptoms suggestive of autonomic dysfunction, particularly urogenital problems like diarrhea and impotence [28].

Erectile impotence has been reported to be associated with HIV infection and AIDS [6]. Its etiology has been described to be a neuropathic abnormality in the male genital organs and/or vascular changes in corpora cavernosa [15]. However assessment of the neuropathic factor has been discontinued in lack of an objective laboratory test.

As it is known innervation of penis is autonomic and somatic. After arising from hypogastric and pelvic splanchnic nerves sympathetic and parasympathetic fibers are distributed in corpora cavernosa. However many histochemical fluorescent techniques have been developed in the recent years to demonstrate the involvement of these fibers during penile erection [19], very limited information is available regarding the electrophysiological evaluation of these fibers. The pudendal nerve which contains somatic sensory and motor fibers innervating the urogenital organs and perineum, has been demonstrated to have an important role in the sexual function, not only in erection but also in ejaculation and orgasm [11]. Since the dorsal nerve of penis, one of the branches of pudendal nerve, runs in the midline and dorsum of penis from base to glans, by cutaneous stimulation it is accessible easily for the electrophysiological studies [7, 24]. In the present study we have evaluated the nerve conduction velocity of the dorsal nerve of penis in HIV-1 infected asymptomatic and AIDS positive impotent men and we discuss its diagnostic value compared to bulbocavernosus reflex latency and penile brachial index which may be useful in defining penile neuropathy in these patients.

Methods

Nerve conduction velocity of the posterior tibial and sural nerve and dorsal nerve of penis, bulbocavernosus reflex latency as well as penile brachial index was measured in 50 HIV-1 infected asymptomatic and 50 AIDS positive impotent men and in 50 age matched HIV-1 sero-negative impotent control subjects. The age of patients ranged from 18 to 40 years (mean: 32) with a 1–3 year the duration from the onset of the disease (mean: 1.5). HIV-1 antibodies were detected by ELISA/Western blot, and the patients were classified according to the Center for Disease Control (CDC) criteria: 20 has AIDS, 2 had AIDS related complex (ARS) [Not included in the study] and 28 were HIV-1 sero-positive asymptomatic individuals, 14 of them to classes II and III and 14 to class IV of the CDC's 1987 classification scheme [3]. All the subjects were independently screened by a careful neurological history and examination and admitted to the study only if they were found to be neurologically asymptomatic and free from dementia. Patients were declared impotent in case they had difficulty for at least six months in obtaining and/or

maintaining erection for a minimum period. Subjects were not asked about their sexual behavior. All the subjects were definitely abstained from neuropsychoactive substances (including alcohol) for at least six moths.

All the conduction velocities were measured by an established method [22]. Bulbocavernosus reflex latency was calculated by putting two disk electrodes 2 cm apart on the dorsum at the base of the penis as stimulus electrodes. Square waves were applied at a rate of 1.7 pulses per second with a duration of 0.3 msec. As a recording electrode, a disk electrode was placed on the skin posterior to the scrotum over the bulbocavernosus muscle as an active electrode and the referential disk electrode was applied on the skin over the anterior iliac crest. A band ground was applied on the posterior aspect of the scrotum. Thirty responses were averaged and recorded. The amplifier band pass was 5 to 250 Hz and the sensitivity was 25 uV/cm.

Penile blood pressure was measured at one of the dorsal arteries and bilateral deep arteries of the penis with a standard digital blood pressure cuff and an ultrasound Doppler system (Model Sharp MB 200, USA). The doppler system deflects the return of blood flow, while the cuff is deflected to systolic pressure. The penile brachial index was calculated by dividing an average value of the 3 penile blood pressures by the brachial arterial pressure in the following manner, penile systolic blood pressure/brachial systolic blood pressure = penile brachial index.

Results

A comparison of the measurement of the nerve conduction velocity of the dorsal nerve of penis, sensory nerve conduction velocity of the sural nerve and posterior tibial nerve, bulbocavernosus reflex latency and penile brachial index in HIV-1 infected asymptomatic and AIDS positive patient and their respective controls is presented in Table I. This comparison revealed a significant decrease ($P<0.0005$) in the values of the nerve conduction velocity of the dorsal nerve of penis and penile brachial index in both HIV-1 infected asymptomatic and AIDS positive patients. However these values were found to be less significant ($P<0.025$ and $P<0.005$) for the nerve conduction velocities of the sural nerve and posterior tibial nerve, respectively, in these patients. The latency of the bulbocavernosus reflex showed no significant difference and was within the normal limits. These findings clearly indicate a neuropathic involvement in the pathway of the erotic reflex in both of our asymptomatic and AIDS positive impotent patients. The greater impairment of the nerve conduction velocity of the dorsal nerve of penis and penile brachial index in these patients may be due to overall greater severity of neuropathy in these individuals with sympathetic as well as parasympathetic damage.

A comparison of the measurement of the nerve conduction velocity of the dorsal nerve of penis, sensory nerve conduction velocity of sural nerve, motor nerve conduction velocity of posterior tibial nerve and penile brachial index between HIV-1 infected asymptomatic and AIDS positive patients is presented in Table II. A non-significant difference in the values of all of the above-mentioned parameters was observed among these individuals, although the values were found to be slightly greater in AIDS positive patients than their respective HIV-1 infected asymptomatic

Table I

Comparative studies of the measurement of the different nerve conduction velocities, bulbocavernosus reflex and penile brachial index in HIV-1 infected asymptomatic and AIDS positive impotent men and in age matched HIV-1 sero-negative controls

Subjects	Measurement of different nerve conduction velocities/bulbocavernosus reflex/penile brachial index (mean±S.D)					
	Post. tibial nerve (m/s)	Sural nerve (m/s)	Dorsal nerve of penis		Bulbocavernosus reflex latency (m/s)	Penile brachial index
			Take off	NI		
HIV-1 infected asymptomatic n=28	37±7**	41±8*	37±6***	28±5***	38±5	0.83±0.14***
AIDS positive n=20	38±4**	42±4*	38±6***	29±4***	39±6	0.84±0.13***
HIV-1 sero-negative controls (n=50)	44±6	47±2	46±5	35±4	38±5	0.98±0.08

n = Total number of subjects investigated. HIV-1 infected and AIDS (with and without neuropathy) values are compared with age matched HIV-1 sero negative controls for t-test

* = $P<0.025$, ** = $P<0.005$, *** = $P<0.0005$

NI = Negative peak of the first wave

Table II

Comparative studies of the measurement of different nerve conduction velocities, bulbocavernosus reflex latency and penile brachial index in HIV-1 infected (Asymptomatic) and AIDS positive (Symptomatic) impotent men

Parameters	Measurement of different nerve conduction velocities/bulbocavernosus reflex latency/penile brachial index (Means+S.D)	
	Asymptomatic (n=28)	Symptomatic (n=20)
Post. tibial nerve, (m/s)	37±7	38±4
Sural nerve, (m/s)	41±8	42±4
Dorsal nerve of penis		
Take off *	37±6	38±6
N1	28±5	29±4
Bulbocavernosus reflex latency, (m/s)	38±5	39±6
Penile brachial index	0.83±0.14	0.84±0.13
Statistical difference	Not significant	Not significant

N = Total number of subjects examined

HIV-1 infected and AIDS values are compared with age matched control subjects for t-test

* First negative peak

patients. These results thus suggest an exclusively apparent sexual pathway for the dorsal penile nerve conduction and penile brachial index which seems to be affected by neuropathic conduction in these patients irrespective of their type of disease.

Discussion

Human anatomical studies indicate that the dorsal nerve of penis supplies axons to corpora cavernosa as well as to the skin of glans and penile shaft. Physiological investigation in animals and human conform the role of the dorsal nerve of penis in 3-aspects of the male sexual expression, that is libido, erectile function and ejaculation [14, 22, 25]. Therefore neuropathic changes in the nerve result in loss of sexual responsiveness, erectile dysfunction and a depressive effect upon orgasm/ejaculation. Since the dorsal nerve of penis, one of the branches of pudendal nerve, runs in the midline and dorsum of penis from the base of the glans, by cutaneous stimulation it is easily accessible for electrophysiological studies. Since neuropathy is an important feature of impotence and HIV-1 infected and AIDS related peripheral neuropathic conditions have been described to affect initially afferent (sensory) pathways [2, 18, 19] in which most distal portion of the peripheral nerve is involved first [8], the conduction velocity of the dorsal nerve of the penis is directed as initially the most

affected site. We have therefore evaluated the nerve conduction velocity of the dorsal nerve of penis in HIV-1 infected asymptomatic and AIDS positive impotent men and discussed its diagnostic value compared to bulbocavernosus reflex latency and penile brachial index.

In our results we have found a significant decrease ($P<0.0005$) in the nerve conduction velocity of the dorsal nerve of penis both at the take off and at the first negative peak (N1) in both HIV-1 infected asymptomatic and AIDS positive men as compared to age matched HIV-1 sero-negative controls. In addition, differences found for the nerve conduction velocities of the posterior tibial nerve and sural nerve were less significant ($P<0.005$ and $P<0.025$, respectively). Therefore, the present findings indicate that the measurement of the nerve conduction velocity of the dorsal nerve of the penis is the most sensitive test to demonstrate neuropathic conditions in these patients among these electrophysiological studies. We thus conclude that the nerve conduction velocity of the dorsal nerve of penis has an exclusively apparent sexual pathway and in HIV-1 infected asymptomatic and AIDS positive patients it is affected by neurophathic conditions analogous to sural nerve.

Penile neuropathic involvement in the pudendal nerve has been detected previously by the demonstration of delayed bulbocavernosus reflex latency [16, 17]. The normal bulbocavernosus reflex latency ranges from 27 to 45 msec in men [10]. The bulbocavernosus reflex latency consists of many factors, such as conduction in the afferent and efferent fibers of the pudendal nerve, synapses in the spinal cord, transmission at the neuromuscular junction in the bulbocavernosus muscle and contraction of the muscle. Further more, some investigators have suggested supraspinal inhibition of the bulbocavernosus reflex [13, 16]. All of these factors are presumed to cause wide spreading of mild or moderate neuropathic conditions in the afferent reflex pathway. In our studies the afferent nerve impairment was considerable to increase bulbocavernosus reflex latency to an abnormal value. A comparison between HIV-1 infected asymptomatic and symptomatic impotent men in our studies further indicated more or less non-significant difference in the values of the nerve conduction velocities of the above-mentioned parameters. It is thus evident from our study that the slowness of conduction indicates neuropathic changes in the peripheral nerve function. We therefore suggest that an impaired electrical activity in our patients is purely due to a penile/pudendal neuropathic condition irrespective of their disease state. We further suggest that although this test for the measurement of the nerve conduction velocity of the dorsal nerve of penis has great value not only in the diagnosis of HIV-1 associated neuropathy but also in the interpretation of the bulbocavernosus reflex and the pudendal evoked responses however the nerve conduction velocity should be calculated with the latency at the onset of evoked action potential if possible, since the stimulation artifact sometimes make it difficult to identify the accurate onset of the evoked response.

Our results further indicated a significant decrease in penile brachial index ($P<0.0005$) in both types of our HIV-1 sero-positive impotent men compared to their respective non infected controls.

Similarly a non-significant association in the values of penile brachial index was also observed when these values were compared among HIV-1 infected asymptomatic and AIDS positive subjects respectively. These finding should be interpreted as an indication that an underlying neuropathic factor itself causes vascular changes in our both HIV-1 infected asymptomatic and AIDS positive patients in addition to the effect of impotence. The present study therefore suggests that a primary defective neuropathic mechanism may play an etiological role in the pathogenesis of erectile impotence in these patients. Altered pattern of the dorsal penile nerve conduction and penile brachial index in these patients thus provides strong evidence towards the possibility of a defect in the motor nerve function in this disease.

Although penile brachial index has some value for the clinical diagnosis of erectile impotence, it still has widely ranging values in patients with penile arterial disease [26, 27] and its clinical usefulness is limited. In view of this, a detailed and thorough investigation of the autonomic and peripheral nervous system is required for the understanding of a probable defect in the autonomic and peripheral pathway in HIV-1 sero positive asymptomatic and symptomatic impotent patients. Since it seems specific for HIV-1 associated neuropathy and if it could be confirmed on a larger group of HIV-1 infected asymptomatic and AIDS positive patients, then it may possibly be developed as an important diagnostic test for HIV-1 associated penile neuropathy.

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Ultrastructural changes in rat paneth and goblet cells after the administration of interferon-alpha

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Electron microscopic examination was performed to show the effects of interferon-alpha (IFN-alpha) on Paneth and goblet cells in rat intestines. After the administration of IFN-alpha (70 000 IU/kg), many cells of both types were depleted of secretory granules and their apical membranes had the deep cavitation that accompanies recent compound exocytotic activity. These results may indicate the involvement of these cells in the inflammatory reactions via IFN-alpha and this model provides a powerful tool to study differential effects of IFN-alpha on Paneth and goblet cells.

Keywords: Paneth cell, goblet cell, interferon-alpha

Interferons (IFNs), which are produced in response to a variety of stimuli are some of the central players in the host resistance mechanisms [5]. The major subgroupings of IFNs include IFN-alpha, IFN-beta and IFN-gamma [15, 28]. It has been shown that IFN-alpha and IFN-beta are produced by nearly all cells in response to viral infections, whereas type IFN-gamma is produced by lymphocytes in response to mitogenic or antigenic stimuli [16]. Studies from a number of different investigators have demonstrated that IFNs have immunomodulatory activities and may enhance local recruitment and anti-microbial functions of host macrophages [1, 6, 10].

The secretory activity of Paneth cells has remained an enigma. What does appear certain is that their secretory activity is closely related to the bacterial milieu in the intestine [21]. It was suggested that cholinergic mechanisms in the intestine are

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susceptible to change in the bacterial milieu and affect directly or indirectly the secretory activity of Paneth cells [22]. Goblet cells are modified columnar cells which synthesize and secrete mucus. The excreted mucus serves to lubricate and protect the surface of the epithelium [13]. It is known that goblet cells continuously secrete mucin at a slow baseline rate [27] and they can be totally depleted of mucin when exposed to bacterial enterotoxins [9].

The involvement of both cell types in the inflammatory reactions of the intestine has led us to examine the possibility that a relationship may exist between these cells and IFN-alpha, a cytokine known to play an important role in the inflammatory reactions. The aim of the present study was to investigate the ultrastructural changes in the Paneth and goblet cells after the administration of IFN-alpha.

Materials and Methods

Six male Wistar albino rats (280–300 g) were fasted 24 hours prior to examination. Three rats were injected IFN-alpha 2b (Intron A, Schering Plough, USA) at doses of 70.000 IU/kg intraperitoneally. Saline (1 ml per rat) was injected to the remaining three rats which were considered as controls. Six hours after the injections, the animals were perfused with 4% paraformaldehyde via the left cardiac ventricle. After perfusion, small tissue pieces were taken from the ileum, and immersed in 1% glutaraldehyde. Specimens were then postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in araldite. We randomly selected 3 tissue blocks from each animal and cut them so as to give transverse sections through the intestinal crypts. Ultrathin sections (600 nm thick) were placed on grids (200 mesh) and were doubly stained with uranyl acetate and lead citrate. Five different sections from each block were examined in a Carl Zeiss 9S2 electron microscope and the number of granules in every visible Paneth and goblet cells were counted by two observers. Data were presented as mean $\pm$SEM. We used Student's *t*-test for statistical analysis and a $P<0.05$ was considered to be significant.

Results

The general structures of Paneth cells from control rats were almost identical to those in normal rats (Fig. 1). The crypt lumen was empty and short microvilli projected outwards. The profiles of secretory granules showing bipartite substructures, electron-dense cores and electron-lucent haloes, lay in the supranuclear region. The average number of granules was 24.4 ± 2.3 for each Paneth cell profile in control rats. Exocytotic figures were rare. In rats treated with IFN-alpha, profiles of the Paneth cells showed 16.6 ± 1.8 secretory granules in average. The difference between the groups was significant ($P<0.05$). Some of the secretory granules in these cells fused with each other. Most of the cells had large vacuoles (Fig. 2). Many vacuoles were seen in open contact with the crypt lumen and sometimes contained electron-dense material. Exocytotic figures on the luminal surface of the cell and secreted electron-dense material in some crypt lumina were frequently noted. Microvilli on the degranu-

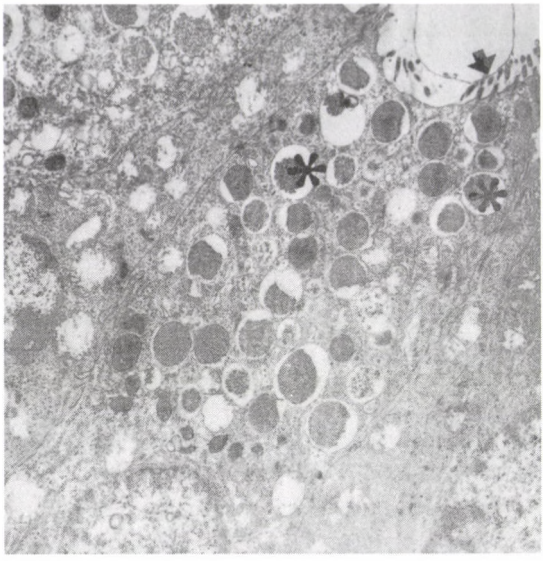

Fig. 1. An electron micrograph showing Paneth cells of a control rat. In the apical cytoplasm, secretory granules (asterisks) showing the bipartite substructure are observed. Arrow: Microvilli. ×4000

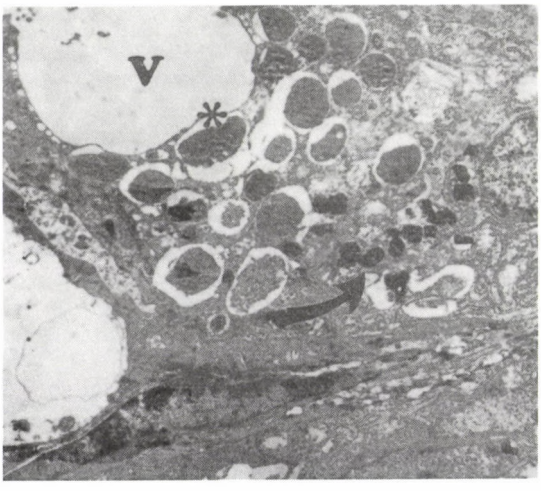

Fig. 2. A Paneth cell from a rat treated with IFN-alpha. Massive exocytosis and apical vacuole (V) opening into the crypt lumen are visible. Some secretory granules appear to be fusing with the vacuole (asterisks). Lysosome-like bodies (arrow) are also visible. ×4000

lated cells were often scarce and sometimes absent. In addition, lysosome-like bodies appeared in the cytoplasms. Nucleus and other cell organelles showed almost normal ultrastructures.

In ileal goblet cells of saline-treated rats, numerous granules with highly electron lucent homogeneous contents were observed throughout the cell cytoplasms (Fig. 3). In rats treated with IFN-alpha, the apical plasma membranes of goblet cells (Fig. 4) became deeply cavitated, and clearly became depleted of intracellular granules indicating that they had accelerated granule secretion. Exocytotic figures on the luminal surface of the cells and secreted material in crypt lumina were also noted. The difference in the number of granules of goblet cells between two groups was also significant ($P<0.05$). It was 47.7 ± 4.7 for control rats and 33.1 ± 2.8 for IFN-alpha administered ones.

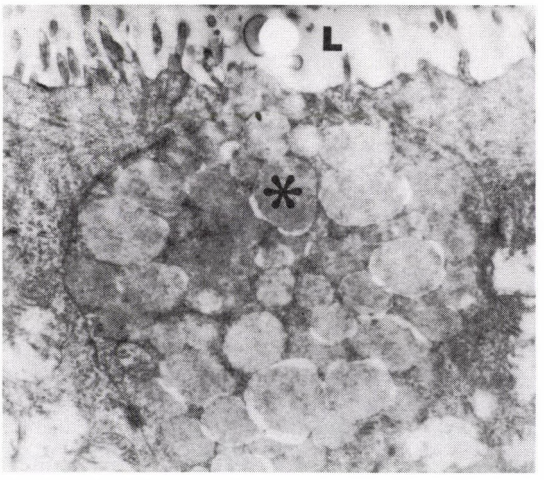

Fig. 3. A goblet cell of a control rat. Many secretory granules (asterisks) are seen in the apical cytoplasm. L: Lumen. ×4000

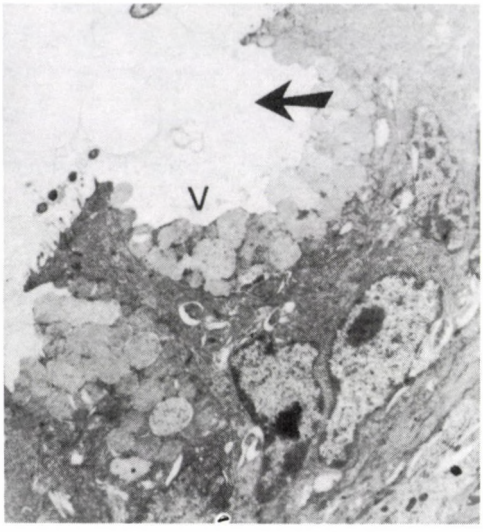

Fig. 4. Goblet cells of a rat treated with IFN-alpha, showing large vacuoles (V). Secreted material (arrow) in the crypt lumina are also noted. ×3000

Discussion

Intestinal Paneth and goblet cells might play a primary role in the local defense system in the intestine [8]. Inter-relationships were reported to exist between the secretory activity of these cells and the intestinal microbial milieu [25]. Paneth cells are found only in the deepest part of the intestinal glands. Histochemical studies have revealed that they possess the following antibacterial agents: lysozyme [4] which dissolves the polysaccharide component of bacterial cell walls, immunoglobulin A [20] and defensin/corticostatin-like peptide [14]. Therefore, it has been suggested that these cells play a role in controlling the bacterial milieu of the intestine by releasing antibacterial agents [22]. It is unclear whether the effect of the bacteria on Paneth cells is direct or indirect. As cholinomimetic drugs (acetylcholine, pilocarpine, muscarine) stimulate Paneth cell secretion, it was suggested that cholinergic mechanisms participate in the changes in Paneth-cell secretion in response to the intestinal microbial milieu [24]. On the other hand, when stimulated goblet cells also release their entire mucigen contents by compound exocytosis [17]. Compound exocytosis is easily recognized in transmission electron microscopy by the deep cavitation of the apical cell surface [18]. It is known that cholinergic stimuli can also elicit mucus secretion from goblet cells [27], that the mucus can protect the intestinal epithelia against bacteria [8], and that the mucus secretions in the intestine may increase when

bacteria are present [7, 26]. Mucus protects the surface of the epithelium and functions as substrate for degradative enzymes produced by the luminal bacteria; this behavior may spare the epithelial surface from degradation, but also necessitates that the mucous blanket be constantly replenished [18]. However, until now, only acetylcholine has been reported to enhance secretion of these cells, whereas other neurotransmitters (catecholamines, serotonin, somatostatin, substance P, vasoactive intestinal peptide) show no direct short-term effects [11]. In our study, we found that administering IFN-alpha also induces a secretory response in both Paneth and goblet cells in rat intestine. The effect of IFN-alpha on these cells could be a direct or an indirect effect. So far, there has not been any report of evidence on the presence of IFN-alpha receptor sites on these cells. IFN-alpha binds to discrete cell surface receptors on its target cells, and thereby alters gene expression [19]. Similar mechanisms may be present for Paneth and goblet cells.

It is known that the membranous (M) cells overlying the subepithelial lymphoid follicles can endocytose and transport many macromolecules, viruses and bacteria from the lumen to the underlying lymphoid tissue [12, 29]. These cells may therefore react to changes in the bacterial milieu of the intestine and transmit information to the cells which can secrete IFNs. IFN may in turn stimulate Paneth and goblet cells indirectly. Therefore, IFN-alpha may play a pivotal role in controlling the secretion of the Paneth and goblet cells that regulate the bacterial milieu in the intestine by releasing lysozyme or mucus into the intestinal lumen. However, to date, the exact mechanisms involved are not known.

Various exocrine cells show vacoule formation after stimulation [2, 3]. After stimulation, the granules sometimes fuse with each other, and large granules are occasionally being formed. Since the proportion of the secretory granules to cytoplasm is high in Paneth and goblet cells [24], it is likely that, after massive exocytosis, supranuclear regions of the cells became empty, thereby forming large vacuoles. In the cell apices, the rapid increase of the surface area of the membrane accompanying massive exocytosis may generate unusually long microvilli-like structures, the exact mechanism of formation of which is unknown [25]. The long microvilli-like structures in the vacuoles indicate that the vacuole membrane is in continuity with the apical cell membrane.

In a previous experiment, after the inoculation of facultative anaerobes into the intestine of germ-free mice, the area occupied by the core of the secretory granules decreases, but the halo area does not change [23]. The discrepancy in the changes of areas of core and halo can be explained if the core-substances (composed of protein) and halo-substances (composed of mucus) are produced separately and packed together in secretory granules. Since, antigen-antibody complexes facilitate the secretory activity of goblet cells [7], it is also possible that bacteria-antibody complexes contained in feces influence the production of mucus substances in the halo of the secretory granules in Paneth cells.

The present study demonstrates interrelationship between secretory activity of the Paneth and goblet cells and IFN-alpha and that the presently used experimental

model is well suited for examining the histophysiology of these cells. Our results may also indicate the involvement of these cells in the inflammatory reactions via IFN-alpha.

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Signal transduction in human myometrial cells

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Calcium plays a pivotal role in the contraction-relaxation cycle of uterine smooth muscle. We investigated the effects of three uterine agonists; prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), oxytocin and platelet activating factor (PAF) on intracellular levels of Ca ($[Ca^{2+}]_i$) in intact human myometrial cells and $^{45}Ca^{2+}$ efflux from permeabilized myocytes. We observed that, whereas oxytocin and PAF activated the phosphoinositide cycle, generating inositol triphosphate to mobilize intracellular Ca^{2+}, $PGF_{2\alpha}$ acted mainly to enhance Ca^{2+} influx. Oxytocin-, but not $PGF_{2\alpha}$-elicited responses were suppressed by pertussis toxin. It is concluded that all three agonists act by regulating $[Ca^{2+}]_i$ in myometrial cells, but the signal transduction mechanism of $PGF_{2\alpha}$ differs from that of oxytocin and PAF.

Keywords: human myometrium, prostaglandin $F_{2\alpha}$, platelet activating factor, signal transduction, calcium mobilization

The two main functions of the pregnant uterus are to provide optimal conditions for the successful development of the products of conception and, when this is accomplished generate sufficient force, combined with cervical dilatation, to expel its content in the process of parturition. The elucidation of the complex mechanism(s) responsible for the maintenance of relative uterine quiescence during human gestation and the identification of the multiple factors involved in the initiation of parturition are essential prerequisites for designing therapeutic strategies for prevention of preterm labor and delivery. Despite considerable progress in recent years, with respect to the

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cellular and molecular events that regulate smooth muscle contraction/relaxation, a cohesive picture of the onset of labor has not as yet emerged. There is, however, general agreement that the concentration of intracellular free Ca^{2+}($[Ca^{2+}]_i$) plays a pivotal role in the molecular events underlying myometrial function. Simply put, calcium, bound to calmodulin, the multifunctional Ca binding protein, activates myosin light-chain kinase which, in turn, phosphorylates myosin, allowing it to interact with actin and the activation of the myosin Mg^{2+}-ATPase generating ATP, the driving force of contraction. Dephosphorylation of actomyosin by myosin light-chain phosphatases reverses the biochemical pathway and relaxation ensues [1].

There are a number of potent, naturally occurring substances that promote uterine contractility. Of these, the uterotonic prostaglandins, $PGF_{2\alpha}$ and PGE_2, as well as oxytocin, have been studied extensively with respect to their possible roles in the regulation of parturition. More recently, platelet activating factor (PAF) and the potent vasoactive peptide, endothelin-1 (ET-1), have also been implicated as extracellular signals promoting myometrial activity. Assuming that the regulation of cellular calcium levels represents a step common to such uterotonic agents, we have conducted experiments designed to evaluate the effects of $PGF_{2\alpha}$, oxytocin and PAF on either $[Ca^{2+}]_i$ or on mobilization of Ca^{2+} from intracellular stores in primary cultures of human myometrial cells [11, 12]. Here we shall present a brief account of these studies.

Materials and Methods

We employed two experimental approaches. Either intact myometrial cells, isolated by enzymatic digestion from tissue fragments obtained at hysterectomy and grown to confluence in Waymouth's MB 752/1 culture medium were used to assess $[Ca^{2+}]_i$ or permeabilized cells were used to measure the release of Ca^{2+} from intracellularly bound compartments. In the first type of experiment, myocytes were loaded with the sensitive fluorescent Ca indicator Fura-2 AM and the concentration of $[Ca^{2+}]_i$ was determined fluorometrically [5]. Generation of inositol phosphates in myocytes prelabeled with [3H]myoinositol was quantitated after chromatographic separation of IP, IP_2 and IP_3, according to Berridge et al. [2]. In some experiments total inositol phosphates were eluted as a single fraction and the radioactivity determined by scintillation counting [12]. In the second approach, confluent monolayers of myometrial cells were harvested, permeabilized with digitonin and loaded with ^{45}Ca in the presence of 5 mM ATP and ruthenium red, in order to determine ^{45}Ca release from non-mitochondrial compartments. Preliminary experiments have shown that ^{45}Ca uptake under these conditions is entirely an ATP-driven mechanism, since, in the absence of added ATP, cells failed to accumulate ^{45}Ca.

Cell cultures supplies were purchased from GIBCO (Grand Island, NY), isotopes from Dupont-NEN (Boston, MA) and other reagents were obtained from Sigma Co. (St. Louis, MO). U-73,122 was a gift from Dr. J. Bleasdale, The Upjohn Co. (Kalamazoo, MI).

Results

Studies with intact cells

When myocytes were stimulated with $PGF_{2\alpha}$ (10^{-7}M) or oxytocin (10^{-7}M) in the presence of 1 mM extracellular calcium, basal levels of $[Ca^{2+}]_i$ were raised several fold within 15 sec (Fig. 1). However, when calcium was omitted from the incubation medium, only oxytocin was able to generate a calcium signal, although a reduced one compared to that obtained in calcium-containing medium (Fig. 1).

In view of the fact that inositol-1,4,5 triphosphate (IP_3) has been identified as the intracellular signalling molecule responsible for Ca^{2+} release from the sarcoplasmic reticulum in target cells stimulated with extracellular receptor agonists, we attempted to correlate oxytocin and $PGF_{2\alpha}$-provoked calcium responses with inositol phosphate generation. Thus, when myometrial cell cultures prelabeled with [3H]myoinositol to isotopic equilibrium were exposed to oxytocin there was a dose-related increase in the production of IP_3, compatible with the observed rise in $[Ca^{2+}]_i$. However, $PGF_{2\alpha}$-induced breakdown of phosphoinositides reached significant levels only at 1–10 μM concentrations of the agonist, which was 1–2 orders of magnitude greater than that required to raise $[Ca^{2+}]_i$. Moreover, when extracellular Ca^{2+} was clamped at 0.1 μM, $PGF_{2\alpha}$-induced inositol phosphate release was abolished, whereas that elicited by oxytocin was not affected. These experiments suggested that $PGF_{2\alpha}$ and oxytocin regulate $[Ca^{2+}]_i$ by discrete mechanisms, the former relying on the influx of extracellular Ca^{2+}, whereas oxytocin appeared to affect both the entry of extracellular Ca^{2+} and the mobilization of intracellular Ca^{2+} via the agency of IP_3.

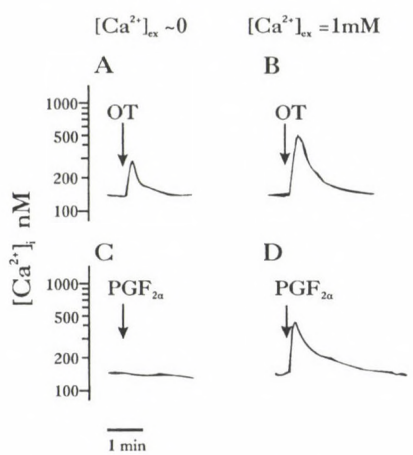

Fig. 1. Effects of oxytocin (OT; 100 nM) and $PGF_{2\alpha}$ (100 nM) on $[Ca^{2+}]_i$ in cultured human myometrial cells. Fura-2-loaded cells were washed and suspended in medium containing 1 mM EGTA without added calcium (A and C) and without EGTA plus 1 mM $CaCl_2$ (B and D). Each panel shows a representative trace from one of six experiments. Arrows indicate the introduction of agonist

This notion was further supported by the following observations: (i) raising $[Ca^{2+}]_i$ by means of incubating [^{3}H]myoinositol prelabeled cells in the presence of the Ca ionophore, A23187, was sufficient to increase the production of inositol phosphates seen in response to high concentrations of $PGF_{2\alpha}$; (ii) pertussis toxin, that inactivates certain GTP binding proteins involved in receptor-coupled signal transduction, suppressed oxytocin-, but not $PGF_{2\alpha}$-induced phosphoinositide hydrolysis, as well as increases in $[Ca^{2+}]_i$, (iii) TPA, a protein kinase-activating phorbol ester, abolished oxytocin-, but not $PGF_{2\alpha}$-induced inositol phosphate generation, and (iv) verapamil, a calcium channel blocker, suppressed $PGF_{2\alpha}$-induced phosphoinositide breakdown without affecting oxytocin-provoked responses.

Studies with permeabilized cells

This cell model, which allows the introduction of various nucleotides (ATP, GTP), as well as IP_3 and other substances, has been successfully used to explore signal transduction in various cell types, including vascular and uterine smooth muscle cells [8, 10]. We employed this model to study and compare the effects of $PGF_{2\alpha}$, oxytocin and PAF on Ca^{2+} mobilization in primary cultures of human myometrial cells. Digitonin-permeabilized cells in the presence at ATP rapidly accumulated ^{45}Ca, reaching apparent equilibrium within 20–30 min. Inclusion of ruthenium red prevented ^{45}Ca uptake by mitochondria, thus ^{45}Ca released in response to agonist was derived primarily from the sarcoplasmic reticulum. When such ^{45}Ca-loaded cells were stimulated with $PGF_{2\alpha}$, oxytocin or PAF, there was a rapid, biphasic release of ^{45}Ca (Fig. 2). The first phase peaked at 1 min and the second at 5 min, after which the rate of ^{45}Ca efflux declined. The calcium-mobilizing effect of all three agonists was dose-dependent. Maximal responses for oxytocin, PAF and $PGF_{2\alpha}$ were observed at 10^{-8} M, 10^{-7} M and 10^{-6} M concentration, respectively, releasing about 35–40% of the total ionophore-releasable ^{45}Ca (Fig. 3).

To corroborate our observations on intact cells, we next investigated the involvement of IP_3 in ^{45}Ca release from intracellular compartments. We made use of two pharmacological tools; U-73,122, a selective inhibitor of phospholipase C and heparin that has been shown to block IP_3-mediated Ca^{2+} release [3, 14]. As summarized in Fig. 4, U-73,122 inhibited oxytocin- and PAF-induced ^{45}Ca release, without influencing $PGF_{2\alpha}$ and, of course, IP_3-stimulated efflux. Similarly, heparin failed to suppress $PGF_{2\alpha}$-promoted ^{45}Ca efflux, while blocking this response to the other two agonists. That heparin acted at the IP_3 receptor site was demonstrated by abolishing the Ca-mobilizing effect of exogenous IP_3.

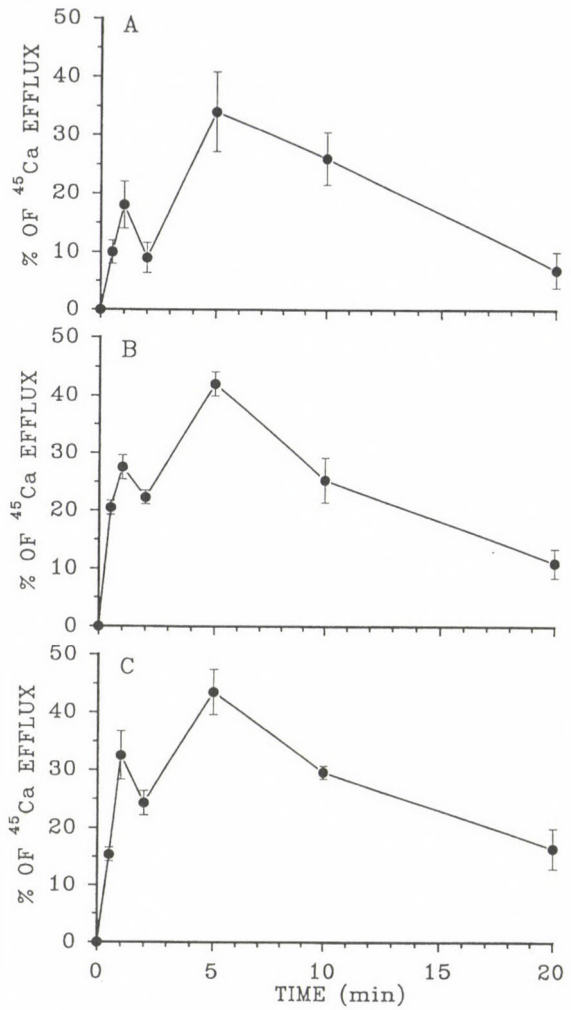

Fig. 2. Kinetics of agonist induced ^{45}Ca efflux in human myometrial cells. Human myometrial cells were isolated and cultured according to Casey et al. [5], with some modifications [6]. Digitonin permeabilized cells were preloaded with ^{45}Ca (5 Ci) in the presence of ATP (5 mM) and ruthenium red (6.5 M) for 30 min and then stimulated with 100 nM oxytocin (panel A), 1 μM $PGF_{2\alpha}$ (panel B) or 1 M PAF (panel C). Values are expressed as percent of total releasable calcium and represent mean+SEM of six experiments [10]

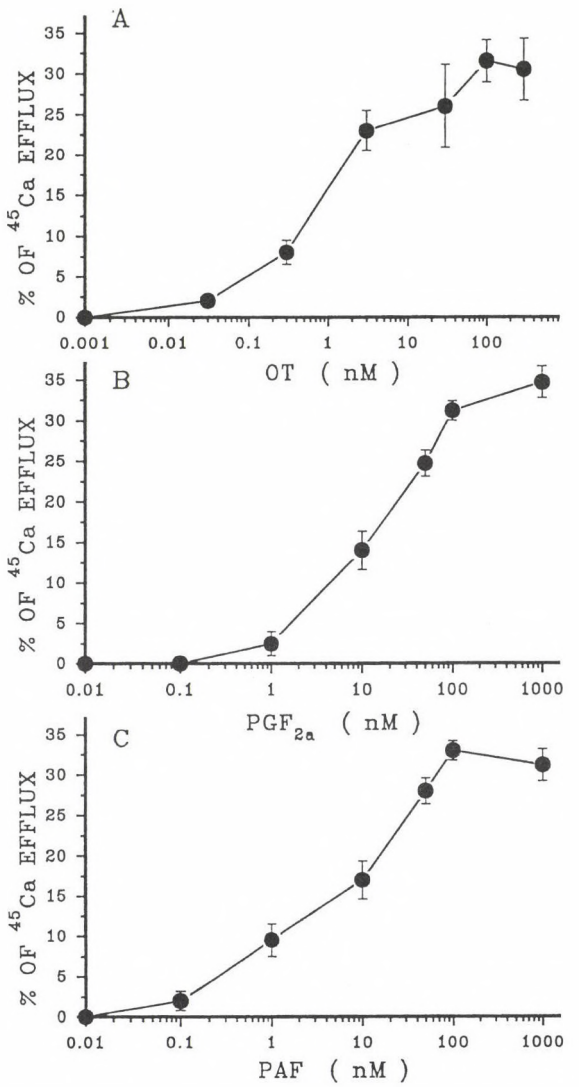

Fig. 3. Dose response of Oxytocin-(A), $PGF_{2\alpha}$-(B) and PAF-stimulated (C) ^{45}Ca efflux from ^{45}Ca-loaded human myometrial cells after 5 min of stimulation. Results are mean ±SEM of three experiments

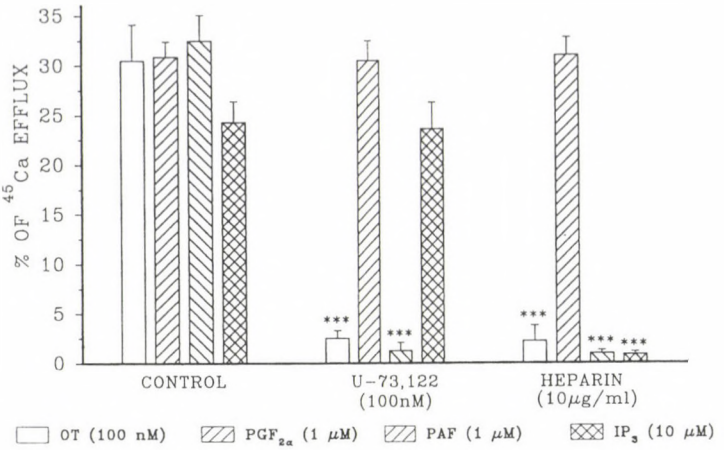

Fig. 4. Effect of phospholipase C inhibitor (U 73,122) and heparin, an inhibitor of IP_3-induced calcium release, on oxytocin, $PGF_{2\alpha}$, PAF and Ins(1,3,4)P_3 stimulated ^{45}Ca efflux in permeabilized human myometrial cells loaded with ^{45}Ca. Data are from four experiments (mean ±SEM). ***$P < 0.005$ by ANOVA

Discussion

Intracellular free calcium levels are tightly controlled. In uterine smooth muscle cells, resting $[ca^{2+}]_i i$ is about 10^{-7} M. Here we have shown that when these cells are stimulated with the classic uterotonic agents, oxytocin and $PGF_{2\alpha}$, in a medium containing physiological concentrations of Ca^{2+}, this value may approach $5–10^{*}10^{-7}$ M. But even at these high, activating levels, the concentration of Ca^{2+} in the extracellular compartment is, at least, three orders of magnitude higher. It is not surprising, therefore, that extracellular calcium represents the major source of intracellular calcium. It has been long recognized that uterine smooth muscle contractility cannot be sustained *in vitro* in the absence of added Ca^{2+}. What has puzzled cell physiologists is the mechanism by which extracellular Ca^{2+} enters the cell and, ultimately, how $[Ca^{2+}]_i$ is regulated by extracellular signals. There is general agreement that the two main avenues of calcium entry are via voltage-gated and receptor-operated channels. In addition, moment-to-moment oscillations in $[Ca^{2+}]_i$ may depend on endogenous sources of calcium, a mechanism that is influenced by intracellular signalling messenger molecules, such as IP_3 and cyclic nucleotides. While the results of our studies indicate that all of these mechanisms are operative in human myocytes, significant differences were uncovered, with respect to the intimate mode by which $PGF_{2\alpha}$, on the one hand, and oxytocin and PAF on the other, influence these processes. First, whereas both $PGF_{2\alpha}$ and oxytocin induced a rapid and dose-related rise in $[Ca^{2+}]_i$ in intact cells, as well as ^{45}Ca efflux in permeabilized myocytes, $PGF_{2\alpha}$-elicited response depended entirely on the presence, and presumably, the entry of extracellular Ca^{2+}. On the other hand, part of the oxytocin-induced calcium signal

in intact cells could be attributed to the activation of the phosphoinositide cycle and the generation of IP_3. This was further substantiated by demonstrating that inhibitors of phospholipase C and heparin, that blocks IP_3 receptors in the sarcoplasmic reticulum, suppressed oxytocin-, but not $PGF_{2\alpha}$-promoted rise in $[Ca^{2+}]_i$, as well as ^{45}Ca efflux. Second, the inhibition by pertussis toxin of oxytocin, but not $PGF_{2\alpha}$-induced responses, suggests that oxytocin signaling proceeds via a G-protein-coupled activation of the phosphoinositide cycle, culminating in intracellular Ca^{2+} release and myometrial contraction. Phaneuf et al. [15], on the other hand, reported that oxytocin action in human myometrial cells is mediated by both a pertussis toxin-sensitive and insensitive mechanism. Nonetheless, our results are in agreement with those of the Oxford group with respect to the relative sensitivity of human myometrial cells to oxytocin and $PGF_{2\alpha}$, in terms of inositol phosphate production. In contrast, $PGF_{2\alpha}$ relies on its ability to enhance Ca^{2+} influx without raising, at physiological concentrations, IP_3 levels and the concomitant release of intracellular Ca^{2+}. Although, at high concentrations that could be viewed as pharmacological levels, $PGF_{2\alpha}$ was also able to promote phosphoinositide hydrolysis, this response may be attributed to a sustained rise in $[Ca^{2+}]_i$ that has been observed to active the inositol cycle in other type of cells, as well [7]. Alternately, human myometrial cells may possess a high and a low affinity class of $PGF_{2\alpha}$ receptors; the former regulating Ca^{2+} channels, whereas the latter is coupled via a pertussis toxin-insensitive G-protein to phospholipase C.

The mechanism of $PGF_{2\alpha}$-promoted Ca^{2+} influx may involve both voltage-gated and receptor-operated calcium channels. It should also be pointed out that $PGF_{2\alpha}$-induced ^{45}Ca release in permeabilized cells is probably due to a direct, ionophore-type action of this prostaglandin [16]. Carsten and Miller [4] have also observed PG-promoted Ca efflux from bovine myometrial microsomes. It would appear, therefore, that this uterotonic prostaglandin may regulate $[Ca^{2+}]_i$ in a paracrine fashion by receptor-coupled and/or voltage-gated ion channels, as well as by acting as an intracrine agent by enhancing Ca^{2+} release from intracellular compartments directly in the cells where it is produced. This aspect of the mechanism may play a significant part in response to agonists that activate the arachidonic acid cascade, raising intracellular levels of prostaglandins. It may be pertinent to point out here that arachidonic acid itself may act as an intracellular signaling molecule, capable of mobilizing calcium in human myometrial cells without it being metabolized to various biologically active prostanoids [9]. Although we did not evaluate the effects of PAF in intact myocytes in the present studies, our results exposed a close analogy between the mechanism of action of this potent naturally-occurring phospholipid and the uterotonic peptide, oxytocin. Both of these substances act via receptors-G-protein-phospholipase C-mediated calcium mobilization.

In conclusion, our studies and those of other investigators have provided compelling evidence that the regulation of $[Ca^{2+}]_i$ in myometrial cells by oxytocin, $PGF_{2\alpha}$ and PAF, represents the common denominator in the mode of action of these uterotonic agents. Additional or complementary pathways cannot, of course, be ruled out. Indeed, recent studies by Japanese workers indicate that oxytocin activates the

mitogen-activated protein kinase cascade in human myometrial cells [13]. The "crosstalk" between these and other potential signaling pathways leading to uterine contraction/relaxation have yet to be identified.

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Phosphatidylcholine cycle: An intracellular signaling mechanism in the primordial human placenta[1]

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The effects of 4β-phorbol-12-myristate-13-acetate (PMA) and 1,2-sn-dioctanoylglycerol (DOCG) on the rate of labeling of phosphatidylcholine (PC) with (^{32}P)phosphate and the rate of formation of (^{3}H)phosphatidylethanol (PET) from PC labeled with (^{3}H)myristic acid were investigated *in vitro* in minced placentae obtained from first trimester human pregnancies. Maximally effective concentrations of PMA (1 μM) or DOCG (125–250 μM) stimulate PC-labeling with (^{32}P)phosphate along different time courses: responses to DOCG and PMA require 30 and 60 min, respectively. The early response to DOCG is attended by a rapid accumulation of ^{32}P)PC_{DOCG} followed by a decline from the peak value in the second 30 min. The PMA effect is accompanied by increased rate of formation of (^{32}P)phosphatidic acid (PA). Importantly, the effects of PMA and DOCG on PC-labeling are additive and PMA does not have any effect on the labeling of PC_{DOCG}. These findings indicate that PMA stimulates degradation and the attendant turnover of PC, whereas a greater part of the DOCG-effect comes from the stimulation of PC synthesis *de novo*. Consistent with this notion is the finding that PMA enhances the PC-selective phospholipase D activity (measured by the formation of PET) 2.4-fold, whereas the effect of DOCG is smaller (1.4–1,8-fold) and not additive with that of PMA. The results provide evidence for the presence of functional PC-cycle in the primordial human placenta. The cycle can be triggered by a single addition of PMA and to a lesser extent by DOCG. The smaller effect of DOCG may be related to its short lifetime in the tissue, which is sufficient, however, to stimulate the activity of the regulatory enzyme (CTP: choline cytidylyl transferase) of PC synthesis. Since the effect of PMA on PC-labeling is diminished by protein kinase C inhibitors, this enzyme appears to be involved in the stimulation of PC-cycle by DAG and its analogs.

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Study of the signaling mechanisms of rapidly proliferating cells of the early human placenta may lead to better understanding the control of growth and differentiation of human cells as well as the biochemical basis of certain abnormalities of placental function. Evidence accumulated in the last decade support the view that cellular phosphatidylcholine is an abundant source of 1,2-diacylglycerol [2,9–12,19,30] which in turn is able to activate protein kinase C, an enzyme thought to mediate signals for cellular growth and differentiation [6, 18, 21, 22]. Importantly, certain isoforms of protein kinase C are activated by diacylglycerol even at resting concentrations of Ca^{2+} present in the cytoplasm [17]. Generation and attenuation of the diacylglycerol signal from phosphatidylcholine can be described by a series of reactions called the phosphatidylcholine-cycle [2, 8, 19, 23, 27]. The effector enzymes responding to extracellular agonists and promoting the specific and selective degradation of phosphatidylcholine are the phosphatidylcholine-selective phospholipase C and D. The reaction of the phosphatidylcholine-cycle which terminates diacylglycerol signal is believed to be catalyzed by CDP-choline: diacylglycerol phosphocholine transferase. Phospholipase C liberates diacylglycerol in one step from phosphatidylcholine, whereas phospholipase D acts in two consecutive reaction steps: first phosphatidic acid is formed and then a second enzyme, phosphatidate phosphohydrolase is needed to cleave phosphatidic acid further into diacylglycerol and phosphate.

Activation of phosphatidylcholine-selective phospholipase C or D may occur either with a direct, receptor and G-protein mediated or an indirect, protein kinase C-mediated mechanism [2, 11, 12, 24]. Protein kinase C, therefore, seems to exhibit a dual role in signaling through phosphatidylcholine: it activates the enzyme degrading phosphatidylcholine to form diacylglycerol and it is activated by diacylglycerol released from phosphatidylcholine (positive feed-back). In fact, activation of the kinase leads to a self-amplifying mechanism capable of producing increasing concentrations of diacylglycerol in a prolonged fashion [2, 12].

One possibility to demonstrate the presence and operation of phosphatidylcholine-cycle is to stimulate protein kinase C activity with a diacylglycerol-analog tumor-promoting β-phorbol ester or a synthetic diacylglycerol, such as dioctanoylglycerol (DOCG) and detect the phospholipolytically triggered increase of the turnover rate of phosphatidylcholine as well as the concomitant release of phosphatidic acid or diacylglycerol [5, 16]. Alternatively, advantage can be taken of the finding that phosphatidylcholine-selective phospholipase D catalyzes the transfer of phosphatidyl group from phosphatidylcholine to primary alcohols to produce phosphatidylalcohols, such as phosphatidylethanol [2, 5, 9, 16, 20]. This approach makes it feasible to determine the stimulation of phospholipase D activity by β-phorbol esters or synthetic diacylglycerols [5, 16, 20]. Bearing in mind that the phosphatidylcholine-cycle could be important to transmit signals in intensively

growing tissues, in the present communication we studied the effects of a β-phorbol ester (PMA) and DOCG on the phosphatidylcholine turnover, the labeling of phosphatidic acid and the formation of phosphatidylethanol in the primordial human placenta.

Materials and Methods

Radioactive isotopes: Carrier-free (^{32}P)phosphate was obtained from IZINTA (Budapest, Hungary), [9, 10 (n)-^{3}H] myristic acid (54 Ci/mmol, 2.00 TBq/mmol), was the product of The Radiochemical Centre (Amersham, UK).

Chemicals: 4 β-phorbol-12-myristate-13-acetate (PMA), 4 α-phorbol-12,13-didecanoate (PDD), 1,2-dioctanoyl-sn-glycerol (DOCG), staurosporin, 1-0-hexadecyl-2-0-methylglycerol (HMG), D-sphingosine and various neutral and phospholipids were purchased from Sigma (St. Louis, MO, USA). 1,2-dimyristoyl-sn-glycero-3-phosphoethanol (sodium salt) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Silicagel H-60 was from Merck (Darmstadt, Germany).

Tissue collection, incubation and lipid extraction: Details are described in previous papers [14, 27–29]. Briefly, placenta pieces were collected during legal instrumental interruption of normal 8–10-week-old pregnancies and placental mince (500 mg) was incubated with 25 or 50 μCi (^{32}P)phosphate or 10 μCi (^{3}H)myristic acid in 2 ml modified Hanks medium [29] buffered with 40 mM HEPES-Na (pH 7.4) in open vials shaken continuously at 37 °C for the time periods indicated. PMA, PDD, HMG, staurosporin and sphingosine were added from stock solutions prepared in DMSO, controls were given the solvent only. Lipids were extracted using a modified method [29] of Bligh and Dyer [4], and small aliquots of the extract were withdrawn to determine total extracted radioactivity.

Radio TLC of phospholipids: Details of the methods have been described [14, 27–29]. The solvent systems applied were as follows: (I) For separation of phosphatidic acid: chloroform/pyridine/formic acid = 100/60/14 (v/v/v); (II) For separation of phosphatidylcholine, lyso-phosphatidylcholine, PC_{DOCG} and phosphatidylinositol: chloroform/methanol/acetic acid/H_2O = 100/50/14/6 (v/v/v/v); (III) For separation of diacylglycerol and triacylglycerols: petrolether or n-hexane/diethylether/acetic acid = 70/30/1 (v/v/v); (IV) For separation of phosphatidylethanol: the upper phase of isooctane/ethylacetate/acetic acid/water: 2/13/3/10 (v/v/v/v) mixture [5].

In separations (II) and (IV) the TLC tank was lined with filter paper soaked in the solvent and TLC started after an at least 24 hour equilibration of the inner space with solvent vapors. Separation (IV) were done twice with intermittent drying [5]. For identification of various lipids, authentic standards were chromatographed in separate lanes. Each lane used for separation of extracted lipids was divided into identical segments. The segments were scraped off into counting minivials and after adding 2 ml scintillation solution (15) the radioactivity was counted using a Beckman LS 7800 liquid scintillation spectrometer.

Calculation of results: The percent incorporation into each lipid species was calculated from the cpm values associated with silica-gel segments and considering the relative position of standards. From the percentage value and the total extracted cpm, the cpm associated with a particular phospholipid was computed as cpm/500 mg wet weight of incubated tissue [14, 27–29]. When (^{3}H)myristic acid was used as label, cpm/mg tissue values were determined on the basis of the sample volume applied for TLC analysis. Experiments were repeated 3–6-times using different pools of tissue. Results obtained from several experiments were normalized relative to the mean value of control incubates. For statistical analysis, Student's "t"-test was used. A difference was regarded statistically significant when $P < 0.05$.

Results

Dose-response studies on the effect of PMA and DOCG on the incorporation of (^{32}P)phosphate into phosphatidylcholine revealed that maximum effects are reached at 1 μM PMA and 125 μM DOCG (final concentrations), whereas the α-isomer of phorbol ester was ineffective. DOCG species of phosphatidylcholine (PC_{DOCG}) accumulated label dose dependently in the range of 125–500 μM DOCG (results are not shown). Based on these findings 1 μM PMA and 125–250 μM DOCG were selected for further studies. Figure 1 demonstrates that PMA and DOCG increases the labeling of phosphatidylcholine with (^{32}P)phosphate along different time courses. The effect of PMA is seen at 60 min after adding it to the incubate, whereas no effect is detectable after 30 min. DOCG elevates the rate of labeling at 30 min and no further effect is seen in the second 30 min. The effects of PMA and DOCG measured at 60 min are additive, suggesting different mechanism of action. Figure 2 shows that PMA promotes the accumulation of labeled phosphatidic acid between 30 and 60 min after adding it to the incubates and DOCG does not have such an effect. Labeling of lysophosphatidylcholine and phosphatidylinositol did not show reproducible effects in response to either PMA or DOCG (data not shown).

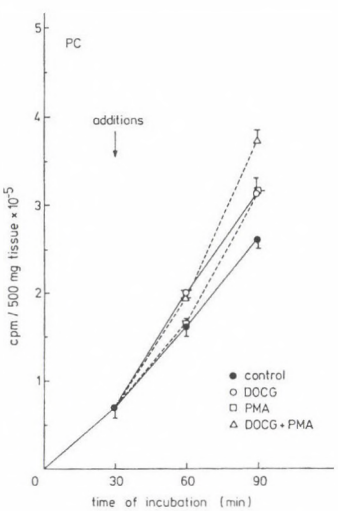

Fig. 1. Effect of PMA and DOCG on the time course of labeling of phosphatidylcholine with (^{32}P)phosphate.

Placental mince (500 mg) was incubated under the standard conditions with 50 μCi (^{32}P)phosphate. PMA (1 μM) and/or DOCG (250 μM) were added to the respective incubates after 30 min preincubation, while controls were given the solvent (DMSO) only. Each point represents a mean value $\pm$ deviation from the mean (error bars) obtained from duplicate incubations. Solid circles: control, open circles: DOCG, squares: PMA, triangles: PMA + DOCG. Time courses obtained with PMA in the incubates are drawn with dashed line. The data are representative out of three experiments yielding similar results

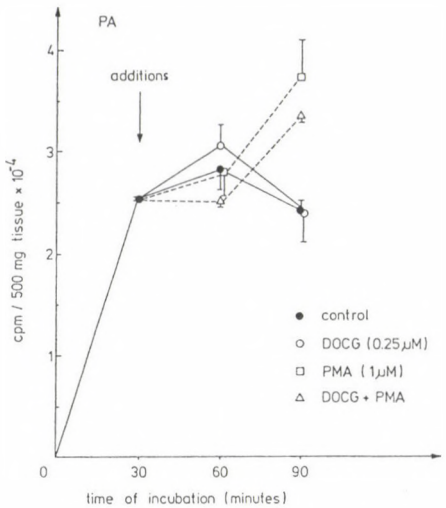

Fig. 2. Effects of PMA and DOCG on the time course of labeling of phosphatidic acid with (^{32}P)phosphate. Experimental details and symbols are described in legend to Fig. 1. Time courses obtained with PMA are shown by dashed lines. Representative results out of three experiments yielding similar result are presented

In a series of experiments PMA was added at the onset, whereas DOCG at 20 min of the incubation and the labeling of phosphatidylcholine, PC_{DOCG} and phosphatidic acid with (^{32}P)phosphate was determined after 60 min incubation. It is shown by Fig. 3 (left panel) that PMA (1 μM) and two different final concentrations of DOCG (125 and 250 μM) enhance the rate of labeling of phosphatidylcholine by 20–25% relative to control, whereas PMA augments the effect of DOCG up to 50–55% over the control value. It should be noted that PMA increases significantly ($P < 0.05$) the labeling of phosphatidylcholine in the presence of DOCG relative to the incubates containing DOCG alone. As it is shown in the right panel, PMA does not influence the labeling rate of PC_{DOCG} measured in the presence of either 125 or 250 μM DOCG. Time-course studies revealed an abrupt rise in the labeling of PC_{DOCG} within 30 min after the addition of DOCG, followed by a rapid decline in the next 30 min (data not shown). Such a time course is consistent with a rapid metabolism of DOCG and PC_{DOCG} in the placenta tissue. In the experiments shown by Fig. 3, PMA increased significantly ($P < 0.05$) the labeling of phosphatidic acid either in the presence or absence of 250 μM DOCG. On the other hand, no change in the labeling of lyso-phosphatidylcholine was observed (data not shown).

In order to present direct evidence for the presence of phosphatidylcholine-specific phospholipase D activity in the placenta tissue, we first labeled the phosphatidylcholine with (^{3}H)myristic acid and then investigated the formation of (^{3}H)phosphatidylethanol in response to PMA and DOCG added either individually or together to the incubates (Fig. 4). The experimental protocol allowed a comparison of the results with that shown by Fig. 2. PMA increases the rate of phosphatidylethanol

formation about 2.4-fold over the control either in the absence or presence of 125 or 250 μM DOCG (Fig. 4). When added alone, these concentrations of DOCG resulted in smaller increases (40 and 80%, respectively) and these effects were not additive with those exerted by PMA. These findings indicate that both PMA and DOCG affects the same enzyme activity. This enzyme must be a phosphatidylcholine-selective phospholipase D, because phosphatidylcholine was the only phospholipid that accumulated significant amount of label from (^{3}H)myristic acid and the labeling of phosphatidylcholine tended to decrease selectively in response to PMA (data not shown).

Finally, we wished to gain evidence whether protein kinase C activity was involved in the stimulatory effect of PMA on phosphatidylcholine turnover measured by labeling with (^{32}P)phosphate. We found that various protein kinase C inhibitors, including staurosporin (2 μM), sphingosine (250 μM) or 1-0-hexadecyl-2-0-methyl-sn-glycerol (HMG, 250 μM) inhibit the effect of PMA (data not shown).

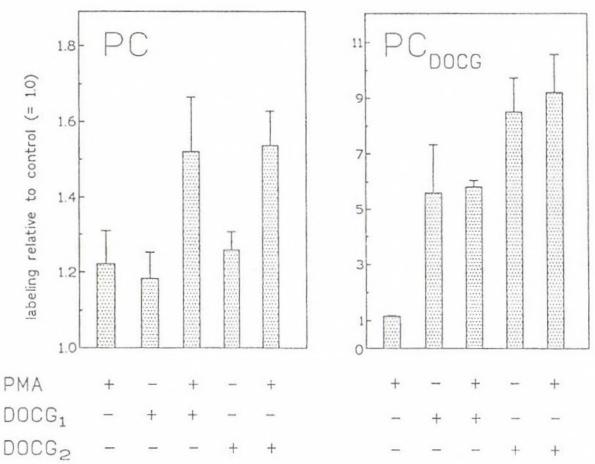

Fig. 3. Effect of PMA in the presence and absence of two different concentrations of DOCG, on the labeling of phosphatidylcholine (left panel) and PC_{DOCG} (right panel) with (^{32}P)phosphate.
Placental mince (500 mg) was incubated in 2.0 ml modified Hanks solution for 60 min, under the standard conditions, with 25 μCi (^{32}P)phosphate. Where indicated PMA (1 μM) was present throughout the incubation period. DOCG was added at 20 min in 125 μM ($DOCG_1$) or 250 μM ($DOCG_2$) final concentrations. Controls were given the solvent (DMSO). Data are from 4–6 experiments with triplicate incubations each, except those for PMA + $DOCG_1$ which were obtained from 2 experiments. Results in each experiment were normalized relative to the average of control values obtained in the same experiment (average = 1.00). In the case of PC_{DOCG}, average of the background cpm values measured in the PC_{DOCG} regions of the TLC lanes of control incubates served as reference for normalization. Mean values of normalized data ± SEM, or ± deviation from the mean (if n = 2), are presented. Average of control values are 139.926 and 4.131 cpm/500 mg tissue for phosphatidylcholine and PC_{DOCG}, respectively. Statistical analysis for phosphatidylcholine values: $DOCG_2$ vs. control: $P < 0.01$; PMA, $DOCG_2$ vs. control: $P < 0.01$; PMA, $DOCG_2$ vs. $DOCG_2$: $P < 0.05$; PMA, $DOCG_2$ vs. PMA: $P < 0.05$

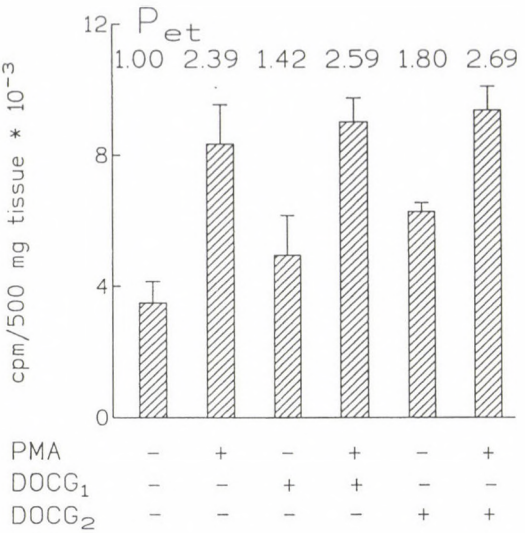

Fig. 4. Effects of β-phorbol ester (PMA) and two different concentrations of dioctaoylglycerol ($DOCG_1$, $DOCG_2$) on the rate of formation of phosphatidylethanol from phosphatidylcholine labeled with (^{3}H)myristic acid.

Minced placenta (500 mg) was incubated in 2 ml Hanks medium at 37 °C under continuous shaking in open vials with 5 μCi/ml (^{3}H)myristic acid. PMA (1 μM final conc.) or the solvent (DMSO) was added after 30 min incubation. After 50 min incubation to some of the incubates DOCG was added in 125 and 250 μM final concentrations, whereas the rest were given only the solvent (DMSO). At 60 min all incubates received 1.5% ethanol (final concentration). Then incubations were continued for a total time of 90 min. Labeling of phosphatidylethanol was determined by TLC procedure. Results from 3 experiments with triplicate incubations each are presented as mean ± SE (error bars) or for the control, mean ± SD. Ratios related to control (=1.00) are shown above the columns. All results obtained with PMA present in the incubate are significantly different from control ($P < 0.02$) and from incubates with $DOCG_1$ or $DOCG_2$ ($P < 0.05$).

Discussion

In previous studies from our laboratory [27, 28] we have reported that conversion of diacylglycerol into phosphatidylcholine and an attending stimulation of phosphatidylcholine synthesis represent a more efficient diacylglycerol signal-attenuator mechanism than the formation of phosphatidic acid by diacylglycerol-kinase in the primordial human placenta. This conclusion has been reached on the basis of the findings that (1) a synthetic diacylglycerol, dioctanoylglycerol (DOCG) is metabolized into PC_{DOCG} rather than PA_{DOCG}, (2) DOCG stimulates labeling of phosphatidylcholine with (^{32}P)phosphate without an effect on phosphatidic acid labeling, (3) formation of phosphatidic acid and PA_{DOCG} is not inhibited by the diacylglycerol-kinase inhibitor diacylglycerol-analog, dioctanoylethyleneglycol

(DOEG) but (4) DOEG inhibits the basal rate of labeling as well as the DOCG-stimulated labeling of endogenous phosphatidylcholine and the formation of (^{32}P)PC_{DOCG}. The present findings provide supportive evidence that PMA, a β-phorbol ester, can promote via a protein kinase C mediated mechanism, the turnover of phosphatidylcholine with an attendant rise of phosphatidic acid level. Moreover, it can stimulate more than twofold a phosphatidylcholine-selective phospholipase D activity. Collectively, these findings strongly suggest the presence of a mechanism in the rapidly growing primordial placenta that degrades phosphatidylcholine into phosphatidic acid and synthetizes rapidly phosphatidylcholine from free diacylglycerol. Since the release of phosphatidic acid is accompanied by the increase of diacylglycerol formation (Tóth, M., unpublished observation), this mechanism may well be responsible both for the generation and termination of diacylglycerol signals in this tissue.

The PMA or DOCG-induced increased labeling rate of phosphatidylcholine with (^{32}P)phosphate is not related to an enhanced formation of phosphatidylcholine from glycolytic or lipolytic intermediates, since neither PMA (Tóth, M., manuscript in preparation) nor DOCG [27] is able to increase the labeling of phosphatidylcholine from (^{3}H)glucose or (^{3}H)glycerol. Furthermore, the present results clearly indicate that in placenta tissue incubated with DOCG and (^{32}P)phosphate, PMA does not exhibit any effect on the rate of formation of (^{32}P)PC_{DOCG}, therefore a PMA action occurring via the de novo biosynthetic pathway of phosphatidylcholine can be ruled out. On the other hand, the additive stimulatory effect observed with PMA and DOCG on the labeling of phosphatidylcholine (Fig. 3) and the difference between the time courses of stimulation by these agents (Fig. 1) favor the view that PMA promotes phosphatidylcholine turnover, whereas DOCG stimulates primarily the incorporation of (^{32}P)phosphate into phosphatidylcholine via the activation of CTP: phosphatidylcholine cytidylyl-transferase activity [26]. The lack of additivity of PMA and DOCG effects on phosphatidylethanol formation (Fig. 4) confirms this conclusion.

The present findings indicate that DOCG is metabolized rapidly in placenta tissue and one of the major products is PC_{DOCG}. In addition, DOCG also increases the labeling of endogenous phosphatidylcholine with (^{32}P)phosphate. It should be noted that increased labeling of phosphatidylcholine with (^{32}P)phosphate can stem from the enhanced activity of cytidylyl transferase even if there is no change in the net synthesis of phosphatidylcholine. DOCG has been found to be metabolized rapidly in a number of tissues [1, 3, 13, 25] and the time course of PC_{DOCG} formation indicates that most of the DOCG are metabolized within 30 min in the early placenta, too. The rapid metabolic clearence may be the cause of the inability of DOCG to stimulate phosphatidylcholine turnover in a fashion similar to that provoked by PMA (Fig. 1), that is, following a lag period of about 30 min. Our recent finding supports this view: when DOCG has been added intermittently in two doses, 30 min apart, there is a stimulatory response after 60 min incubation in the phosphatidylcholine labeling which does not show additivity with the PMA effect (Tóth, M., manuscript in preparation).

In summary, the present and previous results furnish supportive evidence for the presence and putative signaling role of phosphatidylcholine-cycle in the human primordial placenta. These findings warrant further studies on the role of phosphatidylcholine-cycle in diacylglycerol generation for the control of growth and differentiation of the human early placenta.

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Regulation of prostaglandin endoperoxide H_2 synthase in term human gestational tissues

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Increased production of prostaglandins by the gestational tissues is pivotal for the initiation and maintenance of human labour. A major source of prostaglandins in the pregnant human uterus is the amnion membrane, which synthesizes increased amounts of prostaglandin E_2 (PGE_2) at parturition. We have found that the activity of prostaglandin endoperoxide H_2 synthase (PGHS), the enzyme catalyzing the committing step of prostanoid biosynthesis, increases significantly in the amnion at term and preterm labour, and also prior to the onset of clinical labour at term. Furthermore, the abundance of the mRNA encoding the inducible PGHS-2 isoenzyme was higher in the amnion after spontaneous delivery that before labour. The level of the constitutive PGHS-1 mRNA remained unchanged. In addition, we found a significant positive correlation between PGHS activity and the level of PGHS-2 mRNA, but not of PGHS-1 mRNA, in the individual tissue samples, also indicating that PGHS-2 was selectively induced in the amnion membrane at labour. The regulation of PGHS expression by agonists was studied using primary cultures of amnion cells. Glucocorticoid treatment enhanced the activity of PGHS and the level of PGHS-2 mRNA in the cultured cells, without affecting PGHS-1 mRNA abundance. The stimulation was glucocorticoid specific and was blocked by the glucocorticoid receptor antagonist RU486, suggesting that it was mediated by the glucocorticoid receptor. Inhibition of protein synthesis did not block the accumulation of PGHS-2 mRNA showing that the steroid acted directly, without inducing an intervening protein. Protein kinase C activator and protein phosphatase inhibitor compounds and epidermal growth factor also promoted PGHS-2

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mRNA expression, demonstrating the involvement of protein kinase dependent mechanisms in PGHS-2 regulation. However, the role of these effectors in the in vivo control of PGHS-2 expression remains to be determined.

Keywords: parturition, amnion, prostaglandins, cyclooxygenase, glucocorticoids

Prostaglandins are a group of oxygenated derivatives of the polyunsaturated fatty acid, arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid (all-Z)-). They are produced by many different cells and in a variety of organisms, and act as local regulators of tissue function. One of the earliest recognized effects of the prostaglandins was the modulation of myometrial contractility [1]. Two of the naturally occurring, primary prostaglandins, Prostaglandin E_2 (PGE_2, prosta-5,13-dien-1-oic acid, 11,15-dihydroxy-9-oxo-, (5Z,11α,13E,15S)-) and Prostaglandin $F_{2\alpha}$ ($PGE_{2\alpha}$, prosta-5,13-dien-1-oic acid, 9,11,15-trihydroxy-(5Z,9α,11α,13E,15S)-), are powerful stimulants of myometrial contractions, while Prostacyclin (PGI_2, prosta-5,13-dien-1-oic acid, 6,9-epoxy-11,15-dihydroxy-(5Z,9α,11α,13E,15S)-) is relaxant [1]. Research during the past 10–15 years firmly established prostaglandins as key regulators of uterine function at parturition in a number of species.

In women, three lines of evidence point to a pivotal role of prostaglandins in the initiation and maintenance of the parturition process [2]. First, systemic or local administration of PGE_2 or $PGF_{2\alpha}$ induces labour. Prostaglandins are used clinically for promoting labour, especially to enhance cervical ripening [3]. Second, drugs that inhibit prostaglandin biosynthesis, such as indomethacin or aspirin, significantly prolong gestation and protract labour itself [4]. Indomethacin is employed in the obstetrical practice to delay preterm delivery and provide time for exogenous glucocorticoids to expedite lung maturation improving the chances of the premature infant for survival. Third, PGE_2 and $PGF_{2\alpha}$ accumulate in the amniotic fluid shortly before and during active labour suggesting that parturition is associated with an increase of prostaglandin production by the gestational tissues [4, 5]. In agreement with this, the concentration of the predominant $PGF_{2\alpha}$ metabolite increases in maternal blood during labour [4, 5].

Experiments with isolated gestational tissues indicated that the major source of intrauterine PGE_2 is the amnion membrane, while the decidua produces PGE_2 as well as $PGF_{2\alpha}$ [5, 6]. The chorion (laeve) contains high levels of prostaglandin metabolizing activity. The amnion is essentially devoid of prostaglandin inactivating enzymes. Importantly, term amnion tissue was found to synthesize significantly more PGE_2 after delivery than before the spontaneous onset of labour. Measurements of phospholipase (A_2 and C) and lipase activities, responsible for arachidonic acid release in the amnion membrane, showed no abrupt increases concomitant with labour onset and enhanced prostaglandin output [6]. Therefore, we have focused our studies on the irreversible, committing step of prostanoid biosynthesis subsequent to arachidonate release, which is catalysed by the enzyme prostaglandin endoperoxide H_2 synthase (PGHS, cyclooxygenase; E.C. 1.14.99.1) [7]. PGHS is a membrane bound enzyme that oxygenates arachidonic acid to form the prostaglandin endoperoxides PGG_2 and

PGH_2, the immediate precursors of the primary prostaglandins and the thromboxanes. PGHS has two isoforms, which are the products of separate genes [8]. PGHS-1 is expressed constitutively in diverse cell types, while the PGHS-2 isoenzyme may be induced by mitogens, cytokines and endotoxin. In this article, our studies concerning the labour related regulation of PGHS in the human amnion, an archetypal gestational tissue, are summarized.

Methods

Patients

Tissues were collected from patients who delivered either spontaneously (SL), or by elective Caesarean section (CS) before the onset of clinical labour. The mean gestational age of the term SL and CS patient groups was not different, with a range of 37–41 weeks. Delivery after less than 36 completed weeks of gestation was defined as preterm. The mean gestational age of the preterm SL group was 31.3 ± 1.4 weeks, and that of the preterm CS group was 29.1 ± 2.4 weeks (not significantly different, $n=10$ for both groups). The tissues were examined histologically, and those which showed no signs of infection and inflammation were included in the studies. The tissue collection protocol was approved by the University of Alberta Ethics Review Committee.

PGHS enzyme activity

PGHS activity was measured as PGE_2 produced by the particulate (microsomal) fraction of amnion tissue homogenates, as described [9]. Assay conditions were established with PGE_2 production proportional to the amount of microsomal protein added to the reaction mixtures. PGE_2 was determined by RIA after extraction using Sep-Pak C18 cartridges. Enzyme (specific) activity was expressed as pg PGE_2 production/μg microsomal protein/min.

PGHS protein

PGHS protein abundance was determined in the microsomal fractions by immunoblotting. A polyclonal antibody raised against a highly conserved and isoform specific C-terminal peptide of the murine PGHS-2 was used to detect PGHS-2 protein. The polyclonal antibody recognizing both PGHS-1 and PGHS-2 was generated using purified sheep seminal vesicle PGHS as antigen. The specificity of the immunoreactions was established in control experiments with purified ovine PGHS-1 and recombinant chicken PGHS-2 as standards. The complete description of the immunoblotting procedure has been published elsewhere [9].

PGHS mRNAs

PGHS-1 and PGHS-2 levels were measured by ribonuclease protection. Radioactively labelled antisense riboprobes were generated by the *in vitro* transcription of subcloned human PGHS-1 and PGHS-2 cDNA sequences corresponding to ORF positions 1066–1374 and 1516–1925, respectively. Specific recognition of the homologous mRNAs was ensured by selecting highly divergent sequences for subcloning, experimental testing of the probes by Northern blot analysis, and lack of protection of probes transcribed in the sense direction. Total RNA was extracted from tissues using acidic guanidium thiocyanate, while cytoplasmic RNA was isolated from cultured cells following lysis with non-ionic

detergent. For reference, γ-actin mRNA levels were determined in each total RNA sample with a separate RNAse protection assay. The detailed description of our procedures of mRNA analysis has been published previously [10, 11].

Cell culture

Amnion membranes from spontaneously delivered term placentas were digested in a mixture of collagenase, dispase and hyaluronidase, followed by trypsin. The dispersed cells, cultured in medium supplemented with 10% (v/v) fetal bovine serum (FBS), cortisol and EGF (epiderman growth factor), grew to confluent monolayers within 6–8 days. The confluent cultures were incubated in medium containing 1% FBS for 2 days before experiments in order to achieve quiescence. The amnion cell culture system has been described [11].

Statistical analyses

Band intensities were determined by laser densitometry and integration. Statistical comparisons between average band intensities, enzyme activity values and gestational age distributions were performed by t-tests, and, where appropriate, by analysis of variance (ANOVA). Differences were considered significant at $P<0.05$.

Results

PGHS activity and protein

PGHS activity in the microsomal fraction of amnion tissue at term before the onset of labour was 18.2 ± 3.7 pg $PGE_2/\mu g$ protein/min (mean$\pm$SE, $n=19$ patients). Following spontaneous labour, the activity increased to 38.9 ± 6.0 pg/μg protein/min ($n=19$ patients; $P<0.05$). Preterm labour was also associated with a significant, nearly 5-fold increase of PGHS activity (5.9 ± 1.8 vs. 28.3 ± 6.8 pg $PGE_2/\mu g$ protein/min, $n=9$ CS and 10 SL patients, respectively, $P<0.05$). Figure 1 shows the level of PGHS activity in the CS (no labour) group, as the function of gestational age. The scatergram suggests that there is an increase of enzyme activity at term before the spontaneous onset of labour.

PGHS protein levels were assessed by immunoblotting microsomal proteins using a polyclonal antibody raised against a unique C-terminal peptide sequence of PGHS-2. Immunoreactive PGHS-2 or 70 kDa as well as 50–53 kDa degradation products were detected in most patients, however, no correlation was found between the level of enzyme activity and the amount of immunoreactive enzyme protein. Immunoblotting with antibody recognizing both PGHS isoforms also failed to demonstrate a significant correlation between enzyme activity and protein levels. These observations suggested the possibility that inactive enzyme protein was present in the tissue preparations in addition to active PGHS, perhaps as a result of the well-documented property of the enzyme for autocatalytic inactivation [7].

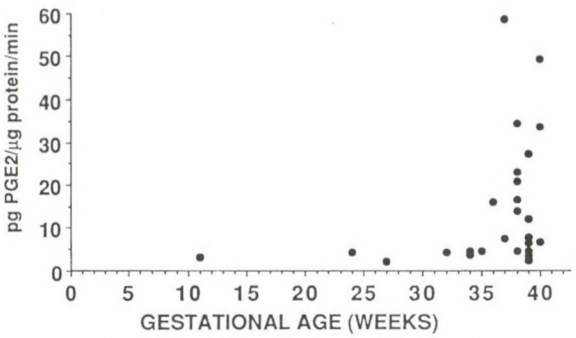

Fig. 1. PGHS specific activity in the human amnion during pregnancy. Points represent individual patients who were delivered by elective Caesarian section at different times of gestation. (Modified from Teixeira et al. [9], with permission.)

PGHS mRNA

The expression of the two PGHS isoenzymes was further evaluated by measuring the levels of PGHS-1 and PGHS-2 mRNAs in amnion membranes collected before and after spontaneous term labour. Highly selective and sensitive ribonuclease protection assays were used to determine the abundance of the isoform specific PGHS mRNAs in the tissues. The antisense RNA probes recognized their cognate 2.8 kb and 4.8 kb PGHS-1 and PGHS-2 mRNA species, respectively, on Northern blots of total or polyA-enriched RNA fractions isolated from amnion and decidua. Ribonuclease protection of the PGHS-2 prove and a reference probe (hybridising with γ-actin mRNA) by total RNA from amnion in two representative series of samples is shown in Fig. 2. The intensity of the autoradiographic bands generated by the protected fragment of the antisense probes (400 bases in the case of PGHS-2) is proportional to the abundance of the respective mRNAs. The signal intensity was measured by densitometry, followed by statistical analysis. While gamma actin mRNA levels were relatively constant, PGHS-2 mRNA abundance was approximately 2.3 times higher in tissues collected after than before labour ($P<0.02$, by two-way ANOVA, $n=15$ and 16 CS and SL patients, respectively). PGHS-1 mRNA was also detected (not shown), however, its abundance did not change with labour. Further, PGHS activity was determined and matched with PGHS mRNA abundance in the same tissues (Fig. 2). There was a significant positive correlation ($P<0.05$) between PGHS activity and PGHS-2 mRNA levels. PGHS-1 mRNA levels did not correlate with enzyme activity.

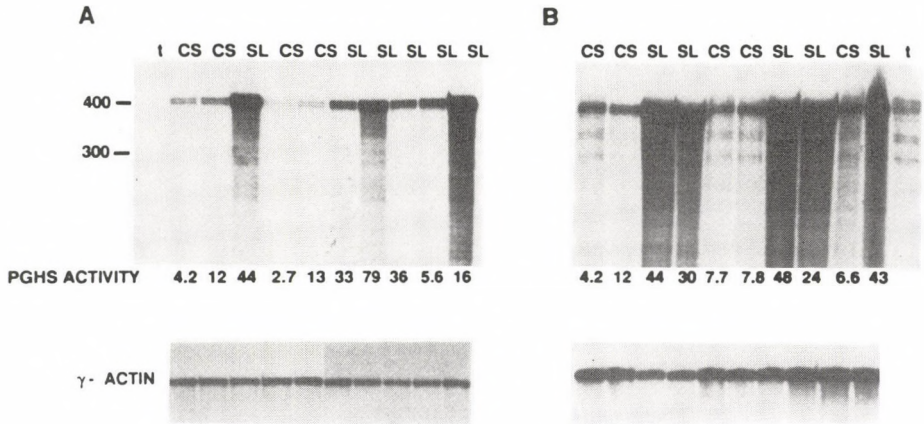

Fig. 2. PGHS-2 mRNA levels in the human amnion. Total RNA was isolated from amnion membranes after elective Caesarian section at term (CS), or term spontaneous labour (SL). PGHS-2 mRNA abundance was measured by ribonuclease protection. The positions of the 300 and 400 base size markers are indicated on the left. The band protected by PGHS-2 mRNA is approximately 400 bases long. Assay backgrounds were determined by hybridizing yeast tRNA with the radioactive probe (t). Gamma actin mRNA levels in the same tissues, measured with separate ribonuclease protection assays, are shown at the bottom of the panels. Each lane represents tissue from a single patient. PGHS activity values in the same tissues are also presented (pg $PGE_2/\mu g$ microsomal protein/min). Panels A and B show the autoradiograms of separate ribonuclease protection assays. (Reproduced from Hirst et al. [10], with permission.)

Regulation of PGHS

The regulation of PGHS expression by agonists was studied in primary amnion cell cultures. Cells released from amnion membranes by enzymatic treatment grew to confluence in media supplemented with 10% fetal bovine serum within 6 to 8 days. The confluent cultures were kept in medium with 1% serum for 2 days before agonist treatments. The PGE_2 production of these quiescent cells was very low, often unmeasurable with RIA even in the presence of exogeneous arachidonic acid. This indicates that the cultured amnion cells lost their PGHS activity that was present in the fresh tissue.

Estrogen and progesterone failed to restore PGHS activity in the cell cultures. Cortisol and the synthetic glucocorticoid dexamethasone (DEX) increased PGHS activity in the cultured cells with EC_{50} values of approximately 45 nM and 5.5 nM, respectively. The stimulation was blocked by the protein synthesis inhibitor cycloheximide and the RNA synthesis inhibitor actinomycin D. Pretreatment of the cells with acetylsalicylic acid, a specific irreversible inhibitor of PGHS, did not prevent dexamethasone from inducing PGHS activity. These observations suggested that the glucocorticoids stimulated *de novo* enzyme synthesis. The subcellular mechanism of the enzyme induction was studied by measuring the level of PGHS mRNAs in the glucocorticoid treated cultures. Figure 3 shows the effect of DEX on

the abundance of mRNAs encoding PGHS-2 (Panel A), PGHS-1 (Panel B), and, for reference, γ-actin (Panel C). Untreated (control) cells contained low, often undetectable levels of PGHS-1 and PGHS-2 mRNAs, which was in agreement with the diminished prostaglandin output. Dexamethasone (100 nM) caused a slight increase of PGHS-2 mRNA abundance after 4 h, and marked accumulation of this message at 8 h and 16 h of treatment. PGHS-1 mRNA remained undetectable during the glucocorticoid treatment. The level of the constitutively expressed γ-actin mRNA was also unaffected by the steroid. The induction of PGHS-2 mRNA was concomitant with an increase of PGHS activity in the cells. Arachidonic acid-stimulated PGE_2 output was enhanced marginally at 4 h, and significantly ($P<0.05$) after 8 h and 16 h of DEX treatment (not shown). Thus, DEX selectively increased the expression of PGHS-2 mRNA, which was utilized by the cells to produce active enzyme protein.

Cortisol also induced PGHS-2 mRNA expression, but with lower efficacy than DEX. Progesterone and estradiol were ineffective. The glucocorticoid receptor antagonist RU 486 (Mifepristone) abolished the stimulation of PGHS-2 mRNA level and PGHS activity when it was added to the treatment media in tenfold molar excess of DEX. These observations indicated that the glucocorticoid receptor was involved in the induction process.

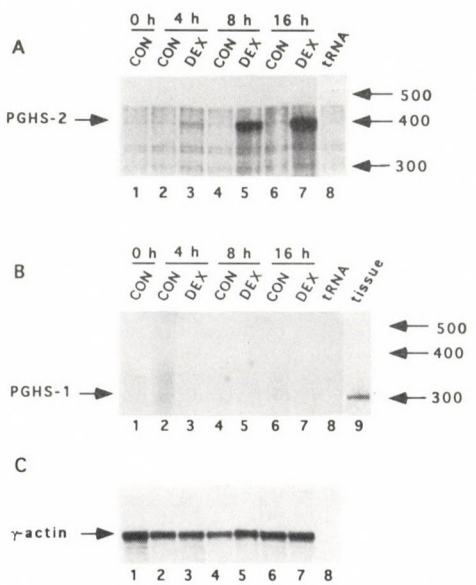

Fig. 3. Time course of dexamethasone action in primary cultures of amnion cells. The cells were treated with dexamethasone (DEX, 100 nM), or vehicle (CON) for 0 h, 4 h, 8 h, and 16 h. PGHS-2 mRNA (Panel A), PGHS-1 mRNA (Panel B), and γ-actin mRNA (Panel C) levels in the cytoplasmic RNA fraction were determined by ribonuclease protection assays. The assay backgrounds (tRNA) are also shown. The tissue sample of Panel B, Lane 8, was used as positive control. The positions of the protected probes and the molecular size markers, measured in bases, are indicated by the arrows on the left and right side, respectively. (Reproduced from Zakar et al. [11], with permission.)

Cycloheximide potentiated the effect of DEX on PGHS-2 mRNA expression. Slight non-specific stimulation of the basal levels of PGHS-2 and PGHS-1 mRNAs as well as the γ-actin mRNA was also detected in the presence of the drug (not shown). The cycloheximide concentrations used in these experiments (10 μg/ml an 40 μg/ml) inhibited the incorporation of amino acids into amnion cell proteins by more than 95% [12]. Therefore, it may be concluded that DEX stimulated PGHS-2 expression in an immediate fashion, without the induction of an intervening protein. In agreement with this, no secreted factor(s) fostering prostaglandin production were detected in media conditioned by glucocorticoid treated cells [11].

Activators of the calcium and phospholipid dependent protein kinase C (PKC) such as the phorbol ester TPA, also enhanced PGHS activity in the cultured amnion cells in a protein and RNA synthesis dependent manner [12]. Preliminary data indicate that this effect was due to the selective induction of PGHS-2. Epidermal growth factor (EGF) and the phosphoserine and phosphothreonine specific protein phosphatase inhibitor okadaic acid induced PGHS-2 expression as well, suggesting that this enzyme is under the control of protein kinase-phosphatase system(s) in addition to glucocorticoids. Work is in progress in our laboratory to identify the individual steps of this regulatory cascade.

Discussion

Our results demonstrate that a major mechanism responsible for the labour-associated increase of prostaglandin production by the human amnion is the induction of PGHS. PGHS activity increased significantly during term and preterm labour, and the steady state level of the mRNA encoding PGHS-2, but not PGHS-1, was elevated following spontaneous delivery as compared to prior to the onset of clinical labour. Moreover, PGHS activity exhibited positive correlation with PGHS-2 mRNA levels, further indicating that the PGHS-2 isoenzyme was induced during labour. The lack of correlation of PGHS-2 immunoreactive protein versus enzyme activity or mRNA levels suggests that varying amounts of inactive PGHS protein was present in the tissues. Rapid autocatalytic inactivation is an intrinsic property of PGHS-1 [7], and possibly PGHS-2 as well. Such "suicide" reactions do not result in the loss of immunoreactivity of the enzyme protein. Since free arachidonic acid is mobilized in the amnion and accumulates in the amniotic fluid at labour [6, 13], inactive enzyme protein is likely to be present in the tissue. Newly synthesized, active PGHS therefore may correlate well with the level of its respective mRNA, but not necessarily with the total amount of immunoreactive enzyme protein. Metabolic labeling and immunoprecipitation of PGHS proteins will be performed to validate this possibility.

Labour-associated increases of prostaglandin production and PGHS expression may be causative as well as consequential to the parturition process. In fact, it has been proposed that the enhanced prostaglandin synthesis of the gestational tissues is a phenomenon accompanying labour, being the result of the exposure of the decidua to

the infectious content of the vagina through the gradually opening cervix [14]. Although this plausible mechanism may contribute to PGHS stimulation during parturition, our finding that enzyme activity increases at term before the onset of clinical labour (Fig. 1) suggests that a distinct, pre-labour induction event might also take place possibly as part of an endocrine or paracrine cascade leading to labour onset.

Agonists involved in the parturient induction of PGHS activity are expected to be selective stimulants of PGHS-2 expression. Several such factors have been identified using primary amnion cell cultures. We have found that glucocorticoids, EGF, and PKC activators enhance PGHS-2 mRNA expression in the amnion cells, while others reported [15] that IL-1 is also capable of this function. Proinflammatory cytokines such as IL-1, and endotoxin may stimulate prostaglandin production by the gestational tissues during labour as pointed out above, and in cases of intrauterine infection. Cortisol, EGF/TGFα and PKC activators (e.g. oxytocin) are present in the pregnant human uterus in increasing amounts at term [16, 17], and thus may play a role in PGHS-2 induction in normal pregnancies. It has to be added as a caveat, however, that with the exception of the PKC activator TPA, none of the stimulants of PGHS expression in cultured cells enhance PGHS levels in fresh amnion tissue or freshly dispersed amnion cells [18, 19]. A possible explanation of this discrepancy is that the gestational tissues are in a maximally stimulated state *in situ*, incapable of responding further to agonists immediately after isolation. Alternatively, the responsiveness of the amnion might be related to the proliferative state of its cells, since cells growth-stimulated in culture respond to a variety of agonists, while the *in situ* growth-arrested cells may be largely unresponsive. Furthermore, the gestational tissues as well as the uterus probably undergo a process of maturation as the pregnancy advances, resulting in changing patterns of gene expression and cessation of cell growth. Oxytocin receptor level and gap junction formation increase in the myometrium [20], CRH production is enhanced in the placenta [21], and PGHS-2 mRNA accumulates in the amnion, decidua, and even in the chorion with advancing gestation. All, or most of these changes could be related to the functioning of a recently postulated "placental clock", controlling the length of pregnancy in women [22].

In conclusion, our results demonstrate that PGHS-2 induction is part of the activation process that takes place in the gestational tissues at term and preterm labour. The agonists responsible for the enzyme induction may include corticosteroids and protein kinase activators. The *in vivo* mode of action of these effectors of gestational tissue activation remains to be established.

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Effect of cadmium on female fertility, pregnancy and postnatal development in the rat

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Adult female rats having regular ovarian cycles were treated with 2.5, 5 or 10 mg/kg cadmium chloride ($CdCl_2$) during estrus or diestrus and mated 32, 80 or 132 h post-treatment. Sperm positivity was checked next day on the predicted estrus. Maternal effects during pregnancy, fetal outcome on day 10 or at term as well as postnatal development of the F_1 generation were recorded.

$CdCl_2$ caused sterility in 40 or 87% of animals at doses 5 and 10 mg/kg, respectively. Influence of Cd on fertility depended on the day of the cycle, and on the time elapsed between treatment and mating.

The Cd-caused overt toxicity in fertile female rats was expressed by dose-dependent decrease in maternal body weight gain and increased progesterone blood levels. No treatment-related alteration in number and weight of conception day 10 of pregnancy or in weight and size of litters, rate of males and females at term and during the 21-day post-parturition study could be seen.

It is concluded that Cd given prior mating may lead to sterility in a dose-dependent fashion. This is suggested to be caused by anovulation resulting from reversible pituitary disfunction. Animals proving fertile in spite of Cd-treatment have developed tolerance against Cd in terms of fetal outcome and postnatal development.

Keywords: cadmium, infertility, pregnancy, prenatal, postnatal

In recent years Cd proved to be a female reproductive toxicant in laboratory rodents. Studies, however, have focused mainly on developmental endpoints following postconception administrations. Knowledge concerning its impact on ovarian cyclic function itself has not been well established.

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In earlier literature Cd-caused serious destructive morphological changes in the ovary [11, 21] or ultrastructurally apparent injury to the microcirculation of the uterus [7], were described in prepuberal or postnatally androgenized [21], i.e. non-ovulating rats, while no morphological, or even less, functional changes of the ovary in adult rats were found [21]. Similarly no fertility effects except superovulation was shown in mature mice [27].

As to more recent evidence $CdCl_2$ in single sc. injection inhibited ovulation when administered close to the time of ovulation having a pronounced but temporary influence on fertility in Golden hamsters [23]. When given to rabbits 1 or 7 days before mating, pregnancy was interrupted in 60–75% of the does [24]. In further investigations 5 mg/kg $CdCl_2$ given as a single sc. injection 48 hour prior ovulation exerted an anovulatory effect i.e. no ova in the ampullae of oviducts could be found in 50% of rats [16]. Moreover in rats cycle-monitored for 48 days Cd was shown to dose dependently disturb the regularity of ovarian function. The ovulatory failure, however, proved to be reversible, for first, a gradual resumption of regular vaginal cornification commenced spontaneously after a 16- to 32-day block [17], second, excess amount of iv. LHRH induced ovulation in Cd-treated anovulatory rats [30].

The present study was performed in order to determine if Cd-caused decrease in ovulation rate experienced in previous studies [17, 30] would be modulated by the process of mating. We have studied if Cd-caused infertility depended on the time elapsed between treatment and mating. In animals getting pregnant in spite of Cd treatment, maternal and fetal effects, parturition and postnatal development of the F1-generation were recorded.

Materials and Methods

$CdCl_2 \times 2.5\ H_2O$ was purchased from Reanal (Budapest, Hungary), pentobarbital from Rhone Poulenc (Paris, France). Radioimmunoassay (RIA) progesterone antiserum was kindly provided by Dr. G. D. Niswender (Fort Collins, CO).

Animals

Adult virgin female and male CFY rats (LATI, Gödöllő, Hungary), weighing 200–250 g, were maintained under controlled light conditions (12 h light, 12 h dark) with free access to rat chow (LATI, Gödöllő, Hungary) and tap water. Ovarian cycle was checked daily by vaginal cytology. Animals displaying at least three 4-day cycles were selected for the experiment.

Fertility study

Experimental protocol (Fig. 1). In Experiment (Exp) A, B and C animals were treated with 2.5, 5.0, and 10 mg/kg bw $CdCl_2$ or 1.0 ml/kg 0.9% NaCl solution on the day of diestrus 2 between 8.00 and 9.00 h a.m. (Exp A and C), or on estrus (Exp B) the same time. After 32 (Exp A), 80 (Exp B), or 128 hours (Exp C) on proestrus (from 4.00 h p.m.) on females were mated with fertile males. Rats having sperm-positive vaginal smears on the next day (estrus) checked between 8.00 and 9.00 h a.m. were designated as day 1 pregnant animals.

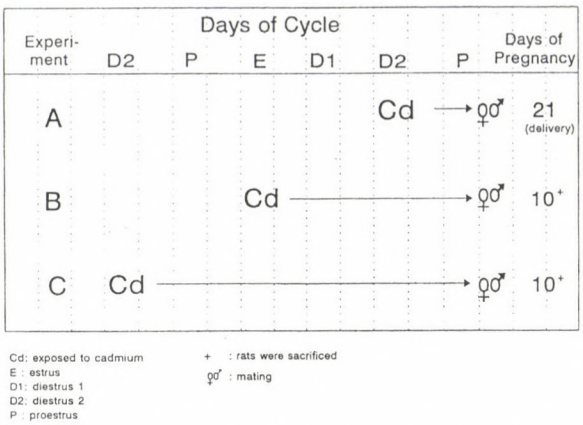

Fig. 1. Treatment protocol. Rats were administered 2.5, 5 or 10 mg/kg $CdCl_2$ sc. on selected days of the ovarian cycle and mated after post-treatment periods of various length. Control groups (not designated) were given 1 ml/kg b.w. 0.9% NaCl solution

Prenatal study

Pregnant animals of Exp B and C were sacrificed 10 days postconception (day 10 of pregnancy) under pentobarbital narcosis (40 mg/kg). Maternal body weights were measured on the day of treatment and on day 1 and 10 of pregnancy. Following laparotomy conception (both uterine horns) were excised and weighed, the number of fetuses and weight of conception were recorded. Blood samples were taken from the maternal aorta and stored at 4 °C for 24 h, centrifuged and sera were stored at -20 °C until analysis. Progesterone (P) levels were determined by RIA.

Postnatal study

In successfully mated rats of Exp A (Fig. 1) pregnancy was maintained till tirm. Pre-treatment, gestational (days 1, 7, 21) and post-delivery body weight of dams, number and weight of litters and rate of males and females were recorded. Average weight of pups on day 1, 5, and 21 post-delivery was calculated.

Statistics

Analysis of variance, Dunn's and Dunnett's multiple range test was used for statistical evaluation. In case of hormone levels calculations were made after logarithmic (ln) transformation of the data. Dose-effect curves were solved using the Litchfield-Wilcoxon method and ED_{50} values were calculated [14]. Effect of Cd on the rate of mating was evaluated by one-sided chi-square test.

Results

Fertility study

Subcutaneous $CdCl_2$-administration reduced fertility of female rats as opposed to that of saline-treated controls (Fig. 2).

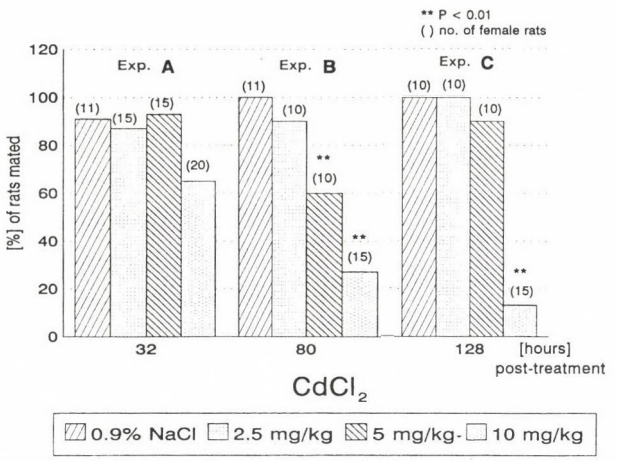

Fig. 2. Effect of $CdCl_2$ given sc. on diestrus 2 (Exp A, and C) or on estrus (Exp B) on the rate of mating in female rats

For comparing the three types of treatment Median Effective Dose (ED_{50}) for Cd to inhibit copulation was calculated on Table I. The lowest ED_{50} was found in Exp B in which treatment was carried out in oestrus followed by cohabitation with males 4 days later. Thus animals exposed according to protocol B proved to be the most susceptible to Cd.

In Exp A (32-h post-treatment interval) a decreasing tendency in the rate of mating was observed, in the 80- and 128-h post-treatment study significant dose-dependent deterioration of mating rate could be recorded.

Prenatal study (Exp B and C)

Maternal body weight gains of successfully mated rats on day 1 and day 10 of gestation (Fig. 3/a and 3/b) were significantly lower in Cd treated animals compared to controls when treatment was carried out on estrus with 10 mg/kg $CdCl_2$ or in animals treated on diestrus 2 with 10 mg/kg and on day 1 of pregnancy in the 5 mg/kg-group.

Table I

Evaluation of dose-effect curves for $CdCl_2$ given sc. to inhibit mating rate in female rats. Median Effective Doses (ED_{50}) and 95% confidence limits are given

Symbol of experiment	Day of treatment	Post-treatment period	ED_{50} [mg/kg b.w.]	Confidence interval [mg/kg b.w.]
A	Diestrus	32	10.8	6.55–17.82
B	Estrus	80	5.9	3.73–9.32
C	Diestrus	128	7.2	5.22–9.94

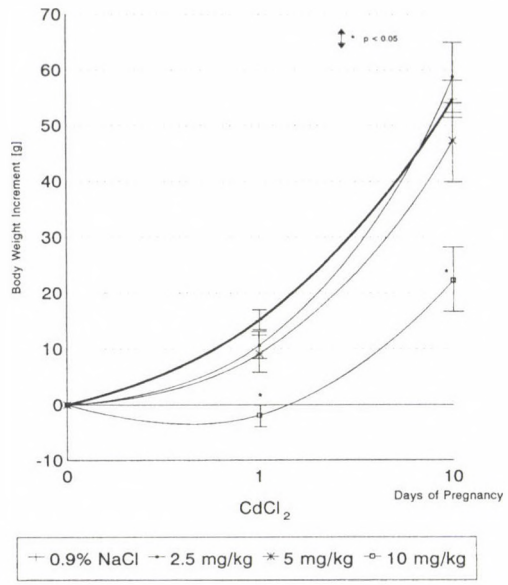

Fig. 3/a. Effect of $CdCl_2$ given sc. on oestrus followed by mating 80 h later on maternal body weight until day 10 of gestation. $\bar{x} \pm SE$ values are shown; *: significant difference from control dams

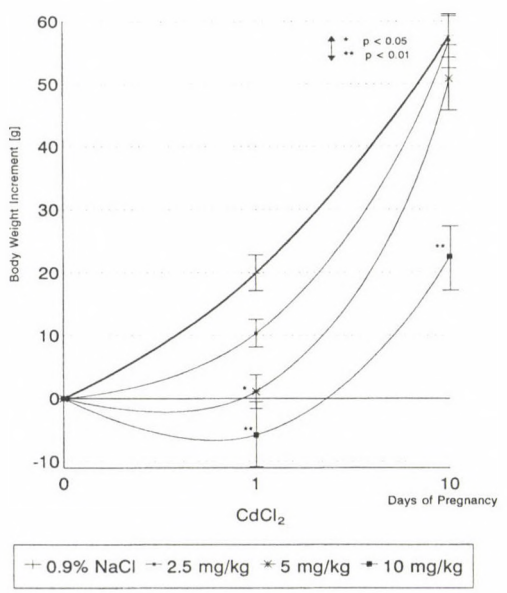

Fig. 3/b. Effect of $CdCl_2$ given sc. on diestrus 2 followed by mating 132 h later on maternal body weight until day 10 of pregnancy. $\bar{x} \pm SE$ values are shown; *,**: significant difference from control dams

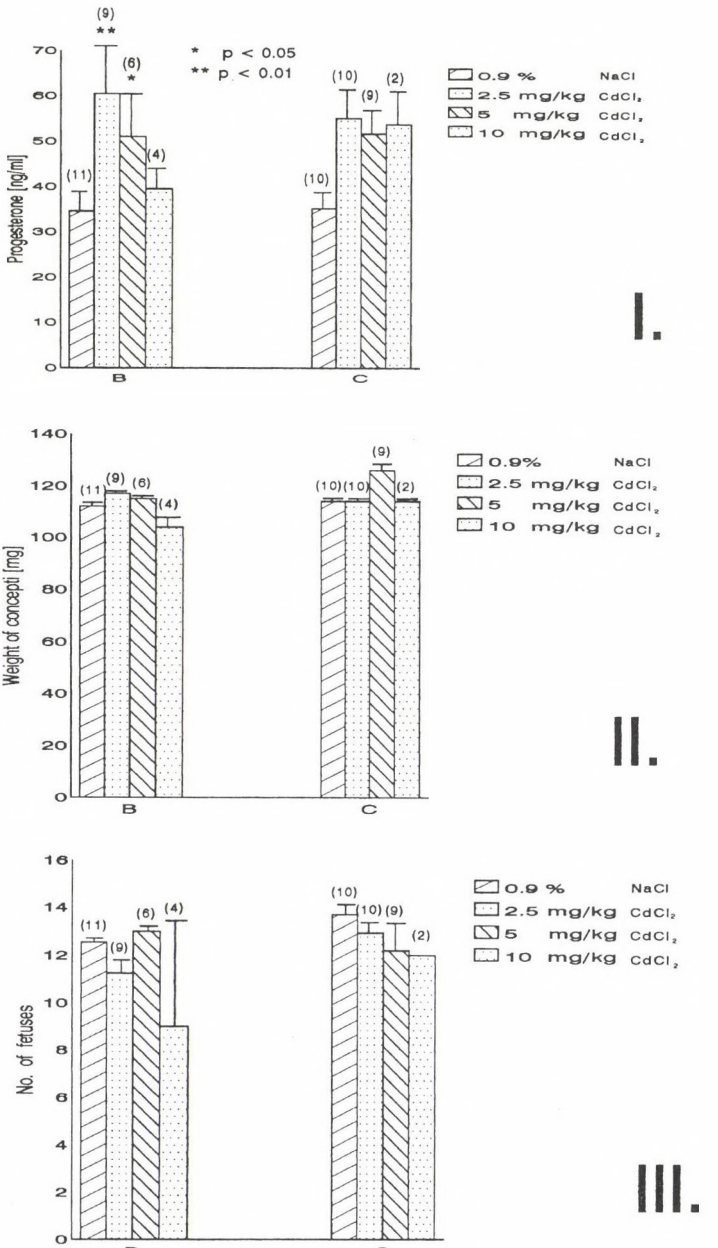

Fig. 4. Influence of sc. $CdCl_2$ given on estrus (Exp B) or on diestrus 2 (Exp C) on progesterone levels in maternal aorta blood (I), on weight of conception (II) and fetal number (III) on day 10 of pregnancy. (At the dose of 10 mg/kg no statistical evaluation was made due to the low number of pregnant animals.)

Table II

Effect of $CdCl_2$ on the postnatal development of F_1 generation until day 21 ($x \pm SE$)

DAY 1

Treatment	n	Number of litter	Number of males	Number of females	Weight of litter [g]	Weight of pup [g]
0.9% NaCl	10	9.60	4.90	4.70	59.9	6.25
1.0 ml/kg		±0.43	±0.66	±0.56	±2.59	±0.10
$CdCl_2$ [mg/kg]						
2.5	13	11.53	6.07	5.46	72.69	6.32
		±0.43	±0.50	±0.61	±2.55	±0.15
5.0	13	11.31	6.69	4.62	68.92	6.08
		±0.77	±0.54	±0.59	±3.95	±0.13
10.0	13	12.08	5.85	6.23	69.85	5.76
		±0.52	±0.58	±0.61	±4.62	±0.26

DAY 5

Treatment	n	Number of litter	Number of males	Number of females	Weight of litter [g]	Weight of pup [g]
0.9% NaCl	9	9.11	4.56	4.56	96.11	10.56
1,0 ml/kg		±0.10	±0.48	±0.63	±0.71	±0.63
$CdCl_2$ [mg/kg]						
2.5	13	10.92	5.77	5.15	108.23	9.94
		±0.70	±0.58	±0.70	±6.63	±0.23
5.0	13	11.01	6.39	4.62	105.92	9.77
		±0.73	±0.54	±0.39	±3.90	±0.13
10.0	13	11.39	5.23	6.15	107.15	9.01
		±0.65	±0.57	±0.65	±6.88	±0.34

DAY 21

Treatment	n	Number of litter	Number of males	Number of females	Weight of litter [g]	Weight of pup [g]
0.9% NaCl	9	8.44	4.33	4.11	421.40	50.53
1.0 ml/kg		±0.63	±0.65	±0.63	±27.15	±1.53
$CdCl_2$ [mg/kg]						
2.5	13	9.92	5.08	4.85	474.15	48.90
		±0.66	±0.55	±0.69	±24.69	±1.93
5.0	13	10.85	6.08	5.54	485.0	46.02
		±0.72	±0.46	±0.72	±21.40	±2.14
10.0	13	11.00	5.00	6.00	493.23	45.68
		±0.60	±0.53	±0.63	±17.55	±1.59

Concerning fetal numbers, weight of conception and P levels of maternal aorta blood no significant difference between the Cd-treated and control animals could be found (Fig. 4).

Postnatal study (Exp A)

In the Cd-treated groups a slight statistically nonsignificant retardation in maternal body weight during pregnancy and post-parturition could be recorded (not shown).

Number and weight of litters, rate of males and females recorded on day 1 after delivery were not influenced by Cd (Table II).

No change in the above data was found on day 5, and the postnatal development of the young coming from Cd-treated mothers was undisturbed till the 21st day of the study.

Discussion

Fertility studies in the present work have confirmed our earlier findings and those of others that Cd disturbes cyclic ovarian function. A single sc. $CdCl_2$ injection interfered with reproductive behaviour: depending on the dose 13–87% of female rats remained sterile due to failure of copulation. As to recent evidence no Cd-induced alteration in the light microscopic histology of ovary could be observed [16], while pituitary LH content and preovulatory blood LH levels decreased [30]. Moreover pituitary has been reported to accumulate Cd [8, 18]. Thus Cd seems to directly interfere with pituitary function.

Since Cd ions themselves soon after getting into circulation are bound to high molecular weight proteins and do not pass the blood-brain barrier [29] a direct effect on the hypothalamus is not probable. An indirect hypothalamic mediation of decreased functional capacity of the pituitary, however, cannot be excluded. It is well known that steroids stimulate certain neurons in the preoptic/anterior hypothalamic area as well as play a well estabilished potentiating action upon the response of the pituitary gonadotrophs to LHRH [9]. Lower ovarian steroid secretions during the cycle which was shown after Cd treatment could alter these mechanism [16].

Remarkable is that following the 10 mg/kg dose, the longer the interval was between the Cd-treatment and mating, the higher the rate of sterility turned out to be. $CdCl_2$ has been described to enter circulation slowly from the site of sc. injection. After 4 days 7–15% of the doses (2,2–4,4 mg/kg) could still be found there serving as a pool [12]. As to recent data Cd is sequestered by and accumulated in the pituitary and ovarian tissues. Blood levels elevate as long as 2 days following a single sc. injection while tissue concentrations rise as long as 10–12 days [18].

The long-term effect is supported by the evidence that $CdCl_2$ dose-dependently suspends regular ovarian function for 48 days or more [17].

As to our previous study after having received 5 or 10 mg/kg $CdCl_2$ only 50 or 48% of rats ovulated (checked by counting ova shed) 2 days later on the predicted estrus [16]. In the present 2-day posttreatment study being treated as to the same protocol, 93 and 65% of rats have mated and proved to be fertile. Thus presence of males and mating itself seems to have increased mating rate resulting in a higher incidence of pregnancy. However, it has not been the case with the 5-day post-treatment study: in spite of being caged with males a $CdCl_2$ treatment of 10 mg/kg lowered the fertility rate to 13% in this study and lowered the ovulation rate to 20% in the previous one [30]. That block of ovulation in the latter case, however, could be dose-dependently reversed by iv. LHRH inj. (Table III).

Despite the known olfacto-hormonal relationship the primary sensory receptor mediating the sensory signal to those areas of the brain which have been described to control mating behaviour is not well known. LHRH neurons have recently been demonstrated to originate in the epithelium of the medial olfactory pit and migrate into the brain along branches of the terminalis nerve. In the nose LHRH immunoreactivity cells and fibres are found in close association with blood vessels (reviewed by Schwanzel-Fukuda and Pfaft [26]).

Table III

Effect of mating and iv. LHRH treatment on the spontaneous ovulation rate in Cd-treated rats. Comparison of present and previous data

Reproductive function	Dose $CdCl_2$ [mg/kg]	Post-treatment time [hours]	Rate	[%]	Reference
Spontaneous ovulation	5	32	7/14	50	(16)*
	10	32	12/25	48	(16)*
Mating	5	32	14/15	93	**
	10	32	13/20	65	**
Spontaneous ovulation	10	128	2/10	20	(30)*
	10	128	2/15	13	*
LHRH treated	10	128			(30)*
2 mg/kg			4/10	40	
4 mg/kg			7/10	70	

* Data of previous (reference given) studies

** Data of present study

Olfactory environment and coitus-induced ovulation is well documented especially in cases when block of ovulation is reversible. (Pheromones act as a primer on the neuroendocrine mechanisms governing ovulation in the cyclic female rat [1].)

The reversal of Cd-induced block of ovulation with mating or excess amount of LHRH points to the reversibility of Cd caused dysfunction of the pituitary – supposedly in the synthesis of gonadotrophs [lower tissue-LH levels in Cd-treated proestrous rats, [30], or in the release as well. This inhibition at lower doses (2.5–5 mg/kg) could be overcome by the physiological olfacto-hormonal stimuli of mating process whereas at 10 mg/kg $CdCl_2$ already a pharmacologic dose of LHRH was required to counteract the Cd-induced ovulatory block.

The finding that ED_{50} value was the lowest in Exp B (treatment on estrus) can be explained by the evidence that newly formed corpora lutea seqester and accumulate Cd more rapidly and at a higher concentration that the nonluteal compartment [18]. The outcome of the following ovulation may depend on the varying susceptibility of the ever changing short-lived functional units of ovary to Cd treatment.

Cd is a proven teratogenic agent in mammals when it is given in early postimplantation on day 8 to hamsters [8, 10] in midpregnancy [25] and late gestation [22]. At the same time given on day 1 of pregnancy it does not exert deleterious effect on oviductal transport, cleavage number and location of ova in rats during the preimplantation time [19] and fails to cause malformation in the offspring examined postnatally in mice [5]. In the present study Cd was administered before conception. It induced sterility in numerous female rats depending on the dose. The rest of animals, however, were able to conceive, and conception was followed by normal pregnancy and uncomplicated delivery. No treatment-related prenatal and postnatal developmental effects were found.

The symptom of overt maternal toxicity has been the reduction in maternal body weight gain in early gestation and a slight rise in midpregnant P blood levels. Studies have demonstrated that overt maternal toxicity as defined by weight loss or mortality is not always associated with the same defined adverse developmental effect in the rat [4]. The higher blood P levels in Cd-treated rats may be due to Cd-induced decreased activity of hepatic cytochrom P450 [28] enzymes that take part in P metabolism.

Cd is able to inhibit ovulation and decrease fertility rate in rats when given during the cycle. Biological half-life of Cd is rather long (>20 years in humans), though when given prior to mating it does not seem to influence pregnancy. Maternal and fetal tolerance to Cd can partly be explained by its kinetics. It is postulated that following sc. injection superinduction of hepatic metallothionein (MT) mRNA occurs because of the continued influx of Cd over 5–10 days. MT having a high affinity for divalent cations reduces the bioavailability of highly toxic free Cd^{2+}. By the time of the already sensitive early postimplantation phase of pregnancy started the major part of circulating Cd will have been stored in the target organ, in the proximal tubular cells of the kidney.

Maternal liver and fetal portion of placentae accumulate Cd more rapidly than other organs [13]. In the placenta Cd is known to induce rapid synthesis of high amounts of MT in rats [3] and humans [6] which prevent the metal from migrating to the fetus [15]. The elevated levels of female sex hormones during gestation may also contribute to tolerance against Cd, for synthesis of MT has been described to be

induced by estradiol in the liver and kidney of rats [6] and by progesterone in humans [2]. In conclusion, present data – in good accordance with our previous results – demonstrate that female gonadal function can be disturbed by Cd at different levels [16, 18, 20] though it is anovulation to be made responsible for the acute and subacute antifertility effect of Cd. The ovulatory block is suggested to be caused by impaired pituitary function. The impairment is reversible since depending on the dose or the time elapsed between treatment and mating it can be restored spontaneously or as a result of physiological or pharmacological stimuli. The temporary but long-term disturbance of pituitary gonadotrop function leads to a considerable shortening of the reproductive lifespan in the rat. Animals getting pregnant despite the single preconception Cd treatment proved to be fairly tolerant against effects during pregnancy concerning maternal effects, fetal outcomes and postnatal development.

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Benzpyrene treatment decreases the sexual activity of adult rats, what is reversed in neonatally allylestrenol treated animals

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In neonatally allylestrenol treated animals in adult age a single benzpyrene treatment significantly decreases the female and significantly increases the male rat's sexual activity. Three month old females display the negative sexual behavioral effect of neonatal allylestrenol treatment less than the six month old ones. The benzpyrene treatment in adult age decreases the sexual activity of male rats. The experiments call the attention to the modifying effect of perinatal steroid treatments to similar exposure in adult age.

Keywords: aromatic hydrocarbons, sexual behavior, hormonal imprinting, steroids, allylestrenol

Hormonal imprinting takes place perinatally in case of the first encounter of the developing receptor and the adequate hormone [3–5]. After imprinting development of the receptor will be completed and the binding capacity characteristic to the adult age will be reached. Without imprinting the maturation of the receptor is aborted [7]. Nevertheless, in the time of maturation the receptor's discriminating capacity is not complete and because of this, materials related to the appropriate hormone (members of the same hormone family, natural or synthetic molecules etc.) also can bind to the developing receptor. This process provokes false imprinting, causing diminished or increased binding capacity of receptors for life [6].

Allylestrenol, a synthetic steroid preparation used in gynecology for saving endangered pregnancies durably decreases the number of thymic glucocorticoid [11] and uterine estrogen [8] receptors. Sexual hormone levels are also changed [8] as a consequence of the perinatal imprinting of microsomal enzyme system [1, 2, 12, 15]. The fetal or neonatal treatment with this hormone preparation dramatically decreased

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the sexual activity of adult female rats, and also provoked a profound increase in the sexual activity of adult male rats after fetal treatment [9]. Benzpyrene, an aromatic hydrocarbon (having also a steroid structure) continuously present in the polluted urban air also produced the same, except the effect on male rats.

Since any person being imprinted by allylestrenol (e.g. as a medical treatment of mothers), can be exposed to benzpyrene due to its occurrence as a pollutant, in these experiments (as a model) the sexual behavior of rats – the effect of allylestrenol administered neonatally and that of benzpyrene given in adult age – were studied.

Materials and Methods

In the the experiments 40 male and 58 female rats of our Wistar breeding stock were used. They were separated into 4 group in each sex.

Group No 1. consisted of newborn animals receiving only the vehicle (sunflower oil).

Group No 2. These animals were treated with allylestrenol (Organon, Oss, Holland) in the first, 3rd and 7th days s.c. with 17.5 μg/newborn rat, dissolved in sunflower oil.

Group No 3. Animals were treated with benzpyrene (Fluka, Buchs, Switzerland) in adult age (three months old) with a dose of 2.0 mg/1000 g body weight i.m. dissolved in sunflower oil.

Group No 4. Newborn animals were treated with allylestrenol, adults with benzpyrene.

Sexual behaviour was studied 2 weeks after treatment in three months old age, using the method of Madlafousek and Hlinák [13].

In the case of females, indicator males helped to observe their receptivity. Two parameters, the so-called Meyerson index and the lordosis quotient were determined. The former gives a binary answer for the appearance of lordotic response as a result of primary mounting by males. The latter is a ratio of the lordosis percent in ten mountings (L/M). In order to get comparable results the females within the two-week study were screened only during estrus (checked by vaginal smears).

The males were studied in a four-week period, once a week for 30 minutes. Five different patterns of behavior were distinguished. Males without any intention of mounting, intromission or ejaculation within 30 min when cohabitated with a receptive female were taken inactive. Others performed only mounting, and some of the males had intromission, too, without ejaculation. These were the sexually sluggish males. Males were considered as sexually active, when the full scale of male's copulation (mounting, intromission and ejaculation) was performed. The rest had the same characteristics but with multiple ejaculations. Details of the method are described by Dalló et al. [10]. Data obtained from both sexes were evaluated as daily averages for calculating the significance by the χ^2 probe.

Results and Discussion

Neonatal allylestrenol treatment decreased the sexual activity of females, however the difference from controls was not significant (Fig. 1). In a previous experiment in a similar case a very strong and significant decrease could be proven [9]. The only difference between the two studies is that previously six months old, this time only three months old animals were observed. Thus it seems to be possible that the inactivity deepens with the proceeding age.

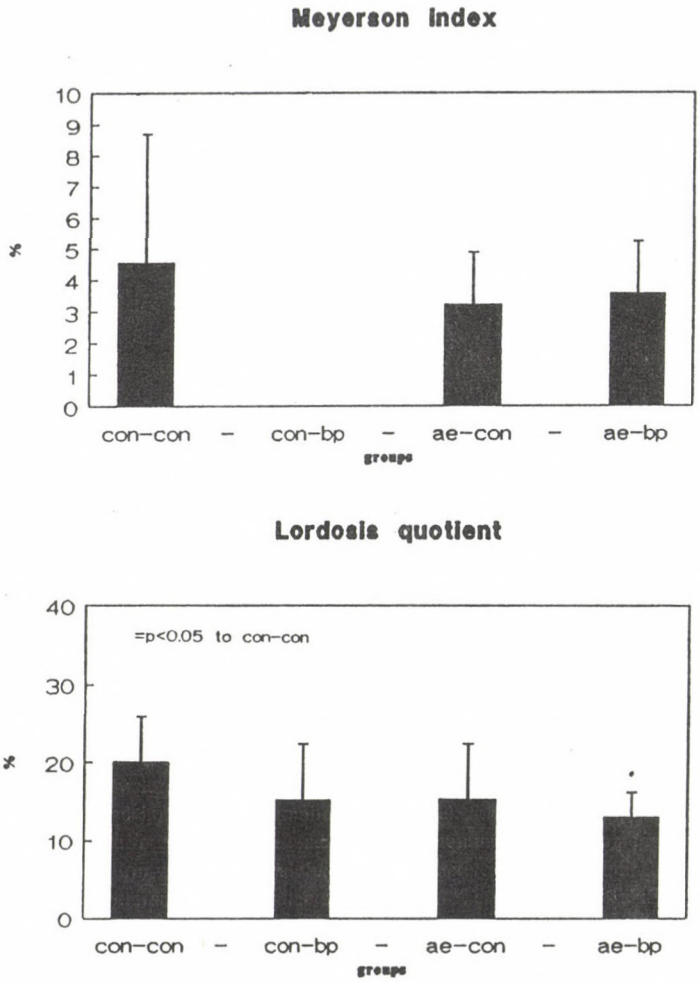

Fig. 1. Meyerson index and Lordosis quotient of female rats. Allylestrenol (ae) and benzpyrene (bp) decreased the sexual activity alike, but only in the case of double treatment was the decrease significant

No significant decrease in the lordosis quotient following benzpyrene treatment of adult females could be seen. Benzpyrene administration of neonatally allylestrenol treated females resulted in a significantly reduced sexual activity (Fig. 1). This means that the neonatal hormone treatment sensitized the animals to a later steroid-like exposure.

Table I

Details of the ejaculations

	Groups			
	con-con	con-bp	ae-con	ae-bp
Number of ejaculations	3	4	7	12
Time of the first ejaculation (min)	9.5 ±4.66	22.2 ±3.32	18.0 ±4.10	14.8 ±1.70
Time between ejaculations (min)	9.1 ±0.83	-	8.3 ±0.40	10.6 ±0.40

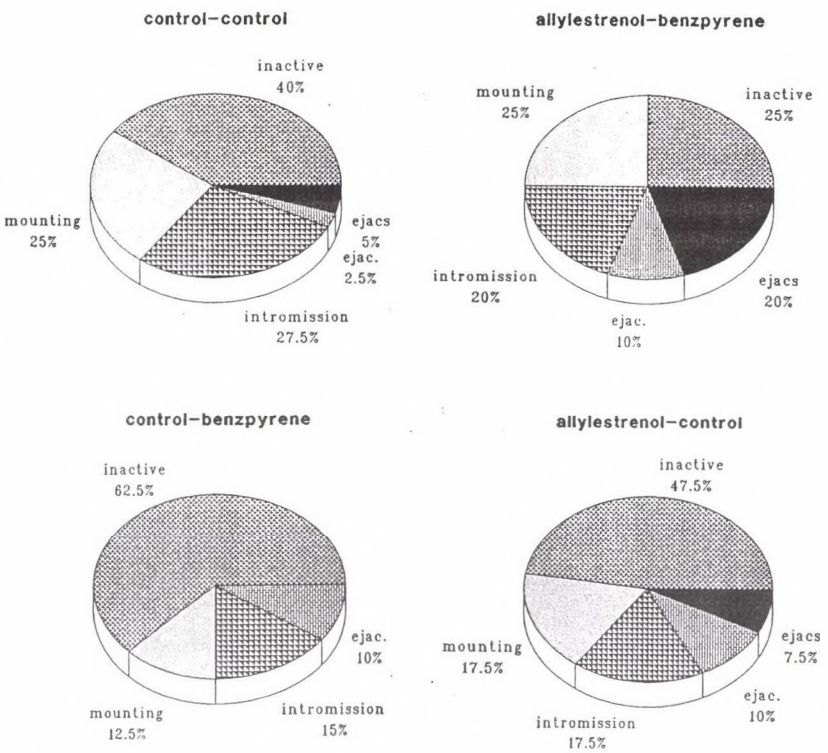

Fig. 2. Pie-diagrams of males' sexual activity. Benzpyrene treatment in adulthood decreased the activity; neonatal allylestrenol treatment was neutral. The neonatal allylestrenol treatment combined with benzpyrene treatment in adult age strongly increased the activity and the number of multiple ejaculators (ejac. = single ejaculation; ejacs = multiple ejaculations)

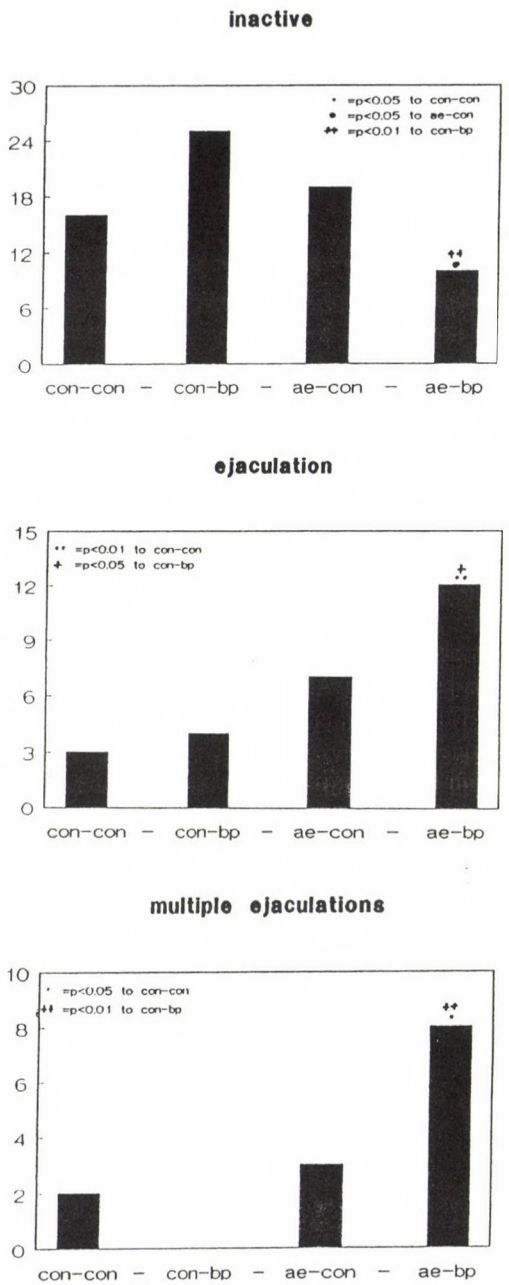

Fig. 3. Column-diagrams of males' sexual activity. There was a significant decrease in the number of inactive rats and a significant elevation in ejaculations and multiple ejaculations in case of double treatment

Benzpyrene treatment in adult age significantly increased the male's inactivity. This manifested in the number of inactive animals as well as in the lack of multiple ejaculations (Figs 2 and 3). The time of the appearance of the first ejaculation was also the longest (Table I). This observation supported our earlier result, when puberal rats were treated with benzpyrene and seven weeks later the sexual activity was decreased. However, in the present experiment the measurement was carried out two weeks after treatment, so rather an early acute effect of benzpyrene was studied.

In males the neonatal allylestrenol treatment was indifferent, like in our earlier experiments [9]. However, the neonatal allylestrenol imprinting reversed the reaction to benzpyrene compared to the only benzpyrene treated (when adult) rats: in this case benzpyrene activated the male animals. The number of inactive rats was significantly lower, the number of ejaculators was significantly higher than in the control group. The number of multiple ejaculators was enormously high (Fig. 3).

Previous experiments demonstrated that a perinatal exposure (imprinting) to a hormone analogue (hormone like material) can cause abnormal receptor binding for life leading to abnormal hormone level and sexual behaviour [5, 9, 14]. The present experiments show that in adult age a steroid-like environmental pollutant (benzpyrene) can diminish the male sexual behaviour and it can provoke a reversed behavioural reaction in animals neonatally misimprinted by a hormone analogue. As allylestrenol was widely used for saving endangered pregnancies, the possibility of apposition by related molecules has to be considered.

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Effect of glucagon and somatostatin on pancreatic secretion in dogs

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The authors investigated the effects of glucagon and a somatostatin preparation (Stilamin) on the secretion volume and on the secreted and absorbed enzyme levels of the pancreas.

Four groups of dogs with an artificial pancreatic fistula were given a single intravenous injection of glucagon (group I, n=8), intravenous drip-infusion of glucagon (group II, n=8), intravenous drip-infusion of somatostatin (group III, n=10) and intravenous drip-infusion of the carrier fluid, physiological sodium-chloride (control, group IV, n=5), respectively. Pancreatic juices were collected and volume, pH, bicarbonate, amilase, lipase, trypsin and proteine contents were determined. Serum amilase and lipase levels before and at the termination of the experiment were also measured.

Intravenous drip-infusion of both Glucagon and Stilamin decreased pancreatic secretion, Stilamin being more effective than Glucagon. On the other hand, a single i.v. injection of Glucagon resulted in an increased secretion.

The authors suggest that based on the observed inhibitory effect on pancreatic secretion, both glucagon and somatostatin could be used to reduce postoperative complications of pancreatic operations in the clinical practice.

Keywords: pancreas, glucagon, somatostatin, animal experiment

Postoperative complications of pancreatic operations are mainly related to the exocrine function of the pancreatic gland. The most common complications are: pancreatitis, pancreatic fistula, ascites, abscess, sepsis. Inhibition of pancreatic secretion in the perioperative period may decrease or prevent these possible consequences. In this paper we examined the effect on the exocrine pancreatic function of glucagon and a somatostatin preparation - Stilamin. According to numerous data both substances have inhibitory effect on pancreatic exocrine secretion [2, 4, 9, 11, 19, 27, 28, 30].

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Methods

The experiments were performed on 31 dogs of similar bodyweight (12–15 kg) without respect to age, sex and subspecies, held under the same conditions. General anaesthesia was performed by a combination of Calypsovet (10 mg/kg) and Rometar (1 mg/kg) administered intramuscularly. Under general anaesthesia an artificial pancreatic fistula was prepared to collect pancreatic juice. After four hours of collecting juice, 16 dogs were given Glucagon (Glucagon Novo Industri A/S, Copenhagen-Denmark), at a dose of 25 μg/kg. Group I (n=8) received a single i.v. injection, whereas group II (n=8) a slow drip-infusion. Group III (n=10) received a somatostatin preparation (Stilamin^R, Somatostatin Serono, Pharmaz. Preparate GmbH, Freiburg, Deutschland) at a dose of 3,5 μg/hour. The control group (group IV, n=5) was given carrier fluid only, physiological sodium chloride at a volume of 500 ml. During the experiment all of the dogs received 2×500 ml physiological saline infusion. In all fractions of the collected pancreatic juice volume, pH, bicarbonate, amilase, lipase, trypsine and proteine content were determined. Serum amilase and lipase levels at the beginning and at the end of the experiment were also measured. Arterial blood pressure was monitored directly through the femoral artery by an Experimetria M.M. Ltd. HG-02 type tonometer.

Results

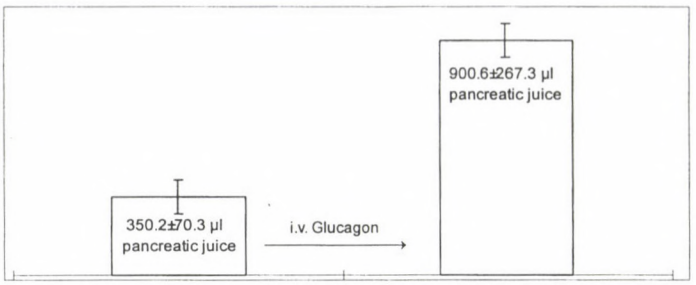
The results are listed in the next four figures. Figure 1 shows that the volume of pancreatic juice increased after intravenously administered Glucagon. The pH did not change at all or showed minimal increase. The bicarbonate content of the pancreatic juice has also increased. The normal level of the amilase, lipase and trypsin concentration showed big differences, but the i.v. Glucagon increased the amilase and lipase levels, while the trypsin concentration decreased. The proteine content was reduced in more than 50% of the animals, while at the rest no change was detected.

The Glucagon drip-infusion reduced the secretion significantly, $p<0.0005$ (Fig. 2). The pH did not change, while the bicarbonate content showed elevation. The amilase and lipase concentration rose, the trypsin secretion decreased after Glucagon drip-infusion. The proteine content was lower during the infusion.

Figure 3 shows the results after Stilamin treatment. The volume of pancreatic juice decreased significantly, $p<0.0005$. The pH did not change or rose minimally. The Stilamin reduced the bicarbonate content, the amilase and trypsin levels did not change, the lipase concentration increased. The reduction in proteine content was not significant. The serum amilase and lipase levels were higher after Stilamin administration.

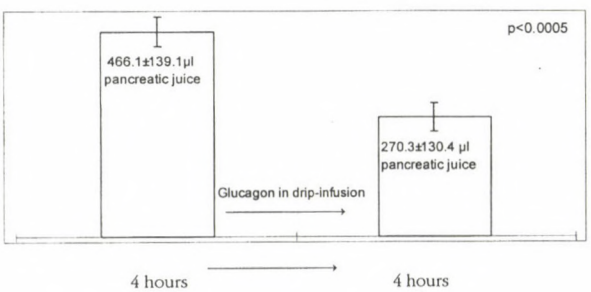
Comparing the effect of Glucagon and Stilamin we can conclude that the reduction in volume of pancreatic juice is significantly greater after Stilamin than after Glucagon drip-infusion ($p<0.01$).

The control group received physiological sodium chlorid infusion. The volume and the enzyme content of the pancreatic juice did not change during the infusion (Fig. 4).



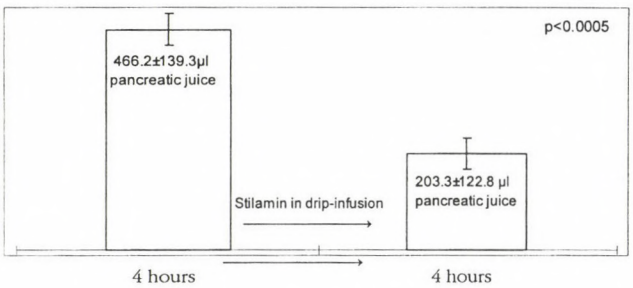
pH	: 7.3	7.4
bicarbonate	: 20.3 mmol/ml	26.6 mmol/ml
amilase	: 74460 U/l	95728 U/l
	(15700-201000)	(17600-233200)
lipase	: 4114 U/l	4444 U/l
	(371-5913)	(957-5935)
trypsin	: 545.4 U/l	131.8 U/l
	(420-890)	(1.0-410)
proteine	: 41.4 g/l	41.2 g/l
	(35-45)	(26-58)

Fig. 1. Composition of the pancreatic juice before treatment and after the administration of a single iv. injection of glucagon



pH	: 7.3	7.3
bicarbonate	: 20.4 mmol/ml	21.3 mmol/ml
amilase	: 93775 U/l	100675 U/l
	(13500-187000)	(13500-208000)
lipase	: 3807 U/l	4043 U/l
	(273-5862)	(377-6914)
trypsin	: 634.5 U/l	166.5 U/l
	(20-900)	(1.0-477.0)
proteine	: 47.5 g/l	29.3 g/l
	(20-64.5)	(10-44.5)

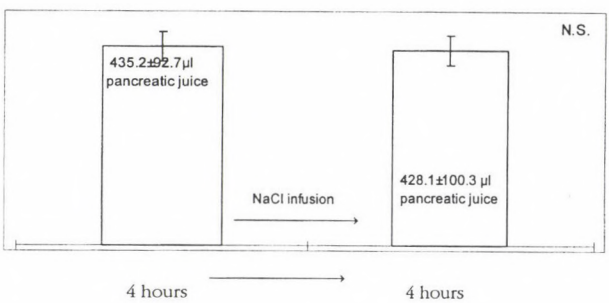
Fig. 2. Effect of Glucagon applied as iv. drip-infusion on the exocrine function of pancreas



pH	: 7.4	8.0
bicarbonate	: 20.6 mmol/ml	17.8 mmol/ml
amilase	: 92300 U/l	89900 U/l
	(13200-18900)	(13600-195000)
lipase	: 2743 U/l	3074 U/l
	(230-5671)	(275-6313)
trypsin	: 502 U/l	546 U/l
	(22-850)	(17-873)
proteine	: 84 g/l	78 g/l
	(25-88.3)	(21-83.5)

Fig. 3. Effect of Stilamin applied as iv. drip-infusion on the exocrine function of pancreas

The systolic blood pressure was between 120 and 140 Hg mm, the diastolic pressure was 80–100 Hg mm with a mean pressure of 100–110 Hg mm. No significant pressure drop was detected.



pH	: 7.3	7.3
bicarbonate	: 20.2 mmol/ml	20.0 mmol/ml
amilase	: 86500 U/l	84000 U/l
	(19600-17900)	(17100-18400)
lipase	: 3405 U/l	3420 U/l
	(390-4250)	(410-4500)
trypsin	: 550 U/l	530 U/l
	(75-710)	(80-640)
proteine	: 49.5 g/l	43 g/l
	(25-62.5)	(21-57)

Fig. 4. Exocrine function of the pancreas in the control group

Discussion

Glucagon is an endocrine polypeptide composed of 28 amino acids. It is produced primarily in the pancreatic alfa-cells, but it can also be found in the intestines and salivary glands. Somatostatin is also a peptide which has been known since the early 1970s. Dubois, Arimura, Polak and his coworkers have shown that cells producing somatostatin can be detected in the gastrointestinal system and in the so-called pancreatic "D" cells as well. In 1981, Miller has found this substance in the nerve endings of the pancreas.

The advantageous effects of glucagon in the gastrointestinal tract are used both diagnostically and therapeutically. It decreases gastric acid secretion and gastric motility and it also leads to the relaxation of the duodenum. These features make this substance particularly effective in radiological examinations of the upper gastrointestinal tract [5, 12, 23, 24, 25]. Its spasmolytic effect on smooth muscle makes it useful in other radioimaging procedures of the gastrointestinal tract: cholangiography, ERCP, irrigoscopy, radiological examination of the small intestine [3, 7, 16, 24, 26].

Contradicting opinions on the use of glucagon in acute pancreatitis have been published in the literature. There are articles showing that glucagon favourably influences the inflammation during acute pancreatitis [13, 18]; others have shown its ineffectivity [8, 21]. Hungarian authors achieved good results by glucagon-treatment of pancreatic fistulas [10]. This treatment was based on experimental data proving that glucagon decreases gastric secretion [5] and has an impending effect on pancreatic juice secretion, on quantity, bicarbonate and enzyme content alike [19]. However Dick et al. have found that glucagon mildly increases baseline pancreatic secretion, and it has an inhibitory effect on juice secretion and enzyme content only in cases in which the pancreas had been previously stimulated by secretin [9].

Our findings, according to which a rapid i.v. injection of Glucagon stimulates basal pancreas secretion, correspond with those reported by Dick et al. [9]. In contrast, Glucagon given in continuous infusion decreased the secretory activity of the pancreas. This controversy could be explained by the results of Unger et al., who found the half life of intravenously given, 134J-labelled Glucagon to be less than 10 minutes [32]. Thus, intravenously given Glucagon may have a rapid inhibitory effect on pancreatic secretion, followed by a rebound effect resulting in an increased pancreatic secretion. This effect may be prolonged and rebound prevented when Glucagon is administered in infusion.

The inhibitory effect of somatostatin on pancreatic exocrine functions has been reported [2, 4], and confirmed in a number of species [1, 11, 20, 28]. In humans, somatostatin also blocks both basal and stimulated pancreatic secretion [6]. There are a number of theories on the action of this hormone. Reichlin suggests that somatostatin has neurohormonal effects in the central nervous system as well as in the peripheral neurons, or it may act as a neurotransmitter modulator. It influences exocrine

pancreatic functions by affecting paracrine and autocrine secretion [27]. In addition it also inhibits the amino acid uptake of pancreatic acinar cells [15].

Because of its favourable effects, somatostatin has been administered several diseases of the pancreas. Usadel et al. applied somatostatin in acute pancreatitis, Strada et al. in pancreatic fistulae, Tulassay et al. in cases when serum amilase concentration was increased after ERCP examinations, while Klempa et al. have given this hormone to prevent complications after pancreas surgery [17, 31, 33]. Somatostatin given in continuous infusion has been effective in certain pancreatic diseases [14, 22].

Our results show that Stilamin, which contains somatostatin, inhibits the secretin-type secretion of the pancreas. Increased serum amilase and lipase levels may result from trauma accompanying the preparation of pancreatic fistulae. However, somatostatin was found to aggravate and not to attenuate inflammatory reaction, which may also lead to increased enzyme production [29].

Our results were compared to those obtained in the IVth (control) group where no changes in pancreas secretion were observed in the absence of the drug.

When comparing the inhibitory effects of the two hormones, Stilamin was found to be significantly more effective ($p < 0.01$).

These data obtained in humans may have clinical relevance. Both glucagon and somatostatin inhibit pancreatic secretion and thus they could be used to prevent complications of pancreas surgery.

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Cytokine level changes in L-arginine-induced acute pancreatitis in rat

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The role of different cytokines in the pathogenesis of L-arginine (Arg)-induced acute pancreatitis in rat, and the ability of KSG-504, a novel cholecystokinin receptor antagonist, to exert protection in this type of acute pancreatitis was evaluated. Male Wistar rats received 250 mg/100 g body weight of Arg intraperitoneally twice, at an interval of 1 h. Control rats received instead the same amount of glycine at the same times. Fifty mg/kg KSG-504 was injected subcutaneously 0.5 h before and 6, 18 and 36 h after the first Arg administration. Rats were examined 12, 24 and 48 h after pancreatitis induction. To assess the severity of inflammation, the edema was quantified, the serum amylase level was measured, and histologic examinations were performed. Serum tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels were determined by bioassay, using the TNF-sensitive WEHI 164 and the IL-6-dependent B9 cell lines, respectively. In Arg-induced acute pancreatitis, the amylase level was increased significantly at 12 h (48.600 ± 3.980 U/l) and 24 h (30.800 ± 3.813 U/l) vs. the control group (6.382 ± 184 U/l). No significant alteration in the ratio pancreatic weight/body weight was found in the different groups. However, in Arg-induced acute pancreatitis, both the TNF-α (15.1 ± 6.9 U/ml) and the IL-6 (39.6 ± 19.2 pg/ml) levels were already elevated significantly at 12 h vs. the controls (3.1 ± 0.8 U/ml and 15.2 ± 3.1 pg/ml, respectively) and remained elevated at 24 and 48 h. Simultaneous KSG-504 administration did not modify the measured cytokine levels. No significant changes in plasma CCK levels were observed. In Arg-induced acute pancreatitis, histological evaluation revealed diffuse but microfocal necrobiotic alterations. No marked protective effects of KSG-504 were observed on histological sections. These results suggest that excessive doses of Arg induce severe acute pancreatitis in rat, with a simultaneous cytokine level elevation. Endogenous CCK does not seem to play an essential role in the pathogenesis of Arg-induced acute pancreatitis.

Keywords: L-arginine-induced acute pancreatitis, KSG-504, tumor necrosis factor-α, interleukin-6, plasma cholecystokinin level

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Excessive doses of basic amino acids such as ethionine, methionine and lysine have been found to injure the pancreas, although the mechanism is unknown [10, 13, 14, 16, 20]. In 1984 Mizunuma et al. reported that a single intraperitoneal administration of Arg (500 mg/100 g body weight) results in selective pancreatic acinar cell damage in rats, without any morphological change in the Langerhans islets [18]. Tani et al. demonstrated the reversible histological characteristics of this new type of necrotizing acute pancreatitis [29]. However, when serial i.p. injections of Arg were given, Delaney et al. observed chronic pancreatitis-like damage of the pancreatic tissue in rat [7]. No plausible explanation of the effect of Arg on pancreatic damage has yet been given.

Some recent studies suggested that cytokines are involved in the pathogenesis of different gastrointestinal diseases [2, 27]. Tumor necrosis factor-α (TNF-α) has been considered to be an important mediator derived from activated macrophages in the development of severe acute pancreatitis complicated by intraabdominal sepsis [25]. In a clinical study, the interleukin-6 (IL-6) level was found to reflect the severity of acute pancreatitis [15]. However, the exact role of the different cytokines in the pathogenesis of acute pancreatitis is unknown.

The gastrointestinal hormone cholecystokinin (CCK) has been shown to be involved in the induction and development of acute pancreatitis in various experimental models [21, 30]. A number of studies of the effects of CCK receptor antagonists on experimental acute pancreatitis have been reported, but the results were controversial [21, 23, 28, 30]. Blockade of the CCK-A receptors by different CCK antagonists decreased the severity of the pancreatic damage produced with supramaximal doses of cerulein, CCK-8 or a closed duodenal loop [28, 31]. The potent and specific CCK receptor antagonist MK-329 did not exert any beneficial effect in choline-deficient, ethionine-supplemented diet-induced hemorrhagic pancreatitis in mice [23].

The present study was planned to investigate the changes in the serum levels of different cytokines in Arg-induced acute pancreatitis in rat and to examine the putative protective effect of KSG-504, a novel CCK receptor antagonist, in this pancreatitis model.

Materials and Methods

Male Wistar rats weighing 280–330 g were used in all experiments. The animals were kept at a constant room temperature of 27 °C, and fed with free access to water and a standard laboratory chow (LATI, Gödöllő, Hungary).

Experimental protocol

Animals were divided in three groups. In groups 1 and 2, animals received 250 mg/100 g body weight of Arg as a 20% solution in saline intraperitoneally (i.p.) twice, at an interval of 1 h. In group 2, 50 mg/kg of KSG-504 (a kind gift from the Kissei Pharmaceutical Co., Masumoto, Japan) was injected subcutaneously (s.c.) 0.5 h before and 6, 18 and 36 h after the first Arg injection. Control rats received instead the same amount of glycine as an 8.6% solution in saline at the same time (group 3). The rats were

examined 12, 24 and 48 h after pancreatitis induction: they were killed by abdominal aorta exsanguination (n=8 rats/group). The pancreas was rapidly removed, freed from fat and lymph nodes, and weighed.

Assays

Serum samples were stored at -20 °C for subsequent analysis of amylase concentration by the Phadebas test method [5].

Plasma CCK-like bioactivity was measured by the highly specific and sensitive bioassay described by Liddle et al. [17]. CCK was extracted from 2 ml of plasma by absorption on octadecylsilylsilica cartridges (Sep-Pak, Waters Assoc.) and eluted in ethanol and trifluoroacetic acid. The quantitation of CCK in the extracts was based on its ability to release amylase from rat pancreatic acini prepared by dispersion with collagenase. The bioactivity of CCK was compared with a standard curve, and the results were expressed in pM CCK-8 equivalents. The assay is sensitive to plasma levels as low as 0.5 pM.

The development of pancreatic edema was quantitated by comparing the pancreas weight obtained immediately after the death of the animals (wet weight) with that of the same sample after incubation at 150 °C for 48 h (dry weight) as described by Ohshio et al. [22].

Serum TNF-α and IL-6 levels were determined by bioassay, using the TNF-sensitive WEHI 164 and the IL-6-dependent B9 cell lines, respectively [9, 1].

Histologic examination

A protein of the pancreas was fixed overnight in a 6% neutral formaldehyde solution and embedded in paraffin. Tissue slices were subjected to hematoxylin and eosin staining and histological study by light microscopy.

Statistical analysis

Results are expressed as means $\pm$S.E.M. Experiments were evaluated statistically with the Student *t*-test for paired or unpaired values, as appropriate; p values of less than 0.05 were accepted as significant.

Results

In group 1, the serum amylase activity was increased 8-fold (48.600$\pm$3.980 U/l) vs. the control group (6.382$\pm$184 U/l) 12 h after the last Arg injection (Fig. 1). Thereafter it decreased, but it remained significantly elevated 24 and 48 h after the onset of acute pancreatitis. Administration of Arg+KSG-504 (group 2) did not modify the serum amylase pattern relative to the Arg-treated group (Fig. 1).

No significant increase in the ratio pancreatic weight/body weight was observed after the administration of 250 mg/100 g body weight of Arg (Fig. 2). Arg+KSG-504 treatment did not cause any significant alteration in the ratio pancreatic weight/body weight vs. the Arg-treated group (Fig. 2).

No significant differences in plasma CCK bioactivity were found between the Arg-treated ($<$0.05 pM), the Arg+KSG-504-treated ($<$0.5 pM) and the control groups ($<$0.5 pM). Slight but not significant increases in plasma CCK level were detected at 48 h in Arg-treated (1.3$\pm$0.5 pM) and Arg+KSG-504-treated (1.1$\pm$0.5 pM) groups.

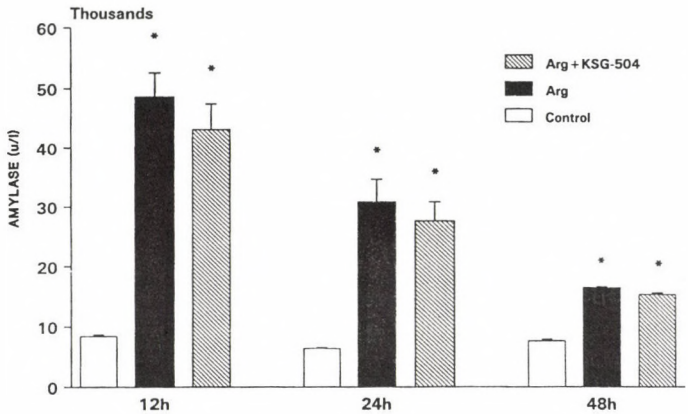

Fig. 1. Serum amylase activity in Arg-induced acute pancreatitis in rat. Rats received 2×250 mg/100 g b.w. of Arg i.p. at 1-h intervals with (group 2) or without KSG-504 (group 1) treatment (50 mg/kg of KSG-504 0.5 h before and 6, 18 and 36 h after the first Arg administration), and were sacrificed at 12, 24 or 48 h. Controls received the same amount of glycine (group 3). Values are means ±S.E.M. in each group involving 8-8 rats. Significant differences from the control values ($p<0.05$) are indicated by asterisks

The serum TNF-α level was already significantly increased at 12 h both in the Arg-induced acute pancreatitis group and in the Arg+KSG-504-treated animals vs. the control rats (Fig. 3).

The serum IL-6 level was also significantly elevated at 12 h both in the Arg-treated and in Arg+KSG-504-treated animals vs. the controls (Fig. 4). Both the TNF-α and IL-6 levels also remained elevated at 24 and 48 h relative to the control group.

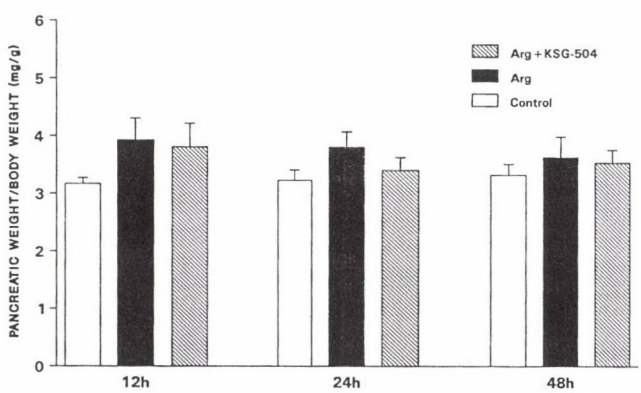

Fig. 2. Pancreatic weight/body weight ratio in Arg-induced acute pancreatitis in rat. Animals were treated, sacrificed and statistically evaluated as indicated in Fig. 1

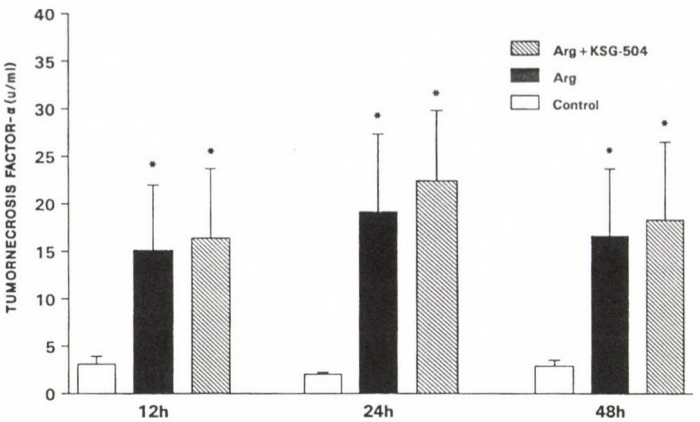

Fig. 3. Serum TNF-α levels in Arg-induced acute pancreatitis in rat. Animals were treated, sacrificed and statistically evaluated as indicated in Fig. 1

As far as the histologic alterations are concerned, Arg treatment resulted in interstitial edema after 12 h. However, 24 h after the onset of acute pancreatitis, a diffuse parenchymal degeneration and multifocal necroses in the acinar cells were seen vs. the control group (Fig. 5). The interstitium was edematous with congested vessels and was infiltrated with polymorphic leukocytes and lymphocytes. The endocrine cells in the islets of Langerhans appeared intact (Fig. 6). At 48 h, the severity of acinar cell necrosis was more marked and the necrotic acini were partially replaced by adipose tissue (Fig. 7). The KSG–504 treatment did not modify these structural changes: the histologic architecture of the pancreas was similar to that seen without KSG–504 administration.

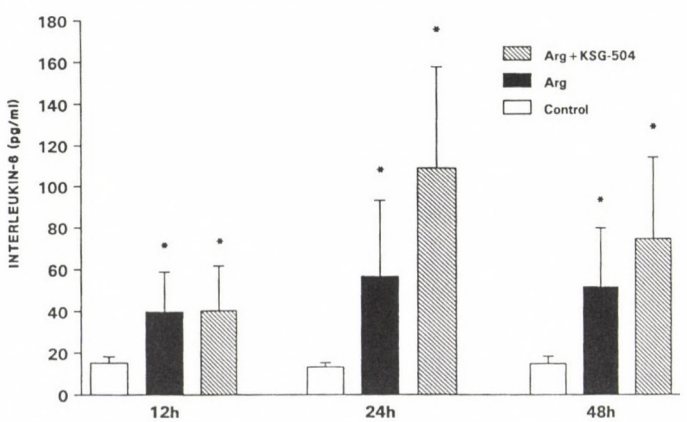

Fig. 4. Serum IL-6 levels in Arg-induced acute pancreatitis in rat. Animals were treated, sacrificed and statistically evaluated as indicated in Fig. 1

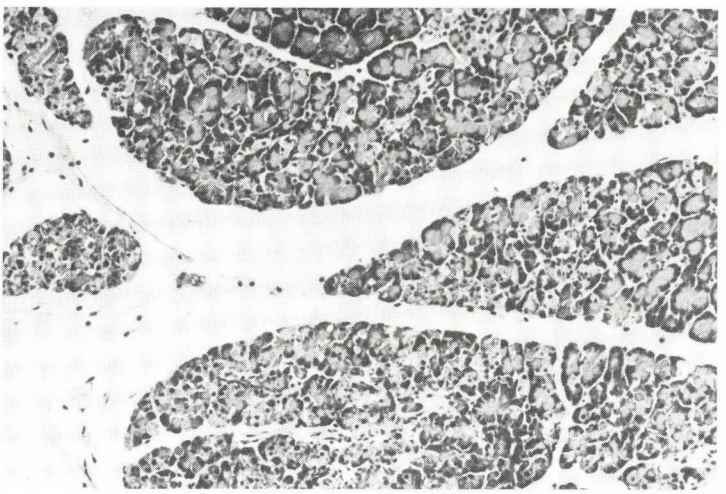

Fig. 5. Representative pancreatic section 12 h after Arg administration. Mild interstitial edema and a few acinar cell necroses are visible. (H and E; ×25)

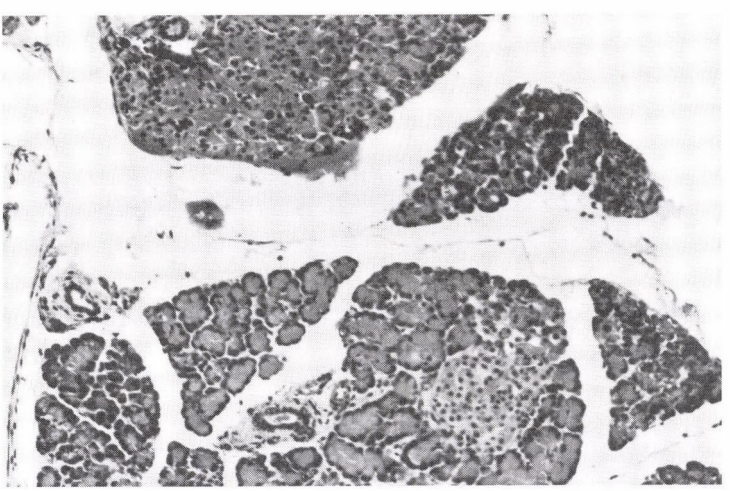

Fig. 6. At 24 h, in Arg-induced acute pancreatitis a diffuse parenchymal degeneration and multifocal acinar necroses are seen. (H and E; ×40)

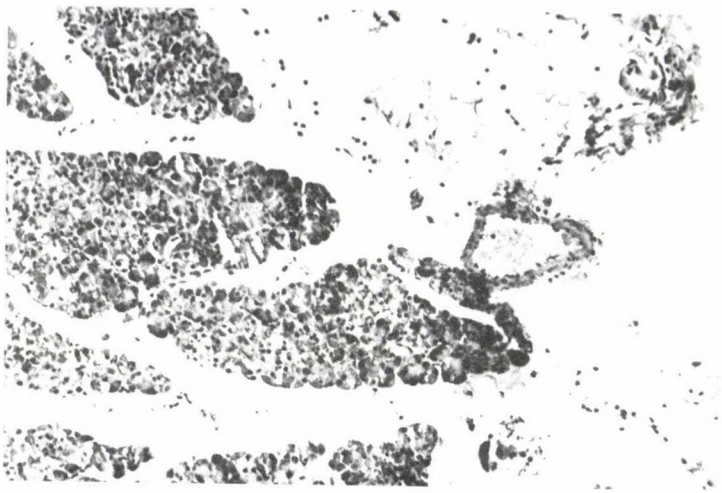

Fig. 7. Representative pancreatic section from a rat 48 h after Arg administration. Severe multifocal acinar cell necroses are visible, partially replaced by adipose tissue. (H and E; ×25)

Discussion

Recent studies demonstrated that Arg takes part in the production of nitric oxide and endothelium-derived relaxing factor, which subsequently leads to hypotension and pancreatic tissue damage [19, 24]. However, no direct body/line/s, pieces of evidence on the role of Arg-derived NO production in the pathogenesis of Arg-induced pancreatitis has been provided. In spite the growing spectrum of studies devoted to this question, no plausible explanation is known concerning the pathomechanism of Arg-induced pancreatic damage.

The present work demonstrated that excessive doses of Arg evoke an acute pancreatitis-like laboratory change (an increased amylase level) and morphologic alterations (acinar cell necrosis) in rat 12 h following pancreatitis induction. The serum amylase activity subsequently decreased continuously. In contrast with the amylase activity, the morphologic alterations seem to worsen during the study (at 24 and 48 h). These results are consistent with the findings of the Otsuki group [29].

Activated macrophages are known to release a number of inflammatory mediators, including TNF-α, IL-1, IL-6 and IL-8 and several growth factors [11]. Among various agents, bacterial compounds, viruses, interferon-gamma and several cytokines, e.g. TNF-α, have been shown to stimulate the synthesis of IL-6 [12]. The actions of these cytokines on the host are controversial: both beneficial and harmful effects have been demonstrated [3, 4]. TNF-α is known to exert marked vascular effects and to aggravate the inflammatory response [11]. In a clinical study, Leser et al. found that the serum IL-6 level reflects the severity of acute pancreatitis [15].

The present results revealed that both TNF-α and IL-6 levels were significantly elevated during the observation period in Arg-induced experimental pancreatitis. However, the exact origin of these cytokines is unknown. They can be released by the activated macrophages/monocytes either from the severely ill pancreas or from peritoneal macrophages stimulated by i.p. Arg injections. In the control rats, the equivalent amount of glycine with the same osmolality did not evoke any change in serum cytokine levels. Laboratory and histologic examinations revealed that the pancreatic tissue damage was preceded by serum cytokine level elevation. It seems, therefore, that cytokines are early markers of the severity of this type of acute experimental pancreatitis. Further studies must be conducted with specific cytokine inhibitors [26] and/or receptor antagonists [8] in order to verify the exact role of the cytokine network in the pathogenesis of acute pancreatitis.

Endogenous CCK has been suspected of participating in the pathogenesis of different forms of experimental pancreatitis [28, 30]. Recent studies with specific CCK receptor antagonists demonstrated the marked protective effect of these agents (CR 1409 and L-364,718) in different acute pancreatitis models [28, 30, 31]. These data provided further evidence on the role of CCK in acute pancreatitis. There are no data, however, concerning the putitative role of endogenous CCK in the pathogenesis of Arg-induced acute pancreatitis.

Our data demonstrated that in Arg-induced acute pancreatitis the plasma CCK level was practically unchanged during the study. At 48 h, the slight but not significant increase in plasma CCK level may have been a consequence of a negative feedback mechanism evoked by the severe pancreatic damage. No effect of 50 mg/kg of KSG–504 on the laboratory and morphologic changes in Arg-induced pancreatitis was found. Recently, the same dose of KSG–504 was demonstrated to prevent pancreatic damage caused by serial injections of high dose of CCK-8 [6]. These results suggest that endogenous plasma CCK is not involved in the pathogenesis of Arg-induced pancreatitis.

In conclusion, the administration of high doses of Arg evokes severe pancreatitis with focal acinar cell necrosis. Both TNF-α and IL-6 were significantly elevated after the induction of pancreatitis and remained elevated during the observation period. These cytokines are suspected on being involved in the pathogenesis of this type of pancreatitis. In contrast, endogenous CCK does not seem to play a role in the pathomechanism of Arg-induced pancreatitis. KSG–504, a potent CCK receptor antagonist, did not modify the pancreatic biochemical or morphological changes induced by Arg administration.

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Comparative study of the circulatory effects of aminoguanidine and N-nitro-L-arginine in hyperdynamic endotoxemia

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We have studied the effects of NG-nitro-L-arginine (NNA) a nitric oxide synthase (NOS) inhibitor, and aminoguanidine (AG) a diamine oxidase inhibitor, on hemodynamic parameters and plasma histamine level using a dog model in which a hyperdynamic circulatory response was elicited with a 2-hour infusion of a low dose (13.75 μg/kg) of *E. coli* 055:B5 endotoxin (ETX). AG (50 mg/kg) or NNA (0.5 mg/kg) was administered intravenously as pretreatment. Hemodynamic variables were studied for 4 hours after the beginning of the ETX infusion. The ETX-elicited hyperdynamic response was abolished by NNA and partially inhibited by AG. AG prevented the increases in cardiac output and heart rate and delayed the early decrease in total peripheral resistance (TPR). The plasma histamine concentration elevation was higher in animals receiving AG than in those receiving only ETX. In the group treated with ETX plus NNA the cardiac output was lower and the TPR was higher than in the ETX plus AG group. In future studies, AG should be considered as one of the possible therapeutic tools in sepsis, as its adverse effect on the compensatory hyperdynamic response is less than that of NOS inhibitors of the L-arginine analog type, while it may favourably influence the deleterious excessive activity of the inducible NOS in the later stages.

Keywords: sepsis, circulation, nitric oxide, histamine

Nitric oxide (NO), together with other vasoactive mediators, has an important role in the regulation of the circulation in sepsis and septic shock. NO is synthesized by the enzyme NOS which has several isoforms [10, 22]. Of these, the constitutive form (cNOS) regulates the normal vascular tone and produces NO continuously under normal conditions. The inducible form of the enzyme (iNOS) is activated by ETX and

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cytokines and displays a much higher rate of NO production than that of cNOS. The production of NO is increased in sepsis and septic shock [17]. The initial phase of sepsis is characterized by a hyperdynamic circulation in which a high heart rate and an elevated or normal cardiac output are associated with a lowered systemic vascular resistance. Studies in recent years suggest the possible role of the overproduction of NO in the transition from the hyperdynamic circulatory setting into the hypodynamic phase, where refractory hypotension and diminished tissue perfusion contribute to the high mortality of late sepsis and septic shock [17, 27].

NOS can therefore be an important target for interventions aimed at modifying the circulatory situation in sepsis/septic shock. Analogs of L-arginine such as NNA or Ng-monomethyl-L-arginine (NMMA) reduce the production of NO by inhibiting NOS [19]. On application of these NOS inhibitors, it was possible to counteract vasodilation and restore the blood pressure in animal experiments [18, 25, 34] and there is a report on the effect of this treatment in two septic patients [28]. Other studies, however, show that these inhibitors cause a decrease in cardiac output in sepsis, the consequences of which include a diminished perfusion of the tissues and a reduced survival rate [5, 19]. The currently available L-arginine analog NOS inhibitors exert approximately the same suppressive activity on both isoenzyme forms, which has a deleterious effect on the outcome of shock [25, 36].

Recent data indicate that the diamine oxidase inhibitor AG has an additional NOS-inhibiting effect and a significant selectivity for iNOS [6, 12, 14]. In the present study, using our previously published model of hyperdynamic endotoxemia [33], we have investigated the effects of AG on the hemodynamic parameters of the hyperdynamic circulation and have compared them with those of NNA. We are not aware of any such previous study. Our results provide an insight into the roles of NO and histamine in the mechanism of the elevated cardiac output in this condition.

Methods

The study was approved by the Ethical Committee for the Protection of Animals in Research of Szent-Györgyi Albert Medical University. The experiments were performed on 60 healthy mongrel dogs with an average body weight of 11.8 kg (range 10–14 kg) under intravenous sodium pentobarbital (30 mg/kg) anesthesia. Supplementary small doses were administered if necessary. Polyethylene cannulas were introduced into both femoral veins and one femoral artery for blood sampling, the infusion of drugs and the measurement of mean arterial pressure (MAP). A silastic catheter was introduced into the vena cava through the external jugular vein for the measurement of central venous pressure (CVP). Electrodes for impedance cardiographic measurement of cardiac output were placed on the shaved skin of the neck and thorax. A 30-min resting period was than allowed for circulatory stabilization.

Five groups of animals were studied.

1. Group ETX (n=14). This group received a low dose of endotoxin (*E. coli* 0.55:B5, SIGMA) in a 2-hour intravenous infusion according to the following schedule: for the first 45 min ETX was infused at a rate of 10 μg/kg/h, and the infusion rate was then decreased to 5 μg/kg/h for the last 75 min; thus the animals received a total dose of 13.75 μg/kg ETX in a total volume of 10 ml saline. ETX was freshly dissolved in saline for each experiment. This group received a 10-min infusion of 10 ml saline, which was

begun 15 min before the commencement of the ETX infusion. ETX was administered in exactly the same manner to the other two ETX-treated groups. This ETX dosage schedule was developed for a previous study involving our model of hyperdynamic endotoxemia [33].

2. Group ETX+AG (n=14). In addition to ETX, this group received 50 mg/kg AG dissolved in 10 ml saline, given as a 10-min infusion. The AG infusion was begun 15 min before the commencement of the ETX infusion.

3. Group ETX+NNA (n=10). In addition to ETX this group was given 0.5 mg/kg NNA dissolved in 10 ml saline, administered in a 10 min infusion 15 min before the ETX infusion.

4. Group AG (n=12). This group was treated in exactly the same way as Group ETX+AG except that they were not given ETX.

5. Group NNA (n=10). This group was treated in exactly the same way as Group ETX+NNA except that they were not given ETX.

Groups that did not receive ETX were given a 120-min infusion of 10 ml saline instead.

Measurements

Hemodynamic parameters were determined at the end of the stabilization period (i.e. immediately before the administration of inhibitors or their vehicle = −15 min), at the beginning of the infusion of ETX (or its vehicle) (0 min), and then every 15 min during the first hour of the infusion (i.e. at the 15th, 30th, 45th and 60th min), and every 30 min during the second hour of the 2-hour infusion (i.e. at the 90th and 120th min). After this, two more measurements were made at hourly intervals (i.e. at the 180th and 240th min). MAP was measured with a Statham P23Db transducer and recorded on a Beckman polygraph. CVP was measured with a saline manometer. Cardiac output and stroke volume were measured non-invasively with the thoracic electrical impedance method [1, 2]. Heart rate was determined from the electrocardiogram. TPR was calculated from the values of MAP, CVP and cardiac output.

In Groups ETX, ETX+AG and AG, the plasma histamine was measured at six time points: at −15 min, at half-hourly intervals during the infusion of ETX (or its vehicle) and at the end of the experiment (= at the 240th min). Histamine was determined with a radioenzymatic method [3].

Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Nonparametric methods were used. Friedman repeated measures analysis of variance on ranks was applied within groups.

Time-dependent differences from the baseline (−15 min) for each group were assessed by Dunn's method. Differences between groups were analysed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In the Tables, mean and median values, and semi-interquartile ranges (difference between upper and lower quartiles divided by 2) [9] are given. P values < 0.05 were considered significant.

Results

In accordance with our previous results a two-stage continuous infusion of ETX initially caused a transient nonsignificant rise, followed by a moderate decrease in MAP, which was statistically significant relative to the baseline value at the 90th and 180th min of the infusion (Table I). After the end of the ETX infusion, MAP rose and reached the initial value by the end of the experiment. In Group ETX+AG, MAP was higher at 0 and 15 min than the corresponding levels in Group ETX but AG did not influence the decrease in pressure due to ETX in the last half hour (90th and 120th min) of the ETX infusion. By the end of the experiment, MAP had normalized in this group, too. In Group ETX+NNA MAP underwent a transient but significant increase from the baseline value in the first 45 min. When given alone, both AG and NNA caused significant increases in MAP.

The heart rate increased significantly from the 30th to the 180th min in Group ETX (Table II). The administration of AG abolished this increase in Group ETX+AG. NNA had a somewhat similar effect in Group ETX+NNA but the heart rate rose towards the end of the experiment in this group. AG or NNA given alone caused an early short-lived decrease in heart rate.

The low-dose ETX infusion elicited a significant increase in cardiac output between the 15th and 90th min, with a maximum (37% change) at 45 min (Table III). The cardiac output had normalized by the end of the experiment. This increase in cardiac output was abolished by both AG and NNA, but to different extents. AG did not cause a significant fall in cardiac output except towards the end of the experiment (at 240 min in Group ETX+AG and at 180 and 240 min in Group AG. NNA treatment actually decreased the cardiac output at 90 min and 120 min in both Group ETX+NNA and Group NNA. In Groups ETX+AG and ETX+NNA, the cardiac output was significantly lower between 30 min and 120 min than the corresponding values in Group ETX. As compared with its values at 90 min and 120 min in Group ETX+AG, the corresponding cardiac output was significantly lower in Group ETX+NNA.

The stroke volume was lower in general in the groups receiving the inhibitors than in Group ETX (Table IV). The pretreatment with AG in Group ETX+AG did not cause a significant decrease as compared with the baseline value while such a decrease did occur between 90 min and 180 min in Group ETX+NNA. The levels between 90 min and 120 min in Group ETX+NNA were significantly lower than the corresponding ones in Group ETX+AG.

Table I

Mean arterial pressure (mm Hg)

Group		Baseline		Minutes after the start of infusion							
		-15	0	15	30	45	60	90	120	180	240
ETX	Mean	114	113	115	117	112	108	98	93	108	115
	Median	115	115	117	120	117	110	90	88	107	115
	SR	5	4	6	7	11	10	10	12	11	6
ETX+AG	Mean	118	126	125	121	117	110	88	85	110	120
	Median	120	130	125	123	120	115	90	88	110	120
	SR	6	9	8	9	11	10	16	11	6	4
								+	+		
ETX+NNA	Mean	112	120	125	130	124	108	98	99	121	124
	Median	113	120	122	130	122	110	92	96	115	125
	SR	6	4	5	6	10	17	12	8	11	9
			*	+*	+*	+				#	
AG	Mean	113	122	124	125	122	121	123	128	129	133
	Median	112	122	125	125	123	123	120	127	130	132
	SR	7	7	5	7	9	9	5	9	6	7
			+	+	+			+*	+*	+*	+*
NNA	Mean	113	119	124	127	129	133	137	139	143	138
	Median	112	120	123	125	130	132	135	140	140	137
	SR	4	5	8	6	7	5	6	9	9	13
							+*	+*	+*	+*	+*

Abbreviation: SR = semi-interquartile range
Symbols:

+ = $p<0.05$ against baseline value
* = $p<0.05$ against corresponding value for Group ETX
# = $p<0.05$ against corresponding value for Group ETX+AG

Table II

Heart rate (beats/min)

Group		Baseline		Minutes after the start of infusion							
		-15	0	15	30	45	60	90	120	180	240
ETX	Mean	129	130	139	149	149	149	152	153	147	141
	Median	126	129	138	155	147	142	146	147	149	146
	SR	7	8	13	16	17	20	19	19	14	16
					+	+	+	+	+	+	+
ETX+AG	Mean	134	113	116	132	136	141	137	137	141	145
	Median	133	119	118	131	132	138	140	130	139	137
	SR	17	20	19	20	23	25	22	23	17	25
			*	*							
ETX+NNA	Mean	129	118	117	128	140	134	138	148	154	152
	Median	127	119	118	128	141	137	135	144	155	153
	SR	7	9	10	14	9	19	16	13	8	14
			*	*					+	+	+
AG	Mean	137	124	126	131	132	136	130	127	126	130
	Median	134	127	128	132	138	141	134	125	130	141
	SR	8	9	12	13	18	14	11	9	11	14
									*		
NNA	Mean	130	118	116	124	126	131	128	123	127	138
	Median	134	116	117	123	125	127	127	125	120	139
	SR	8	10	7	7	7	10	12	12	19	14
				+*	*	*			*		

Symbols: see Table I

Table III

Cardiac output (ml/min/kg)

Group		Baseline		Minutes after the start of infusion							
		-15	0	15	30	45	60	90	120	180	240
ETX	Mean	118.5	122.2	143.5	150.0	163.0	158.9	139.3	135.7	125.3	120.2
	Median	119.5	121.8	145.0	145.2	151.8	158.0	136.0	137.3	126.4	116.6
	SR	15.6	25.8	34.0	32.8	38.0	34.6	20.0	29.4	26.8	27.7
				+	+	+	+	+			
ETX+AG	Mean	125.8	123.8	118.1	109.7	109.6	110.8	110.7	110.1	111.5	104.1
	Median	125.4	127.3	115.5	103.5	108.1	109.7	111.8	105.5	108.1	97.2
	SR	10.9	27.0	19.0	21.7	15.2	21.0	17.6	16.6	17.5	17.0
					*	*	*	*	*		+
ETX+NNA	Mean	113.1	115.4	114.0	102.1	96.1	88.2	76.8	78.2	92.4	99.7
	Median	111.2	106.2	105.5	97.8	96.9	83.6	70.5	73.7	86.1	101.4
	SR	21.3	27.3	22.9	6.7	18.3	18.7	15.7	13.1	20.9	10.6
					*	*	*	+*#	+*#		
AG	Mean	120.1	113.2	116.1	116.2	111.7	115.2	108.4	106.9	102.0	100.6
	Median	114.2	112.8	115.0	110.5	106.5	110.8	105.4	106.7	94.8	97.1
	SR	21.3	10.4	14.0	11.1	20.1	26.9	25.5	20.5	19.4	23.0
						*				+	+
NNA	Mean	109.5	107.5	110.6	105.8	96.9	95.5	88.1	88.8	99.0	97.3
	Median	106.3	97.3	109.7	104.1	94.4	92.1	92.0	88.7	95.3	90.6
	SR	17.9	17.1	17.3	18.4	11.2	13.9	18.0	21.3	23.2	19.7
					*	*	*	+*	+*		

Symbols: see Table I

Table IV

Stroke volume (ml/beat)

Group		Baseline		Minutes after the start of infusion							
		-15	0	15	30	45	60	90	120	180	240
ETX	Mean	11.2	11.7	13.6	12.5	13.6	13.2	11.3	10.7	10.8	10.6
	Median	10.4	11.1	12.4	11.3	13.4	12.4	10.5	10.6	10.1	9.1
	SR	1.3	1.9	3.1	2.8	2.9	1.5	0.9	1.5	2.2	2.5
						+					
ETX+AG	Mean	11.2	12.4	11.4	8.8	8.7	8.7	8.8	8.8	8.7	7.8
	Median	10.1	11.9	10.6	8.0	8.0	8.8	8.7	8.6	8.4	7.3
	SR	3.2	5.0	3.1	1.8	1.4	2.2	1.8	1.8	1.5	1.5
						*	*	*			
ETX+NNA	Mean	10.6	11.3	11.2	9.5	7.7	7.5	6.5	5.9	6.8	7.7
	Median	10.0	11.0	10.2	8.5	7.8	7.0	6.4	5.5	6.4	7.4
	SR	1.8	1.9	2.8	2.9	1.6	2.0	1.3	1.1	1.1	1.5
						*	*	+*#	+*#	+*#	
AG	Mean	10.8	11.3	11.1	11.2	10.1	10.3	10.4	10.6	10.0	40.0
	Median	10.5	11.0	10.6	11.3	10.8	10.6	10.9	10.5	9.9	9.4
	SR	1.3	1.6	1.5	2.0	1.6	2.0	1.6	1.5	1.8	2.8
NNA	Mean	9.8	9.6	10.4	9.2	8.2	7.7	7.3	7.9	8.5	7.7
	Median	9.4	9.5	10.1	9.1	7.9	7.2	7.1	7.4	9.0	7.1
	SR	1.1	1.7	0.9	1.7	0.6	0.9	1.7	1.9	1.9	1.5
						*	*	+			+

Symbols: see Table I

Table V

Total peripheral resistance (mmHg/ml×min×kg)

Group		Baseline		Minutes after the start of infusion							
		-15	0	15	30	45	60	90	120	180	240
ETX	Mean	991	966	851	848	732	714	722	712	902	1048
	Median	949	886	764	832	698	693	678	675	830	971
	SR	143	208	211	213	151	156	153	109	162	217
					+	+	+	+	+		
ETX+AG	Mean	951	1083	1092	1140	1101	1028	813	799	1024	1214
	Median	942	989	1111	1136	1067	968	785	760	1052	1217
	SR	59	150	161	175	179	219	88	153	167	195
						*	*				+
ETX+NNA	Mean	1021	1125	1141	1313	1351	1315	1305	1350	1408	1269
	Median	1077	1153	1266	1316	1291	1249	1320	1242	1442	1263
	SR	235	235	220	241	359	414	289	262	391	200
				*	+*	+*	*	+*#	*#	+*	
AG	Mean	962	1098	1087	1089	1128	1102	1199	1256	1323	1401
	Median	999	1073	1083	1077	1186	1085	1134	1202	1386	1376
	SR	172	138	217	116	203	286	291	204	312	378
						*		+*	+*	+*	+
NNA	Mean	1055	1213	1162	1245	1346	1473	1680	1659	1542	1484
	Median	1042	1183	1228	1292	1322	1365	1537	1592	1611	1635
	SR	175	231	225	226	147	177	340	425	419	392
				*	*	*	+	+	+*	+*	+

Symbols: see Table I

Table VI

Plasma histamine concentration (nmol/l)

Group		Baseline	Minutes after the start of infusion				
		-15	30	60	90	120	240
ETX	Mean	1.26	2.16	1.95	1.54	1.21	1.06
	Median	1.08	1.97	2.14	1.48	1.31	1.09
	SR	0.36	0.98	0.79	0.43	0.38	0.53
			+	+			
ETX+AG	Mean	1.31	2.94	2.73	2.34	1.92	1.69
	Median	1.11	3.23	2.87	2.36	1.82	1.64
	SR	0.18	0.47	0.79	0.46	0.38	0.55
			+	+	+*	*	
AG	Mean	1.03	2.69	2.30	1.94	1.91	2.11
	Median	1.05	2.46	1.94	2.08	1.93	2.01
	SR	0.16	0.90	0.91	0.43	0.29	0.43
			+	+	+	+*	*

Symbols: see Table I

The values of TPR are shown in Table V. There was a sustained decrease in Group ETX, which was significant between 30 min and 120 min. In Group ETX+AG, TPR showed a tendency to increase, which was nonsignificant except at 240 min. In contrast, TPR increased significantly from the baseline level between 30 min and 180 min in Group ETX+NNA. AG alone increased TPR between 90 min and 240 min as did NNA alone between 60 min and 240 min. TPR at 90 min and 120 min in Group ETX+AG was significantly lower than the corresponding levels in Group ETX+NNA.

Plasma histamine levels are shown in Table VI. The histamine concentration was significantly increased at 30 and 60 min in Group ETX. It had returned to the baseline level by 120 min. As expected, the increase in histamine level was greater in the AG-treated groups than in the ETX-only-treated group. Significantly higher histamine levels were found at 90 and 120 min in Group ETX+AG and at 120 and 240 min in Group AG than the corresponding values in Group ETX.

Discussion

The present results indicate that AG prevents the development of the hyperdynamic circulatory response in the early phase of endotoxemia. Following pretreatment with AG, a low dose of ETX does not elicit an increase in cardiac output and a decrease in TPR. An increase in TPR occurs only at the end of the observation period, at 240 min.

Recent *in vivo* and *in vitro* studies suggest that AG inhibits the generation of NO from L-arginine. AG is structurally similar to L-arginine and could therefore be a competitive inhibitor of NOS [14, 15]. AG has been shown to inhibit interleukin-1b-induced nitrite formation and cGMP accumulation in RINm5F cells, the effect being approximately equipotent with that of L-NMMA [6, 14]. Similar results were obtained in experiments where AG was compared with other NOS inhibitors on the basis of potency and selectivity. Investigations involving blood pressure responses in rats show that L-NMMA is a more potent cNOS inhibitor than AG. The potency of L-NMMA was 40 times higher in one study [6], and 16 times stronger in another one [14]. These cited authors consider that the different blood pressure responses cannot be attributed to differences in the cellular uptake of the drugs, as the peak effect was invariably observed within 2–5 min after intravenous bolus administration in all groups. Other, *in vitro* studies also suggest that the selectivity of AG is greater for iNOS. AG increased the phenylephrine-elicited contractions of pulmonary artery preparations from ETX-treated rats in a dose dependent manner in both intact and endothelium-denuded vessels, but had no effect on control arteries [12]. After ETX treatment, AG causes a concentration-dependent contraction of endothelium-denuded vessels which can be completely inhibited with the precursor L-arginine. This is unequivocal evidence of the participation of NO in this process [15].

The constitutive form of NOS can be found in the endothelial cells, the blood platelets and the brain, whereas the inducible form of the enzyme, which is also present in the endothelium, is located in the vascular smooth muscle, the macrophages and the neutrophils [23, 30]. The question arises whether cNOS activation participates in the early hyperdynamic circulatory response in sepsis. This is a compensatory reaction in which the increased oxygen demand of the septic tissues is met by an elevated cardiac output and peripheral vasodilatation. There are data in the literature to support this hypothesis [16, 32]. On the other hand, an overproduction of NO by iNOS in the late stage of sepsis is responsible for the intractable circulatory collapse and the high mortality [27]. The significance of NO and the possible use of NOS inhibitors in sepsis, however, have not been fully clarified. The available L-arginine analog NOS inhibitors, which inhibit both NOS isoenzymes, restore the systemic arterial pressure and increase TPR, but at the expense of reducing the cardiac output in the face of an increased oxygen demand; these agents therefore have a deleterious effect on the outcome of shock [5, 18, 20, 36].

We have chosen NNA as an inhibitor of NO synthase, because its inhibition potency is relatively stronger than that of other NOS inhibitors [35].

In our experiments, AG, similarly to the effect of the low dose of NNA employed, inhibited the hyperdynamic response. However there were, important differences between the effects of these two inhibitors. In contrast with AG, treatment with NNA increased the TPR of animals receiving ETX and reduced their cardiac output and stroke volume, confirming previous data on experimental animals and patients [8, 11, 21, 29, 31]. The different action of AG may be due to two factors: a selectivity difference for the isoenzymes and the additional effect on histamine

metabolism. Whereas AG has a higher selectivity for iNOS [6, 12, 14], NNA has a higher affinity for cNOS [13]. Our results indicate that the activation of cNOS in the early phase can be inhibited with AG, and this inhibition is sufficient for the prevention of a hyperdynamic response without an unfavourable decrease in cardiac output and an increase in TPR. The present experiments also provide further evidence on the early activation of cNOS in endotoxemia.

The second component of the effect of AG could be its inhibitory effect on diamine oxidase (histaminase). This enzyme participates in the catabolism of histamine [7]. In most species, the main pathway of histamine degradation is methylation by N-histamine-methyltransferase, but in some species and organs (such as the stomach, intestine and uterus) the importance of oxidative deamination is also appreciable [4, 26]. Histamine is released in all types of shock [24]. Our previous results [33] demonstrate the role played by this vasodilator and positive inotropic autacoid in the elevated cardiac output and lowered TPR of hyperdynamic endotoxemia. We hypothesized that through the inhibition of the degradation of endogenously released histamine, cardiac output will be stimulated in endotoxemia. This might indeed be the case in our study if we consider that the additional consequence of AG treatment, NOS inhibition, has an opposite effect and the resultant effect is a moderate suppression of the hyperdynamic response as compared with the more pronounced result of NNA administration. Our plasma histamine measurements support this concept, as the histamine concentrations were higher in Group ETX+AG at the time of lowered TPR (90 and 120 min), while NO synthesis remains inhibited by AG in this period.

In conclusion, our data provide further evidence for the participation of NO and histamine in the early hyperdynamic circulatory response in endotoxemia. While attempts to apply NOS inhibition in the treatment of sepsis have so far failed to demonstrate beneficial results, the special properties of AG make it worthy of further study, especially in view of the known deleterious consequences of iNOS activation in fully developed septic shock.

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Echocardiographic estimates related to various body size measures in athletes

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In comparing subjects of different body size echocardiographically measured data have usually been related to various body measures, mostly to body surface area (BSA). The present study serves to point out an inherent error of this procedure. Two-dimensionally guided M-mode echocardiography was performed in 106 elite male athletes. Left ventricular diastolic wall thickness (LVWT), internal diameter (LVID), and estimated left ventricular muscle mass (LVMM), stroke volume (SV) and cardiac output (CO) were related to body height (BH), body weight (BW), fat-free body mass (FFBM) and BSA. The analysis of the 20 indices has revealed that most indices and above all the BSA-related ones suffer from systematic errors so they are unsuitable to compare subjects independently from body size as they significantly correlate with BSA. These errors arise from the fact that the numerator and the denominator of these quotients differ in their power terms. With higher absolute component values all relativized quotients containing a numerator raised to a smaller power than its denominator is bound to decrease (e.g. LVWT/BSA, LVID/BSA) while quotients in which the numerator's exponent exceeds that of the denominator will increase (e.g. LVMM/BSA, CO/BSA, SV/BSA). To avoid such spurious trends it would be wiser to use indices in which power terms match. When relations to BSA are preferred, its square root appears more suitable for linear numerators (wall thickness, diameters), while for volumes and weights the cube of the square root of BSA is suggested. In the general case, however, a dimensional consistence in choosing the relativizing parameter is recommended.

Keywords: echocardiography, method of calculation, athletes

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In comparing subjects of differing body size it is usual to to relate echocardiographic data by some body dimension. Most of the studies reported have been related to body surface area (BSA) [26, 32].

Many authors employing BSA-related measures seem, however, to foster some suspicion regarding the correctness of this way of calculation. Accordingly, other body dimensions, such as body height, body weight or lean body mass, have also been used, in particular when subjects of very divergent body dimensions were compared, such as youngsters and adults [3, 17] strength-trained athletes [2, 15, 30], extremely obese persons [10, 12, 13], males and females [14], or subjects with cardiac hypertrophy [9].

The purpose of the present study was to find more suitable expressions of body size that would make the echocardiographic indices and data related to them really independent of body dimensions and provide heart/size indices free from systematic or calculation bias.

The criterion chosen for size independence was the significance of correlation between 20 recognised echocardiographic indices and BSA.

Materials and Methods

Subjects

Data of 106 young male elite athletes of different events (cycle racers, pentathlonists, triathlonists, judoists, weight-lifters, body-builders, handball and basketball players) and of comparable training history were used. Their mean age was 23.0 ± 3.5 years, mean height 180.6 ± 9.1 cm (min. 158, max 203 cm), mean body mass 80.8 ± 13.6 kg (58–133), mean fat-free mass 70.9 ± 10.4 kg (52.0–106.7), mean body surface area 2.01 ± 0.19 m^2 (1.60–2.63) and mean relative aerobic power was 60.4 ± 9.7 $ml.kg^{-1}.min^{-1}$.

Anthropometry

In taking height (BH), weight (BW) and skinfolds the recommendations of the international Biological Program [31] were observed. Skinfold sums [18] were used to estimate fat mass. BSA was calculated as by Du Bois and Du Bois [6] the formula of whom has found the widest acceptance and has been evidenced to be the best general predictor [16]. Fat-free body mass (FFBM) was obtained by substracting fat mass from BW.

Echocardiography

A Dornier AI 4800 echocardiograph was used with a 2.5 MHz tranducer. Two-dimensionally guided M-mode measurements were made, as in making consecutive measurements and measuring across several cardiac cycles. For measurements and calculations the M-mode pictures are very popular also today [11]. During echography the subjects lay on their left side.

Left ventricular internal diameter (LVID), interventricular septum thickness (IVST) and posterior wall thickness (PWT) were measured in mm immediately below the mitral leaflets both during systole and diastole on the screen using the Penn convention [5]. The respective means of ten cardiac cycles each were the input data of the analysis. All reported data refer to the end of diastole. Left ventricular wall thickness (LVWT) was obtained as IVST+PWT.

End-diastolic and end-systolic volumes (EDV, ESV) are usually estimated by cubing the internal diameters [7, 25] or by the Teichholz equation [28]. The choice between these methods is not a point of the present study. Since for healthy persons the cubing method is also accepted and is quite often used also in studies with athletic subjects [3, 15, 26], end-diastolic and end-systolic volumes (EDV, ESV) were estimated by cubing the internal diameters [7, 25], similarly to our earlier investigations [19, 20, 24]. Stroke volume (SV) was calculated as EDV–ESV, cardiac output (CO) as SV times heart rate. Left ventricular muscle mass (LVMM) was calculated as: $1.04\ \{(LVID+IVST+PWT)^3-(LVID)^3\}-13.6$ [Devereux and Reichek, 5]. LVWT, LVID, LVMM, SV and CO were related to BH, BW, FFBM and BSA, i.e., altogether 20 indices were obtained.

Statistics

To disclose their body size dependence, all the 20 echocardiographically derived indices were subjected to least square linear regression analysis plotted against BSA for every subject. Regression was considered significant at a P value of less than 0.05.

Results and Discussion

BSA-related LVWT and LVID proved to become the smaller the larger BSA the athletes had (Figs 1 and 2).

When similarly related to BSA, LVMMs behaved unexpectedly in an opposite manner in that LVMM estimates grew with increasing BSA (Fig. 3).

It is quite apparent that such discord between the respective indices calculated from the same data cannot be attributed to any biological or cardiological cause, but has to be explained by an error in the way they were calculated.

A consideration of the dimensions used in the formulas to derive these indices reveals that in these quotients the numerators and denominators differ in the exponents. In the wall thickness index [$LVWTI=(IVST+PWT):BSA$, mm/m^2] and internal diameter index [$LVIDI=LVID:BSA$, mm/m^2], the numerators have an exponent of one. Left ventricular muscle mass index (LVMMI) is calculated by cubing the respective diameters, thus the numerator's exponent is three (mass in grams). The denominator has, however, an exponent of two (surface area in square meters) in all the three formulas.

Consequently, in such quotients any change in the absolute values will necessarily associate with dissimilar changes in the numerator and denominator. When it is the exponent of the numerator that is smaller (LVWTI, LVIDI), the index will become disproportionately smaller with growing body size. On the other hand, when it is the exponent of the numerator that is higher than that of the denominator (LVMMI), the index will spuriously increase with growing body size.

The correct proportion of the numerator and the denominator, as an important principle of the dimensional consistency has already been stressed by Astrand and Rodahl [1].

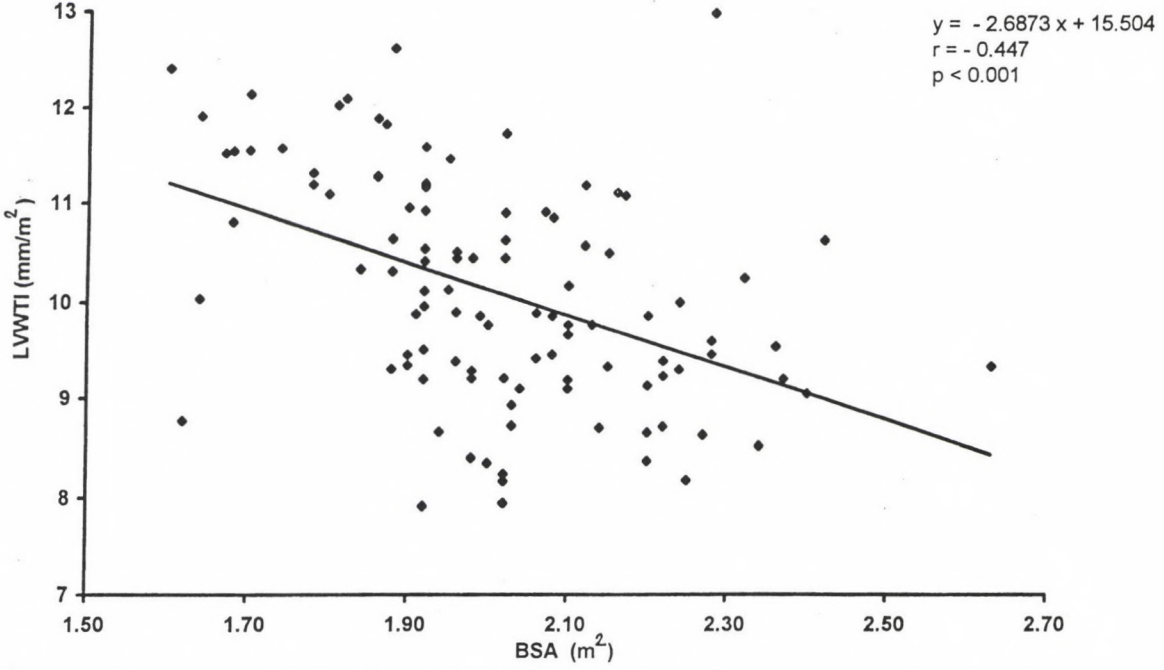

Fig. 1. Left ventricular end-diastolic wall thickness related to and plotted against BSA in athletes (N = 106)

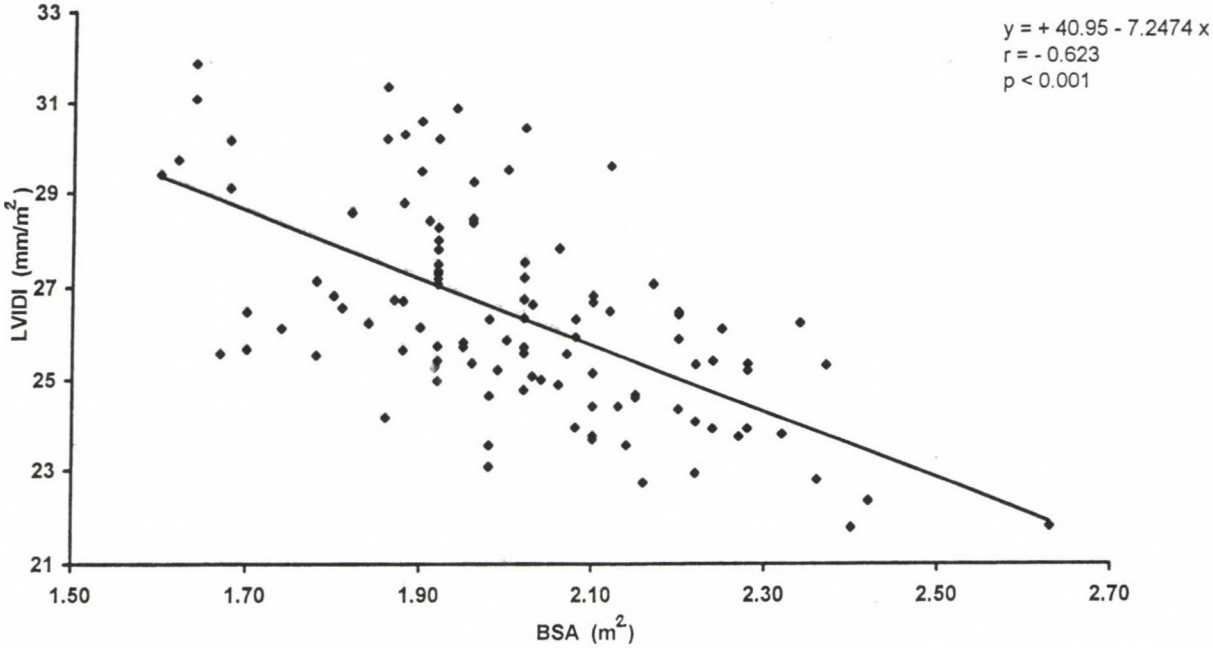

Fig. 2. Left ventricular end-diastolic internal diameter related to and plotted against BSA in athletes (N=106)

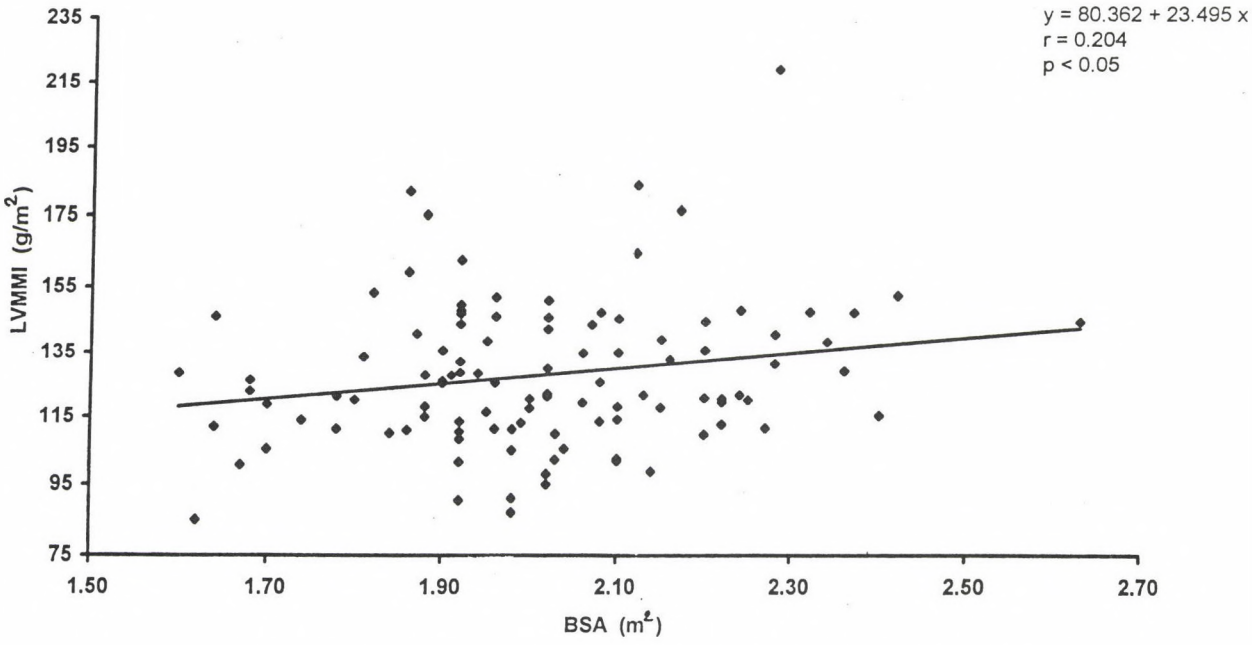

Fig. 3. Left ventricular muscle mass related to and plotted against BSA in athletes (N=106)

Table I

Correlation coefficients of the studied relative end-diastolic indices with BSA

	BH		BW		FFBM		BSA	
LVWT	LVWT/BH	n.s.	**LVWT/BW**	**−0.699**	**LVWT/FFBM**	**−0.638**	**LVWT/BSA**	**−0.447**
LVID	LVID/BH	n.s.	**LVID/BW**	**−0.786**	**LVID/FFBM**	**−0.734**	**LVID/BSA**	**−0.623**
LVMM	**LVMM/BH**	**0.473**	LVMM/BW	n.s.	LVMM/FFBM	n.s.	*LVMM/BSA*	*0.204*
SV	**SV/BH**	**0.439**	SV/BW	n.s.	SV/FFBM	n.s.	*SV/BSA*	*0.220*
CO	**CO/BH**	**0.470**	CO/BW	n.s.	CO/FFBM	n.s.	***CO/BSA***	***0.311***

Abbr.: LVWT: left ventricular wall thickness, LVID: left ventricular internal diameter, LVMM: left ventricular muscle mass, SV: stroke volume, CO: cardiac output, BH: body height, BW: body weight, FFBM: fat-free body mass, BSA: body surface area, n.s.: no significant correlation, $P<0.05$, $P<0.01$, $P<0.001$

In view of this trend, neither cardiac index {CI=[(EDV–ESV).HR]:BSA, litre/m^2.min} nor stroke volume index {SVI=(EDV–ESV):BSA, ml/m^2.beat} can be regarded as independent from body size since the numerator exponent is three in these indices, while the denominator is a square.

Table I contains the correlation coefficients studied and relates to indices with BSA.

As demonstrated in Table I, no significant correlation with BSA was seen in case of the indices in which the exponent of numerator and denominator was the same (LVWT/BH, LVID/BH, LVMM/BW, SV/BW, CO/BW, LVMM/FFBM, SV/FFBM, CO/FFBM), but every index correlated significantly with BSA in which the exponents differed.

Also the magnitude of the correlation coefficients was proportionate to the discord between the exponents. The closest correlation occurred with the quotients the numerator of which was on the 1st power and the denominator on the 3rd (LVID/BW, LVID/FFBM, LVWT/BW, LVWT/FFBM). Slightly smaller coefficients were found when the proportion of the exponents was 1:2 (LVID/BSA, LVWT/BSA) or 3:1 (LVMM/BH, CO/BH, SV/BH), and correlation with BSA was the smallest but still significant for the quotients in which the numerator was on the 3rd and the denominator on the 2nd power (CO/BSA, LVMM/BSA, SV/BSA).

Furthermore, dimensionality differences determine also the direction of the relationships. Indices with a smaller exponent in the numerator showed negative correlations, those with a higher exponent in the numerator displayed positive relationships with BSA.

This kind of calculation may seem a little unusual, especially because denominators of the indices contain either BSA or some other body dimension which basically influences BSA. The method appears, however, a simple yet suitable way to reveal the relative independence of the different indices from body dimensions. In this study BSA was chosen as a reference dimension. It seemed to be especially interesting when the different indices displayed coefficients with sign inversion.

Regarding our present data, it is small wonder that interindividual differences in the BSA-related cardiac dimensions or volumes have embarrassed researchers when they wanted to compare subjects differing in body size. This problem is of particular relevance for studies made in athletes and for sports cardiology.

In our present material, competitors of the lower weight-classes showed too high relative values in the linear dimensions (LVWTI, LVIDI), while poor ones in LVMMI. A similar problem can arise when such data are compared between children, young and adult athletes. This explains why in our previous study [24] child-age and adult athletes disproportionately differed in BSA-related left ventricular wall thickness, internal diameter and muscle mass. The fact that Csanády et al. [3] found no difference between young and adult basketball players in body-size related LVWT and VILD, but significant differences in the respective volumes has a similar explanation. Spataro et al. [27] reported that their athletic subjects had higher, comparable or lower BSA-related LVID, but consistently higher LVMM/BSA when contrasted with non-trained

controls of a definitely smaller BSA. The computation method again is likely to explain why LVMM/BH was consistently higher and LVMM/BW lower in extremely obese subjects than in lean persons in a study of Hammond et al. [8].

SV and the CO are not really echocardiographic parameters. The observation that indices with mismatched exponents cannot be independent from body size suggests, however, that CO ought not to be related to BSA even when measured by any other method. In an earlier study of ours [22] CO was estimated by isotope dilution. In 36 young athletes and non-athletes, CI gave a correlation coefficient of 0.249 (n.s.), but SVI correlated significantly ($r=0.513$, $P<0.01$) with BSA.

Our results suggest that indices of heart size should only be related to body size when numerators and denominators match. Hence, wall thicknesses and diameters may be corrected for BH, while volumes and LVMM for BW. Such attempts are seen in animal experiments, when heart weight, SV of CO are more often related to BW than to BSA [21, 23, 29], or also in echocardiographic studies, when LVMM is related to body weight [4, 9] or to height$^{2.7}$.

In the general case, a dimensional consistence in choosing the relativizing parameter is recommended.

If one prefers to retain BSA as a popular reference body size for practice, a possible way to avoid the above detailed problems would be to modify such indices be modified by matching the exponents of numerator and denominator. To this end, the use of the square root of BSA is suggested for linear measures (wall thickness, diameters) and the cube of the square root of BSA for numerators on the 3rd power (volumes, weights). None of the indices modified in this way correlated with BSA (coefficients in Table II).

Finally, we note that the principle of matching exponents between numerator and denominator should be examined also in other indices used in the medical practice (metabolism, respiration, etc.).

Table II

Correlation coefficients between dimensionality corrected end-diastolic echocardiographic indices and BSA

$LVWT/BSA^{1/2}$	-0.027 $P>0.1$
$LVID/BSA^{1/2}$	-0.089 $P>0.1$
$LVMM/BSA^{3/2}$	-0.078 $P>0.1$
$SV/BSA^{3/2}$	0.004 $P>0.1$
$CO/BSA^{3/2}$	0.137 $P>0.1$

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Effects of hormones on the number, distribution and degranulation of mast cells in the ovarian complex of mice

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The changes in the number and degranulation pattern of mast cells varied with the types of hormonal treatment and ovarian compartment. Luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH) and 17-β estradiol (E_2) treatment caused increase ($P<0.05$) in the number of mast cells in the hilum as compared with the controls. Increase ($P<0.05$) in the number of mast cells in the whole ovarian complex was observed only following FSH and E_2 treatment. All the hormones used in the present study increased the percentage degranulation of mast cells in the hilum. However, only LH, FSH and E_2 increased the percentage degranulation of mast cells in other compartments of the ovary (medulla, bursa and cortex). TSH and ACTH failed to cause any increase in the percentage degranulation of mast cells in these compartments. The present findings indicate E_2 to be the most potent among the hormones tested in causing degranulation of mast cells in all ovarian compartments.

Keywords: mast cells, ovary, mice, hormone

It is well known that tissue mast cells produce and secrete histamine [10]. Further, it is known that the mast cell is not merely a sac that breaks open when acted upon by drugs or other agents, but it is a cell capable of secretion of stored substances through the specific process of exocytosis or degranulation [6]. In fact, a direct correlation between the mast cell degranulation and histamine content of the tissue has been shown in the ovary of hamster [14]. As histamine has been postulated to take part in ovarian processes [10], information on mast cell degranulation following specific hormonal treatment may provide a clue about the involvement of histamine as a physiological intermediary in the ovarian function.

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In most secretory cells there are nervous or humoral factors that regulate secretion. Several hormones (peptides and steroids) have been shown as regulators of the mast cell release of biologically active histamine and other mediators in different endocrine glands and reproductive organs. Among these there are gonadotrophins (Gn), thyroid stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH) and 17-β estradiol (E_2).

Gn surge at proestrus is shown to induce a rapid release of histamine from ovarian mast cells in the hamster [13]. Histamine is also shown to be acutely liberated from the ovary upon stimulation by LH [24]. This LH- or Gn-induced release of histamine, in turn, causes ovarian hyperaemia [12], and in this light it has been implicated as an inflammatory mediator of ovulation [4] and ovarian contractility [17, 19]. TSH is also shown to induce a rapid release of 5-hydroxytryptamine (5-HT) and histamine from these cells in the thyroid gland and these amines, in turn, increase thyroidal blood flow [18]. ACTH is shown to evoke histamine as well as 5-HT release from rat peritoneal mast cells *in vitro* [8]. ACTH has also been shown to cause dramatic increase in the ovarian blood flow [25].

Spaziani and Szegő [23] have reported a marked decrease in uterine histamine content within 4 h following E_2 treatment. This finding has been later confirmed and extended by Shelesnyak [22]. These and other observations form the basis of a hypothesis in which vasodilation, oedema formation and perhaps other responses elicited by E_2 in the uterus are attributed to the action of E_2-induced histamine release [5, 16]. E_2 is also shown to affect thyroidal mast cells [20]. Whether E_2 treatment affects ovarian mast cells has not been demonstrated so far.

Thus, in the present study, LH, FSH, TSH, ACTH, albumin and E_2 were tested *in vivo* for their effects on mast cell degranulation in the mice ovarian complex.

Materials and Methods

Adult cyclic mice of Parke's strain were maintained in a room with a daily lighting schedule of 14 L:10 D; (Lights on, 0600 h), food and water were available *ad libitum*. Vaginal smears were examined daily and the animals which had displayed four consecutive 4–5 day estrous cycles were employed in the experiment. Mice in diestrus stage (selected by vaginal smear) were employed in this study. Anterior pituitary hormones, LH (NIADDK-OLH-26), FSH (NIADDK-O FSH-17), TSH (NIADDK-h TSH-B-1), ACTH (Sigma) and bovine serum albumin (BSA) were dissolved in the physiological saline solution. A dose of 10 μg/0.1 ml saline of each of these peptides was finally selected on the base of preliminary study. All the peptides were administered via the tail vein. E_2 was dissolved in propylene glycol and administered intravenously (i.v.) at the dose of 1 mg/0.1 ml of propylene glycol. Three different types of controls were used in this study. Controls for peptide hormones received either BSA or saline, while control for steroid hormone experiment received propylene glycol. Six mice were used for each treatment group and as controls. Two hours after the injection of peptide/steroid hormone mice were sacrificed by decapitation. The ovary with bursa and the fat pad containing ovarian blood vessels (ovarian complex) were excised and placed in 10% buffered neutral formalin. Serial 6 μm paraffin sections were stained, dehydrated and then mounted in DPX. All sections were stained for approximately 1–2 min with the Quick Toluidine Blue method, the sections were then dehydrated in 2–3 changes of acetone [13].

Mast cell counting procedure

Mast cells were classified as non-degranulated (the intact cells with extrusion of less than 10 granules) and degranulated (cells exhibited dispersion of more than 10 granules). Mast cells were counted with a 10 X ocular and a 45 X objective in every 5th serial sections of the ovary. The percentage of degranulated mast cells were calculated by dividing the actual number of degranulated mast cells by the total number of mast cells (nondegranulated + degranulated), and multiplying this with 100.

Mast cells were counted in each compartment of the ovarian complex viz., medulla, hilum, bursa and cortex as shown in Fig. 1.

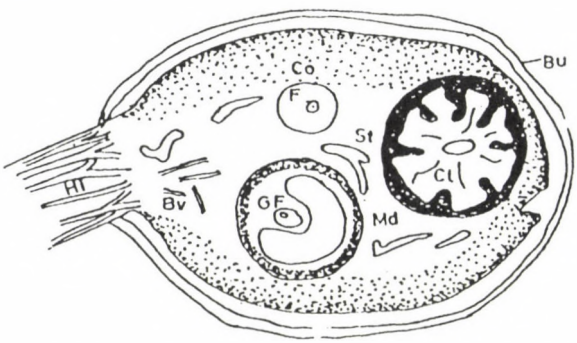

Fig. 1. Diagrammatic representation of different compartments of ovarian complex. A = Atretic Follicle; Bu = Bursa; Bv = Blood Vessel; Co = Cortex; CL = Corpus Luteum; DF = Developing Follicle; GF = Graafian Follicle; Hi = Hilum; Md = Medulla; St = Stroma

Statistics

Data were analysed by the one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. Wherever appropriate, Student's 't'-test was used. Differences were considered significant if $P<0.05$.

Results

Effects of LH, FSH, TSH, ACTH and BSA

The percentage degranulation of mast cells increased considerably in the ovarian complex following administration of LH, FSH, TSH or ACTH treatment as compared with the BSA or saline-treated mice (Table I). Whereas, changes in the number of mast cells following administration of these hormones varied with the ovarian compartment and the type of hormonal treatment.

Table I

Effect of hormones on total number (TMC) and percentage degranulation (% Deg) of mast cells in ovarian compartments of mice

	Hilum		Medulla		Bursa		Cortex		Total number of mast cells in ovarian complex	Average % degranulation of mast cells in ovarian complex
	TMC	% Deg	TMC	% Deg	TMC	% Deg	TMC	% Deg		
Saline	893.0±54.5	16.0±1.2	509.5±30.9	12.0±1.3	215.0±13.4	14.5±0.9	-	-	1617.5±68.4	14.2±0.9
Propylene glycol	872.2±53.9	16.8±1.2	528.8±27.4	14.2±1.1	207.3±14.1	12.7±1.3	-	-	1608.3±80.9	14.6±0.5
		A			A				A	A
BSA	761.5±25.0	19.2±0.9	574.8±33.2	14.2±0.9	192.3±11.5	16.7±1.1	-	-	1528.2±37.8	16.7±0.8
	C	AC	AC	ABC	C	AC			AC	AC
LH	1079.7±40.2	68.7±2.2	334.2±22.7	38.7±2.2	215.5±11.3	35.5±2.1	69.0±7.3	38.2±2.0	1698.3±38.9	45.3±1.0
	AC	ACD	CD	ACDB	ACD	ACD	D		ACD	ACD
FSH	1122.2±34.5	43.0±2.1	479.2±39.5	25.5±2.2	303.3±27.8	24.0±1.8	138.6±17.2	31.0±3.3	2043.3±46.9	30.9±1.4
	E	ACDE	AC	DE	ACDE	DE			CE	ACDE
TSH	971.1±61.6	33.8±1.8	423.3±25.6	16.0±2.0	248.2±9.4	17.0±2.0	-	-	1642.7±58.0	22.3±1.1
	DE	ACDEF	CD	DE	CEF	D			ACDEF	ACDEF
ACTH	734.0±42.3	30.0±1.3	461.5±28.8	14.5±1.7	213.5±15	18.0±2.0	-	-	1409.0±61.1	20.8±1.2
	H	H	ABEFG	H	H	H	D	DE	H	H
17-β Estradiol	1908.8±202.6	62.3±1.9	252.3±25.1	76.5±3.3	315.3±26.9	66.5±2.0	166.1±13.4	55.1±3.1	2642.7±192.8	65.1±1.7
One way ANOVA p value	0.001	0.005	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Data on mast cell number and % degranulation were analysed separately by one way ANOVA and Duncan's Multiple Range Test (Foot notes A–H).

A = $p<0.05$ as compared to saline
B = $p<0.05$ as compared to propylene glycol
C = $p<0.05$ as compared to BSA
D = $p<0.05$ as compared to LH
E = $p<0.05$ as compared to FSH
F = $p<0.05$ as compared to TSH
G = $p<0.05$ as compared to ACTH
H = $p<0.05$ as compared to others

The number of mast cells has increased ($P<0.05$) in the hilum in both LH- or FSH-treated mice as compared with the control or BSA-treated females. The number of mast cells has decreased in the medulla in LH-treated mice ($P<0.05$) while it has not following FSH administration ($P>0.05$) when compared with the control (Table I). No significant variation in the number of mast cells was found in the bursa following treatment with LH. FSH-treatment, however, caused increase ($P<0.05$) in the number of mast cells in the bursa. Interestingly, some mast cells were observed in the cortex region of the ovary in the mice treated with either LH or FSH but no mast cells were found in the ovarian cortex of control or BSA-treated mice. Mast cells were also not found in the cortex region of mice treated with either TSH or ACTH. Percentage degranulation of mast cells increased sharply in all of the ovarian compartments (hilum, medulla and bursa) following LH- or FSH-treatment as compared with the control or BSA-treated mice. Both TSH- and ACTH-treatment caused increase ($P<0.05$) in the percentage degranulation of mast cells too mainly in the hilum. TSH-treatment caused moderate increase in the percentage degranulation of most cells in the medulla and bursa. But a slight decrease in the number of mast cells in the medulla was noticed following TSH-treatment. ACTH-treatment caused significant ($P<0.05$) decline in the number of mast cells in the hilum as compared with the control. But this decline in the number of mast cells is similar to that observed following BSA-treatment.

Effects of E_2

The number of mast cells were considerably changed in all of the ovarian compartments following administration of E_2. The E_2 treatment also produced widespread degranulation in all ovarian compartments (Table I).

The number of mast cells was increased ($P<0.05$) in the hilum and bursa, while it was decreased ($P<0.05$) in the medulla in E_2-treated mice as compared with the control. Mast cells with a high (52.2 ± 3.2) percentage of degranulation were found in the cortex region of the ovary similar to that observed in the LH- and FSH-treated mice. The percentage of mast cells degranulation increased considerably in all of the ovarian compartments as compared with the control (Table I).

Discussion

Changes in the number of mast cells varied with the type of hormonal treatment and ovarian compartment. Both LH- and FSH-treatment caused a significant increase in the number of mast cells in the hilum but they caused a significant decrease in the medulla as compared with the controls (saline as well as BSA). TSH-treatment caused a slight increase in the number of mast cells in the hilum but it evoked a decrease in the medulla. At the same time ACTH- and BSA-treatment caused decline in the number of mast cells in the hilum when compared with the saline-treated control.

However, the total number of mast cells in the entire ovarian complex increased significantly in the females treated with FSH but it did not in the females treated either with LH or TSH. Females treated with ACTH showed decline in the total number of mast cells in the ovarian complex. The significant variation in the number of mast cells in the ovarian compartments were observed following E_2 treatment. Females treated with this hormone showed significant increase in the number of mast cells in the hilum, bursa as well as in the entire ovarian complex as compared with the saline- or propylene glycol-treated controls but mast cell number decreased significantly in the medulla. The differences in the response of mast cells to different hormones in different ovarian compartments suggest the mediation of local factor(s).

Interestingly, in females treated with LH, FSH or E_2, mast cells were seen in the cortex of the ovary. Mast cells are generally not found in this region during normal cycles in the mice [11]. These hormones, thus, stimulate migration of mast cells into the cortex. Such migration of mast cells was not observed in the females treated with TSH, ACTH or BSA. This migration of mast cells to the cortex may also explain the decline in the number of mast cells in the medulla following treatments with these hormones. Migration of mast cells into the ovarian complex has earlier been reported in the rat [9] and was postulated to be related to high E_2 level. The results from present study further confirms the role of E_2 in stimulating mast cells migration into the ovarian cortex. Significantly higher number of mast cells in the cortex was found in the females treated with E_2 as compared with either LH or FSH. We speculate that the action of LH or FSH in inducing mast cell migration may be an indirect effect through steroid synthesis.

The results of this study indicate that LH is the most potent peptide hormone which induces degranulation of mast cells in all ovarian compartments. This finding is consistent with previous reports indicating that LH is the signal for depletion of ovarian histamine [13, 24].

To the best of our knowledge, this is the first study demonstrating the effect of FSH alone in causing extensive degranulation of mast cells in the ovarian complex of the mouse. Unlike LH, FSH induces significant ($P<0.05$) increase in the number of mast cells in the bursa as well as in the entire ovarian complex as compared with the control. This may partially explain the earlier finding of Lipner [15], who has suggested that in the rat, LH tends to deplete histamine in the ovary, whereas Pregnant Mare's Serum Gonadotrophin (FSH and LH) increases ovarian histamine concentration.

TSH- and ACTH-treatment also caused significant ($P<0.05$) increase in mast cell degranulation in the hilum but not in other compartments of the ovary. These findings point to a local mechanism within the hilum being responsible for the extensive degranulation of mast cells. In the hilum mast cells were found mainly in the vicinity of the blood vessels that enter and leave the ovary which might imply that they may be playing some important role in the regulation of blood flow to the ovary [13, 14]. Both TSH and ACTH are known to promote vascular reaction [3, 25].

The present finding indicates E_2 to be the most potent among the hormones tested in causing degranulation of mast cells in all ovarian compartments. This is in accordance with the earlier studies suggesting E_2 as a potent stimulator of mast cell degranulation in the uterus [2]. A recent *in vitro* study has shown that E_2 stimulated induced secretion of histamine from purified rat peritoneal mast cell starting at 1 μm and in a dose dependent manner [26]. The information published on the relationship of mast cell degranulation and/or histamine level to E_2 level in the ovary is scanty.

The finding reported here poses the following intriguing questions: (1) How do these hormones bring about changes in the number and degranulation of mast cells, (2) whether ovarian mast cells contain specific hormone receptors. Answer to these questions are not yet clearly known. Earlier study, however, failed to demonstrate a direct LH/hCG binding to ovarian mast cells as revealed by topical autoradiography using I^{125} hCG [Krishna and Terranova, unpublished]. Thus effect of these hormones may not be a direct action on the ovarian mast cells but it may require the synthesis of specific mediating factors. Factors involved in inducing mast cell degranulation in other systems are IgE, phorbol esters and various neurohormones [1, 7, 21], all of which can be found in the ovary or they are produced by it.

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Management of hemophilias – gene therapy to come?

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Background: Hemophilias are relatively common coagulopathies that require very careful management to prevent bleeding and related complications.

Objective: To review the current management of hemophilias, with an overview of gene therapy.

Summary: Although with the availability of highly purified and recombinant factor concentrates the replacement therapy of hemophilias has become safer and more effective, a therapeutic method providing continuous supply of clotting factors is desirable. Theoretically, this is possible, by transplanting organs, than can express antihemophilic proteins, but in most cases a less invasive method is preferable. Therefore the gene therapy would be revolutionary in treating hemophiliacs.

Conclusion: The treatment of choice for hemophilias is the infusion of highly purified or recombinant factor products. Although still in experimental level, gene therapy for hemophilias is promising and may become the treatment of choice in these disorders.

Keywords: hemophilia, gen therapy

The most common hereditary coagulopathies, hemophilia A (Classic hemophilia) and B (Christmas disease) are classic chromosome X-linked recessive disorders, which occur when factor VIII (in hemophilia A) or factor IX (in hemophilia B) are deficient or functionally inactive. The frequency is about 1 in 5,000 male births in hemophilia A, and 1 in 30,000 live male births in hemophilia B [1, 2]. As part of coagulation cascade, factor IX in its active form factor IXa with its cofactor factor VIIIa, due to its proteolytic activity, activates factor X to factor Xa. Factor VIII has no proteolytic activity, its role is to increase the rate of the reaction [17].

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Clinical features

The clinical features of hemophilia A and B are similar. They include deep tissue bleeding with deep hematomas, hemarthrosis and significant bleeding after trauma or surgical procedures. Repeated hemarthrosis leads to constant synovitis, which can result in severe disabling arthropathy if not treated appropriately. Intramuscular hematomas can cause serious problems by compressing nerves, vessels or airways. Massive soft-tissue bleeding can occur in the retroperitoneal space and the thigh, giving rise to severe anemia. Intracranial bleeding was among the leading causes of death before the AIDS epidemic. Hemophiliacs should avoid situations that increase the risk of bleeding, such as sports, that are frequently associated with injuries.

Screening laboratory tests show a normal platelet count and prothrombin time, and prolonged activated partial-thromboplastin time. Specific factor assays confirm the diagnosis, and differentiate hemophilia A from hemophilia B. The plasma level of factor VIII in hemophilia A and of factor IX in hemophilia B corresponds to the clinical severity of the disease. Hemarthrosis and spontaneous bleeding are frequent, when the circulating factor levels are less than 1% of the normal. Even slight increases of the plasma levels of factor VIII, and especially of factor IX can result in dramatic improvement, and a clinically severe hemophiliac can become asymptomatic.

"Non-gene" therapy
Replacement therapy

The replacement therapy of hemophilias is based on the administration of factor containing products. By giving different kinds of products, replacing the blood clotting factors is the most common and most accepted therapeutic option. The administration of factor containing products can have different, often serious side effects, depending on the method of production and purification of these concentrates.

Plasma

Plasma was the only treatment for bleeding episodes until the 1960s [11]. This therapy was severely limited, since plasma, whether fresh frozen, or stored, contains relatively small amounts of factors VIII and IX, and the quantity of infusable plasma is restricted. Plasma is not generally recommended as a first line therapy, however, it represents an appropriate initial emergency therapy, until the appropriate factor concentrate is available.

Factor VIII and IX concentrates

In the 1960s, the first factor VIII concentrates were developed. Although these products had still relatively low specific activities, and were primarily composed of other plasma proteins, such as fibrinogen and fibronectin, more specific and effective therapy became available.

The older factor IX containing products also contain the other vitamin K-dependent factors and proteins C and S, therefore they are called as prothrombin complex concentrates. While the prothrombin complex concentrates are highly efficient in the treatment of hemophilias, the use of these products has been associated with serious complications, such as thromboembolism, disseminated intravascular coagulation, and transmission of viral diseases, particularly hepatitis B, C, and HIV infection [5, 10]. The thromboembolic complications include venous thrombosis, and myocardial infarction, which can even occur in young, or otherwise low risk individuals. The mechanism of thrombogenesis associated with the administration of prothrombin concentrates is not completely understood, but possibly due to supernormal levels of factors II and X [18]. The advantage of the use of prothrombin concentrates is their relatively low cost, compared to the highly purified and recombinant products.

Highly purified factor concentrates

The highly purified concentrates contain no or minimal amount of other factors. These products undergo processes, such as heating, ultrafiltration, and exposure to sodium thiocyanate to inactivate or remove viral contaminants. Due to these processes the transmission of viral diseases is markedly reduced, but not completely eliminated [13, 16]. Further advantages of using highly purified factor concentrates include less thrombogenicity and decreased exposure of allogenic proteins. Potential hazard of the use of such products is the infusion of minimal amounts of contaminating mouse proteins, although clinical experience with monoclonal factor concentrates indicates that adverse immune reactions are very infrequent. The disadvantage of these products is their high cost.

Recombinant factor products

Recombinant DNA-derived factor concentrates, now available, can be used for the treatment of hemophilias without the risk of transmission of viral infections. These products are not derived from human plasma, therefore they do not contain HIV, hepatitis B or C or any other viruses that could be present in human plasma. They seem to be well tolerated, very effective, but expensive. Although still controversial, it is possible, that these products might provoke a higher incidence of factor inhibitors than do plasmaderived concentrates [4].

Guidelines for replacement therapy

The amount of factor VIII or IX needed for the management of bleeding episodes depends on the site and severity of the bleeding. For less serious injuries or spontaneous hemarthroses factor peak levels of 30% to 50% are usually sufficient, bleeding problems may require peak levels of 70% to 100%. One milliliter of normal plasma contains 1 unit of factor VIII and IX, by definition. The plasma volume is about 50 ml/kg, thus, to increase the plasma level of factor VIII from 0% to 100%, in a 60 kg patient, 3000 units of factor VIII has to be administered. The volume distribution of factor IX is twice that of factor VIII, therefore a 60 kg patient requires 6000 units to raise the plasma level from 0% to 100%. The half-life of infused factor VIII is 12 hours, therefore, after 12 hours the plasma level of factor VIII will decrease by one half. Infusion of one half of the initial dose will restore the plasma factor VIII to the initial level. The same rules apply to factor IX, but its half life is 24 hours [8]. However in the past several years with the highly purified and recombinant factor concentrates becoming available, for the above reason, the treatment with gene therapy would be revolutionary. Since there is not need for tissue specific expression, and there is no precise regulation of the level of factor VIII and IX, the hemophilias are classic examples of diseases, that are most likely treatable with gene therapy [22]. The genes for factor VIII and IX have been cloned, and a number of different vectors, cells, cell lines, and approaches have been used to achieve the expression of these factors.

Vectors

Although both viral and non-viral vectors have been used for genetic modification of different cells, in most experiments viral vectors have been studied [23] and proven to be effective.

Viral vectors

The most commonly used viral vectors are retroviruses [19]. These are very effective in genetic modification of cells, but there might be some significant safety issues regarding their use in humans [7]. Adenoviruses, parvo viruses and herpes viruses also have been examined. When using viral vectors, the critical viral genes are removed, producing replication defective viruses to avoid the possibility of infection. Genes encoding factor VIII and IX can be substituted for viral genes and then delivered into the target cell. After entering the cell, the viral genetic material will be integrated into the cellular DNA.

Continuous delivery of coagulant factors would be preferable to intermittent replacement to prevent bleeding episodes, and preventive therapy is more advantageous

than prompt treatment on demand after hemorrhage. Prophylactic replacement therapy is unfortunately not practical, because of the side effects of the less pure products and the expenses of the highly purified and recombinant concentrates. Thus, such therapy is not commonly practiced in the United States and Europe.

Although the above described therapies are effective in the treatment of hemophilias, none of them provide continuous delivery of factors VIII or IX. Therefore, there always has been an interest to search for other ways to treat hemophilias.

Gene therapy – a potential therapeutic revolution

The goal of the therapy of hemophilias is the continuous and safe delivery of clotting factors. Although the replacement therapy of hemophilias has markedly improved over the last decades. The acquisition of new genetic markers by the incorporation of added DNA would be the best method for correcting the factor abnormality. This process is called transfection. The most common way of transfection is the use of viral vectors (mostly a retrovirus), in which the desired base pairs are inserted. That virus genom will be incorporated into the eukaryotic cell without making the cell able to produce the infectious virus, since the genes required for the replication have been removed. This process is known as transduction.

Non-viral vectors

Genetic modification of cells is also possible without using viral vectors. The processes using physical or chemical methods to introduce DNA into cells are termed transfection. The methods used for this purpose include electroporation, lipofection, and calcium phosphate transfection. The transfected DNA is usually not integrated into the cellular DNA, and therefore it is called as episomal.

Direct gene transfer can be achieved by intravenous injection of a plasmid-liposome complex, and even naked DNA without a special delivery system can be directly injected, resulting in expression of the DNA [24, 27].

Cell and cell lines

Expression of human clotting factor VIII has been reported in a variety of cells and cell lines, such as hamster kidney. Chinese hamster ovary, mouse embryonic mesenchymal cells, and primary human skin fibroblasts [10]. Expression of factor IX has also been observed in many different cell lines and primary cells. These include primary fibroblasts, myoblasts, keratinocytes, hepatocytes, Chinese hamster ovarian cells and endothelial cells [6, 9, 25, 26]. A fully functioning factor IX can be produced

by all of the above cells, although the level and time of expression varies, depending on the cell types and cell lines utilized as well as on the characteristics of the vector employed. A number of different methods have been used to improve the expression of clotting factors in genetically modified cells.

Approaches

The genetic modification can take place in cultured cells (in vitro), or direct in the body (in vivo).

In vitro approaches

When using "in vitro" approaches, the genetic modification is performed ex vivo, in cultures, and than the genetically modified cells are returned into the body. The return of the modified cells is usually the critical step of the in vitro approaches. Providing appropriate perfusion for the implanted cells, without generating immune response can be very difficult. For this purpose, the use of a biocompatible membrane allowing the diffusion of the factor molecules but blocking the entry of cellular mediators to protect the modified human factor producing allogenic cells has been examined and found to be effective [4]. This problem can be eliminated by using marrow cells for genetic modification, which are easy to return into the bloodstream. To achieve more efficient transduction, the proliferation of the primary cells to be transducted can be enhanced by using various growth factors [3]. The in vitro approaches have the advantage that the genetic modification can be well controlled and optimized [15].

In vivo approaches

When using an in vivo approach for expression, the genetic material containing vector is injected directly into the body and the genetic modification takes place "in vivo". To work efficiently, these approaches can be performed by targeting the vectors to a specific cell type by using biochemical or mechanical methods [21]. The limitation of in vivo approaches is generally the number of cells susceptible for genetic modification. A higher incidence of transduced cells can be obtained by stimulating cell proliferation. To achieve this, partial hepatotectomy has been carried out to enhance hepatic cell proliferation [12]. The in vivo approaches are advantageous because of their relative simplicity due to the elimination of the need for cultivation and returning cells into the body. When performed safely and effectively, in vivo approaches will be preferred.

Conclusions

Gene therapy for hemophilias can be revolutionary by providing continuous therapeutic levels of blood clotting factors, resulting in the prevention of bleeding. Although still on experimental level, this therapeutic method has the potential to result in the cure of these coagulopathies. In most cases, the level of expression is still low, but even a slight increase of the plasma level of factor VIII or IX can significantly decrease the incidence of bleeding complications, and is able to change the phenotype to a less severe one where spontaneous bleeding is less frequent. The above discussed in vivo and in vitro approaches are more than promising, and a number of experiments are being done worldwide to make gene therapy available for the treatment of hemophilias, hopefully in the near future.

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Acta Physiologica Hungarica, Volume 84 (2), pp. 199–200 (1996)

Book reviews

Cystic Fibrosis

Lung Biology in Health and Disease Series, Volume 64
Pamela B. Davis, editor
Marcel Dekker, Inc., 1993
560 pp., bound, illustrated, US$ 185

Pamela Davis, herself an authority in the field, has mustered an impressive roster of 25 authors from academe and the industry to write the 13 chapters of this monograph on cystic fibrosis, the most common inherited fatal disease of Caucasians.

Five percent of adult whites are carriers of the cystic fibrosis gene, and consequently, the disease occurs about once in every 2,000 births among Caucasians. Although more than 98% of the affected males are infertile, and fertility is also reduced in women with the disease, cystic fibrosis has a relatively high incidence, which prompted speculations about an insofar poorly characterized selective advantage for the heterozygous individuals.

Cystic fibrosis is a multi-system disorder in which the exocrine glands secrete abnormally thick mucus, leading to obstruction of the pancreas and chronic infections of the lungs, which generally cause death in childhood or early adulthood, the median survival for patients being 28 years. Some mildly affected patients may survive longer, occasionally to age 50 and beyond.

In 1989, the gene responsible for cystic fibrosis was localized to region q21–31 on chromosome 7, thus making cystic fibrosis the first major disease with an unknown basic biochemical defect, for which the gene was found totally by genetic techniques. Since then tests have been developed to detect the most common alterations, tests that can also identify unaffected carriers of the disorder. About 70% of the cases are due to a three base-pair deletion resulting in a loss of a phenylalanine in position 508, and more than 200 different mutations in the cystic fibrosis gene account for the remaining 30% of the cases.

No cure for the disease has yet been found. Patients with pancreatic insufficiency can take pancreatic enzymes with meals. Those with respiratory tract infections are treated with antibiotics, with aerosols that relieve constriction of the airways and liquefy the thick mucus, and by vigorous physical therapy to help patients cough up the obstructing secretions. Intestinal obstruction, which occurs primarily in infancy (meconium ileus), may require surgery. Newer therapeutic strategies aim at complementing the mutated CF gene with a normal CFTR cDNA to establish production of the normal CFTR protein at levels sufficient for normal functioning.

This volume provides a conceptual framework for understanding cystic fibrosis, providing in-depth discussion of major aspects of the disease, particular emphasis being placed upon the lung.

The first chapter provides an overview of the genetics of the disease, including issues such as detection of carriers and gene therapy. As the latter mainly involves manipulation with the lung, a tissue readily accessible for exogenous compounds, this subject is also dealt with in a separate, and later chapter, concerned with the treatment of pulmonary diseases.

The subsequent chapters deal with pathophysiology, infection and inflammation of the lung, and pulmonary complications. As abnormal secretions in the lung are central to the pathology of cystic fibrosis, a special chapter is dedicated to normal and pathologic mucus.

All affected organs other than the lung are also discussed, emphasis being placed upon the pancreas, due both to its major clinical importance and the fact that the implications of the electrolyte defect

are poorly understood. Likewise, the sweat duct is covered in detail, because of the major insight the study of this organ has provided.

A final chapter on the pathology of cystic fibrosis covers the lung, GI tract, pancreas and hepatobiliary system and the genitourinary system.

With more than 2,000 literature citations, tables and figures, the book draws a comprehensive and clear picture of the state-of-the-art at the time of its writing, also presenting major questions, and areas still in debate. It certainly makes an invaluable resource for the student of cystic fibrosis.

L. VÁSZÁR, M.D.

A History of Breathing Physiology

Edited by Donald F. Proctor
Lung Biology in Health and Disease Series, Volume 83
Marcel Dekker Inc. New York–Basel–Hong Kong 1995.
360 pages plus Index.
ISBN: 0-8247-9653-5 $ 150.00

Of course this recent volume of the series represents much more than simply a History of Breathing Physiology. It gives an account of the history of scientific thinking, gathering and interpretation of facts, some details of the life and ideas of great physiologists, and if one wants to find it, it also adequately summarizes the current status of Respiratory Physiology.

There are some classical historical parts. Chapter 1. "Ancient Medicine and the Mystery of Breathing", Chapter 2. "Galen: His Genius and His Shadow (both by Donald F. Proctor, who planned, edited and also wrote most of the chapters in this the book), and Chapter 3. "Leonardo and the Physiology of Respiration" by Wayne A. Mitzner. In this latter one can find some interesting details on Leonardo's familial situation and his personality. Proctor is the author of the next 7 chapters, beginning with Harvey and giving a generous treatment to the work or "the controversial Dr. Mayow".

Francis P. Chinard has written two chapters: one on "Transition and Revolution" and one on "Priestley and Lavoisier: Oxygen and Carbon Dioxide", both of which are full of History of Philosophy (to mention only the Encyclopédie of Diderot and d'Alambert), but also the biography of Joseph Priestley and Antoine-Laurent Lavoisier. More important however is, that it is described how to discoveries were made.

Anybody, who wants to understand fluid mechanics in circulation and respiration should read "Fluid Flow Studies Related to Physiology of Breathing" by David L. Swift. All the important equations are given, not avoiding some calculus, but also references go until 1993. Quite similarly, "The Pressure Surrounding the Lung" goes from Homer and sketches of Leonardo and Dürer (and a reproduction of Chagall) until the recent fluid dynamic model published in 1994. In the next chapter, "Pulmonary Circulation: Mechanics" by Sobert Permutt, the names go from Harvey until André Cournand (but the sequence is M. Vischer – A. Cournand – Claude Bernard, and it is all logical!).

Mary Ellen Avery wrote the chapter on "Lung Stability and Surface-Active Agents". "The Regulation of Breathing" was covered by Roberts S. Fitzgerald. His report on the State of the Art is excellent, could be used in every advanced text of Physiology. The last chapter is on "Nonrespiratory Functions of the Respiratory Tract" by Oomen P. Mathew and Tapan K. Gosh.

There are photographs of Arthur Otis, Hermann Rahn and Wallace Fenn, with whom Donald F. Proctor has been working in his younger years, what clearly had a major impact on him and also on the book he made. The book is dedicated to the memory of the late Philip Bard and it serves well this memory.

ATTILA FONYÓ

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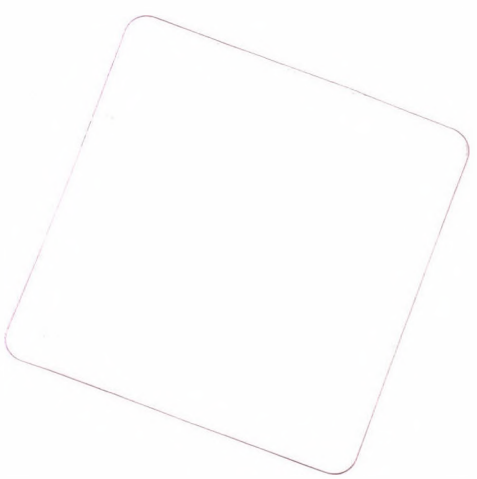

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Dettler J. C.: Biochemical variation. In: Textbook of Human Genetics, eds Fraser, O., Mayo. O., Blackwell Scientific Publications, Oxford 1975, p. 115.

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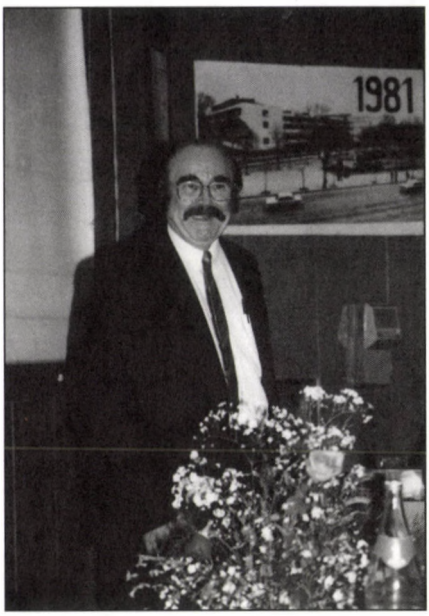
Acta Physiologica Hungarica, Volume 84 (3), pp. 201–203 (1996)

In memoriam Professor A. G. B. Kovách



VALE, PROFESSOR!

We were stunned to hear of the sudden death on 18th January, 1996 of Dr. Arisztid Kovách, founding professor of the Clinical Research Department – 2nd Institute of Physiology, Semmelweis University of Medicine, and until 1990 its director and professor. With his death our university has lost one of its most versatile and interesting figures of the post-war period. After matriculating from the Madách Secondary School, Arisztid Kovách enrolled in the Medical Faculty of the Péter Pázmány University of Budapest in 1938. The next step which he took in his first year at the university when he applied to do research work in the Institute of Physiology was decisive for the whole of his later career. He also studied for two semesters at the Heidelberg University. It was here that he became interested in the question of how the state of circulatory shock becomes irreversible. This period had a lasting influence on his professional career and that of many of his later colleagues. He realised from this experience that successful scientific research work could not be advanced in Budapest without international perspectives and contacts. Later as the director of the Clinical Research Department this led him to make every effort to give his young colleagues the opportunity of going for longer study trips to internationally recognised West European or American universities at the earliest possible stage in their careers.

Akadémiai Kiadó, Budapest

Following completion of his training he began work in the Institute of Physiology and organised a research team. He became a leading figure in international shock research, drawing attention to various disorders of tissue metabolism in shock. He defined the course of changes of sympathetic nervous responsiveness seen with the development of circulatory shock. He proved that survival time can be increased significantly with sympathetic blockers, or by a transfusion of blood from healthy dogs through the brain of dogs in shock. In 1959 the University gave him the task of organising the Experimental Research Laboratory of the External Surgical Clinics. In the following decades countless candidates' and doctoral dissertations were written in the Research Laboratory, and what is perhaps even more important: life-long, mutually enriching professional and personal relationships were formed during the work on experiments. Despite his international reputation, it was not until 1970, at the age of 50 that he was appointed a university professor. Finally, after a break of 13 years, he was again able to teach physiology to medical students. In the early 1970s one of the biggest international medical organisations, the International Union of Physiological Sciences (IUPS) entrusted him to edit its international Newsletters. Shortly after he became a member of the IUPS Council and then from 1974 its secretary. He was instrumental in bringing the IUPS World Congress to Budapest in 1980.

In 1971–72 he spent a year and a half in the research institutes of the Philadelphia Hahnemann University and the University of Pennsylvania. He and Professor Britton Chance developed a non-invasive optical method for monitoring cell metabolism processes now widely used throughout the world. He developed sources of funding to support ongoing research between the University of Pennsylvania and the Semmelweis Medical University which continues to this day.

In the second half of his life he received many awards and distinctions. To mention only the most important of these: Associate Professor of the Institute of Neurology of the University of Pennsylvania; member of the board of the Hungarian Physiology Society; Chairman of the Worldwide Hungarian Medical Academy; Secretary, then Vice-President of the International Union of Physiological Sciences; member of the International Society of Oxygen Transport to Tissue, the International Shock Society and numerous other Hungarian and international scientific societies and committees. He was an honorary member of the Belgian Royal Academy, the Polish Academy of Sciences and the Czechoslovak Purkinje Society, and an honorary doctor of the Kuopio University. His awards included Outstanding Teacher of the Semmelweis Medical University, Prize of the Hungarian Academy of Sciences, Semmelweis Prize 1st degree and the Prize of the Swedish Health Research Council.

Arisztid Kovách was a gentleman and a scholar of international stature who loved and proudly represented the best of Hungarian traditions. His professional influence was felt far beyond the borders of Hungary. He was extremely perceptive in

recognising new research trends and methods. The successes of his students and former colleagues working in Hungary and around the world are a living testimony of his gifted leadership. His death marks the end of a period in the history of Hungarian physiology and of our Institute. We will carefully preserve the professional, spiritual and moral values we received from Arisztid Kovách and pass them on with affection to our successors. Vale, Professor!

Prof. Péter Sándor
Deputy-Director

Prof. Márk Kollai
Deputy-Director

Prof. Emil Monos
Director

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The functional damages of ischemic/reperfused skeletal muscle

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Skeletal muscle is frequently damaged by ischemia-reperfusion when exposed to direct injury or in the surgical practice. The purpose of the present experiments was to examine how the different types of skeletal muscles (fast & slow) react functionally to one and two hours of ischemia followed by two weeks of reperfusion. The fast-twitch (*m. extensor digitorum longus*/EDL) and the slow-twitch (*m. soleus*/SOL) muscle were prepared. They were stimulated, *in vivo*, either directly or indirectly at different reperfusion times following tourniquet ischemia, and the contraction force (CF) was recorded. CF of the EDL was reduced over 40% and 90% of the control value during the first 24 hours of reperfusion after 1 and 2 hours of ischemia, respectively. It was about 50% at the end of the 2nd week in the one-hour group. CF increased significantly during the second week if ischemia lasted for two hours. Reduction of CF in the SOL muscle was over 50% and 90% following one and two hours of ischemia, respectively. It further decreased in the 1-hour group, and it started to regenerate from the second week after 2 hours of ischemia. It is concluded that 1. two hours of ischemia causes significantly more severe damages in both types of skeletal muscles than one hour. 2. There is a reperfusion injury in both muscles during the first week of reperfusion. 3. The two types of muscles regenerate differently, i.e. the SOL starts to regenerate earlier than the EDL.

Keywords: skeletal muscle, contraction force, ischemia-reperfusion

Ischemia (I) and reperfusion (R) of the skeletal muscle are commonplace in trauma surgery. The limb can often become ischemic due to injury, and planned operations are also frequently carried out in the bloodless state. Revascularisation after prolonged ischemia may result in long-lasting or permanent damage. The muscles deplete their energy stores during the bloodless period [14]. Lactate accumulates due to anaerobic glycolysis and the amount of the produced lactate depends on the length of I [11, 12].

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Numerous investigators have studied the effect of I on the skeletal muscle. Patterson and Klenerman [21] studied ultrastructural changes of the EDL and the SOL after ischemia-reperfusion, and found more severe damage (i.e. eroded Z-lines) in the EDL, than in the SOL. Other investigators examined the contractile properties of the skeletal muscle after different times of ischemia. Fish et al. [6] reported that the recovery of the isometric twitch and the tetanic function after tourniquet was inversely related to the ischemic interval. Gardner et al. [7] studied the effect of tourniquet ischemia on the contractile properties of the two types of muscle using a guinea pig hind limb model. They found that 2 hours of ischemia resulted in a marked reduction in twitch and tetanic force production. This reduction was more evident in the fast than in the slow muscle. All these studies focused on a specific, mostly early time (first hours) of reperfusion.

Ischemia itself results in cellular damage and reperfusion can further aggravate it. However, regeneration of the damaged tissues can develop if R lasts long enough.

The fast and the slow muscles have different metabolic patterns and rates [14], and their antioxidant capacities also differ from each other [22]. The EDL is predominantly a fast-twitch muscle and the SOL is predominantly a slow-twitch muscle, the later is more dependent upon aerobic metabolism. It is possible that the two types of muscle react differently to I and R and also there might be differences in their regeneration processes [14].

The aim of the present series of experiments has been to investigate the functional changes of the two types of skeletal muscle during the early and late reperfusion phases following 1 and 2 hours of I which are frequently used in trauma surgery.

Ischemia of the leg is generally elicited by a mechanical strangulation. Nervous supply of the muscle can also be affected during I and R. We wanted to know whether injury of the nervous tissue can aggravate the damage of the muscle tissue, the effect of indirect and direct stimulations of the two types of muscle were also investigated.

Material and Methods

Surgical preparation

Experiments were carried out on anaesthetised (35 mg/kg Nembutal i.p.), male Wistar rats weighing 250–300 grams. Maintenance doses were given for continuous anaesthesia, during the ischemic, and the measuring periods. With the exception of the series in which CF measurements were carried out immediately after I, the rats were allowed to recover from anaesthesia following I, and were reanaesthetized to determine

the contraction force later. All animals were housed in a temperature–humidity-controlled room and fed rat chow and water *ad libitum*.

A tourniquet was applied by means of a standard A30–32 elastic rubber band wound three times around one of the hind limbs of the animals below the hip-joint. The other leg served for control measurements. We wanted to verify that this procedure caused total ischemia to the muscles, Evans-blue was i.v. injected to 5 rats during I. The dye did not stain the occluded leg during the ischemic period but it stained the muscles homogeneously when the occlusion was released.

Surgical preparation started either at the end of I or after reanaesthesia. The sciatic nerve was prepared through a 1 cm long incision on both sides in the mid third of the thigh. The nerve was proximally ligated and it was placed on a bipolar silver electrode. The EDL or the SOL were exposed in their middle parts through a 1 cm long incision. A silver bipolar electrode was placed directly on the surface of the muscle. The respective distal ligaments of EDL or SOL were also prepared. The contraction force of the two types of muscle was measured in different animals.

The rats were placed on an experimental pad designed for the studies (Experimetria Ltd., Hungary). They were laid on a temperature controlled plate to keep rectal temperature at 37±0.2 °C. They were in the prone position for studies on the SOL or in the supine position for studies on the EDL. The knee and the ankle joints were immobilised by fixed clamps. The distal ligament of the muscle was attached to a strain gauge (Type FSG–01, Experimetria Ltd.). The two electrodes for direct and indirect stimulations were also attached to the pad. Each muscle was preloaded by 20 grams. According to our earlier studies this preload produced the best resting length of the two muscles to gain the maximal contraction force.

Experimental protocol

The ischemic period lasted for one or two hours. Duration of R lasted for two hours (2 h), twenty-four hours (24 h) and one (1 w) or two weeks (2 w). The number of animals was 7–10 in every group. Direct and indirect stimulations of the muscles were carried out every 30 minutes during a two-hour measuring period. The muscles received the first stimulation at the 30th and the last at the 120th minute of reperfusion in the 2 h group.

Stimulation was carried out with a biostimulator with the double intensity of the threshold. Optimal supramaximal parameters for stimulation were determined at our laboratory in pre-experiments: Frequency: 75 Hz, Amplitude: 9 mA, Impulse width: 1 msec, Train duration: 3 sec. This pattern of stimulation elicited tetanic isometric contractions. Tetanic contraction forces were recorded on a Brush 260 (Gould) recorder. Four (in the 2 h group), and five (in all other groups) measurements were

carried out during a two hour observation period. The average of these measurements was used, because there were no significant differences between the individual values.

Statistics

Results are expressed as means of 7–10 experiments ± S.E.M. The numbers represent contraction force values expressed in gram or % values of the experimental limb compared to the corresponding nonischemic contra-lateral side. Statistical analyses of the data was carried out by multifactor analyses of variance. Duncan test was used for *post hoc* evaluation. Differences between the two types of muscle were calculated by Student's *t*-test and they were considered to be significant if the p value was less than 0.05.

Results

All animals survived the I–R. The rats in the 24 h, 1 w and 2 w groups had motor deficits. The injured hind limb was oedematous from the very early phase to the 3–4 days of R. They could use their legs freely only on the second week of R.

Ischemia-reperfusion induced injury of the EDL muscle

Tetanic contraction force values of the EDL are presented in Tables I and II and also in Figs 1 and 2.

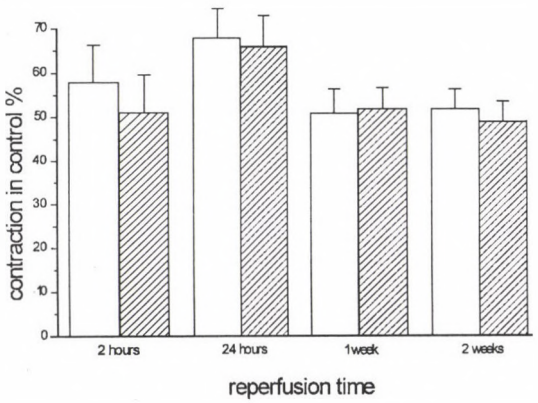

Fig. 1. Contraction force of the EDL muscle due to direct and indirect stimulation after one hour of ischemia. Values are given as % of the control side ± S.E.M. Open bar: direct, pattern bar: indirect stimulation

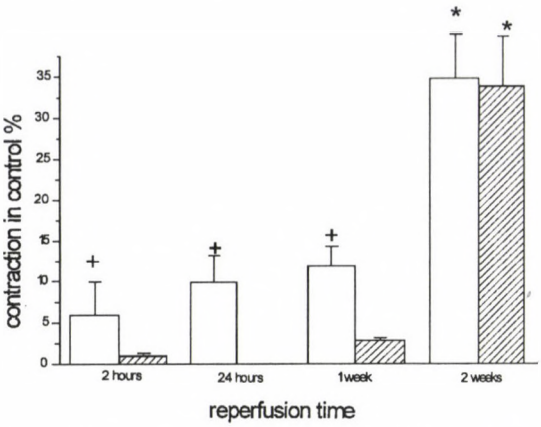

Fig. 2. Contraction force of the EDL muscle due to direct and indirect stimulation after two hours of ischemia. Values are given as % of the control side ± S.E.M. Open bar: direct, pattern bar: indirect stimulation. * significant difference between reperfusion times, + significant difference between direct and indirect stimulation. $p<0.05$

Table I

Contraction force of the EDL muscle elicited by indirect and direct stimulations after 1 hour of ischemia

Duration of reperfusion	Type of stimulation			
	direct		indirect	
	control $\bar{x}$ ±S.D.	occluded $\bar{x}$ ±S.D.	control $\bar{x}$ ±S.D.	occluded $\bar{x}$ ±S.D.
2 hours	69.3±11.7	40.8±14.7 *	72.0±6.5	37.6±18.7 *
24 hours	68.6±10.3	46.7±14.2 *	73.5±8.2	48.5±13.8 *
1 week	69.0±7.11	35.1±10.3 *	72.3±6.0	37.3±8.6 *
2 weeks	66.7±11.1	35.3±11.3	75.5±6.0	37.5±9.8

Values represent the means of the contraction forces expressed as gram ± S.D. * represents significant differences compared to the control values

Table II

Contraction force of the EDL muscle elicited by indirect and direct stimulations after 2 hours of ischemia

Duration of reperfusion	Type of stimulation			
	direct		indirect	
	control $\bar{x}$ ±S.D.	occluded $\bar{x}$ ±S.D.	control $\bar{x}$ ±S.D.	occluded $\bar{x}$ ±S.D.
2 hours	72.3±5.9	4.6±8.2 *	74.8±4.8	0.7±0.8 *
24 hours	75.3±4.3	7.5±6.8 *	76.1±5.2	0 *
1 week	69.6±5.9	8.6±4.5 *	72.6±5.5	2.2±0.6 *
2 weeks	71.7±4.1	25.4±8.9	74.1±3.3	24.9±9.9

Values represent the means of the contraction forces expressed as gram ± S.D. * represents significant differences compared to the control values

One hour of ischemia reduced CF to 50% of control immediately after I and it did not change significantly at later reperfusion times. Direct stimulation resulted in a 40% reduction of CF at early reperfusion, and CF decreased to 50% of control 1 or 2 weeks later (Fig. 1). There was no significant difference between the direct and indirect stimulations.

Two hours of ischemia resulted in a severe reduction of CF both to direct and indirect stimulations, however, the muscle contracted significantly stronger due to direct stimulation during the first week. CF returned to 35% of control at the end of the second week, and it was significantly higher than at earlier reperfusion times (Fig. 2).

CF was significantly smaller at all reperfusion times after two hours of ischemia than after 1 hour.

Ischemia-reperfusion induced injury of the SOL muscle

Tetanic contraction force values of the SOL are presented in Tables III and IV and also in Figs 3 and 4.

Table III

Contraction force of the SOL muscle elicited by indirect and direct stimulations after 1 hours of ischemia

Duration of reperfusion	Type of stimulation			
	direct		indirect	
	control $\bar{x}$ ±S.D.	occluded $\bar{x}$ ±S.D.	control $\bar{x}$ ±S.D.	occluded $\bar{x}$ ±S.D.
2 hours	29.3±8.6	12.7±10.2 *	36.9±12.6	14.4±8.2 *
24 hours	25.1±11.8	8.7±7.7 *	28.8±8.6	10.7±4.3 *
1 week	27.9±5.5	6.3±3.8 *	29.4±5.3	9.2±2.5 *
2 weeks	33.2±7.9	12.8±5.9	34.8±6.5	14.9±5.3

Values represent the means of the contraction forces expressed as gram ± S.D. * represents significant differences compared to the control values

Table IV

Contraction force of the SOL muscle elicited by indirect and direct stimulations after 2 hour of ischemia

Duration of reperfusion	Type of stimulation			
	direct		indirect	
	control $\bar{x}$ ±S.D.	occluded $\bar{x}$ ±S.D.	control $\bar{x}$ ±S.D.	occluded $\bar{x}$ ±S.D.
2 hours	25.0±10.8	2.4±1.9 *	32.6±6.5	3.2±2.8 *
24 hours	26.8±5.5	3.0±4.1 *	26.7±3.5	0.2±0.4 *
1 week	27.0±6.5	6.3±1.5 *	27.1±5.5	8.3±3.7 *
2 weeks	27.0±4.1	6.9±2.1	30.4±4.5	10.9±2.3

Values represent the means of the contraction forces expressed as gram ± S.D. * Represents significant differences compared to the control values

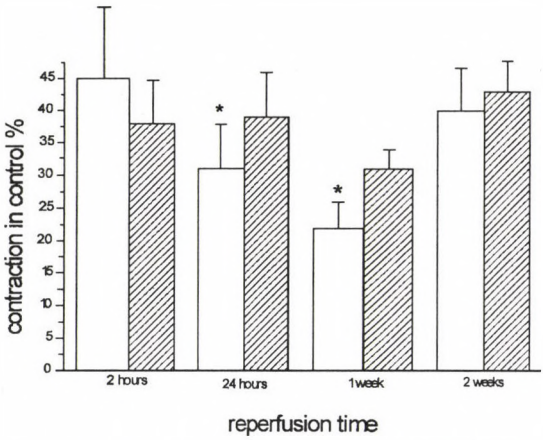

Fig. 3. Contraction force of the SOL muscle due to direct and indirect stimulation after one hour of ischemia. Values are given as % of the control side ± S.E.M. Open bar: direct, pattern bar: indirect stimulation. * significant difference between reperfusion times. $p<0.05$

One hour of ischemia reduced CF to 38% of the control value at the beginning of R, and no significant changes were seen at later times when indirect stimulation was applied. Direct stimulation resulted in a 45% reduction of CF at the beginning and further decreased to 22% of control during the first week of R. A significant recovery to 40%, was found at the end of the second week (Fig. 3). The difference between indirect and direct stimulations was not significant.

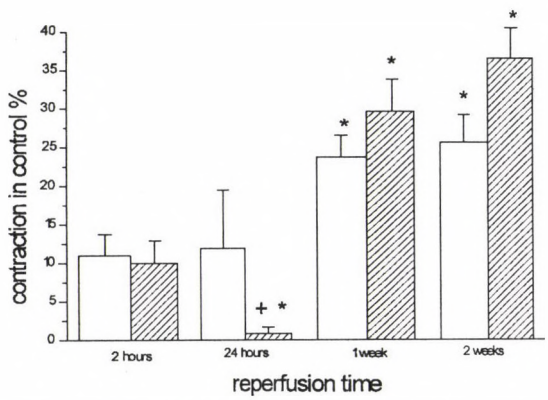

Fig. 4. Contraction force of the SOL muscle due to direct and indirect stimulation after two hours of ischemia. Values are given as % of the control side ± S.E.M. Open bar: direct, pattern bar: indirect stimulation. * significant difference between reperfusion times, + significant difference between direct and indirect excitation. $p<0.05$

Two hours of ischemia reduced CF to 10% of the control value immediately after the onset of R and CF decreased significantly close to 0 at 24 hours when the muscle was indirectly stimulated. It did not decrease further at 24 hours when the SOL was directly stimulated. There was a slow recovery at late reperfusion, however CF was only 37% and 26% of the control value after two weeks of reperfusion due to indirect and direct stimulations, respectively (Fig. 4). CF was significantly higher in the 1 w and 2 w than in the 2 h and 24 h groups. The difference between the direct and indirect stimulation values was significant only in the 24 h group.

Comparison of the two types of muscle (EDL and SOL)

EDL showed less reduction of CF than SOL did at all reperfusion times after 1 hour of ischemia (Figs 1 and 3). Significant regeneration of the CF was seen in the SOL during the first week already. Regeneration started in the EDL only during the second week after two hours of ischemia (Figs 1, 2, 3 and 4).

Discussion

Earlier studies have shown that tourniquet ischemia can cause ultrastructural [21], biochemical [14], and functional [1, 6, 7] changes in the skeletal muscle. Gardner et al. [7] also examined the contractile properties of the ischemic fast and slow muscle following two hours of I followed by 2 hours of R. They have found that the excitation-contraction coupling mechanism of the fast muscle was more sensitive to tourniquet ischemia than that of the slow muscle. This observation is in accordance with our results of this early reperfusion time. Fish et al. [6] examined the recovery of isometric contractile function of rat gastrocnemius muscle following different times of I but they used only two weeks of R. They found that isometric twitch, tetanus, and the rate of rise of twitch tension were inversely related to the ischemic interval, and the time to peak tension, relaxation time, and muscle fatigue were not related to the tourniquet duration. We also share the same opinion of Fish that, in the clinical use, a tourniquet is not without an injury to the muscle function.

Depletion of the energy stores damages the cell membrane which results in a decrease of the membrane potential [14, 12] which leads to a decrease of CF immediately after I. One hour of I is already long enough to reduce significantly the ATP and phosphocreatine (PCR) contents of the tissues [17]. This decrease can explain the reduced contractile capacity of both muscles at the very early reperfusion when the energy stores could not have been repleted yet.

Very little CF was recorded after two hours of ischemia. This period of I is frequently used in planned surgery. The almost complete loss of function immediately after I is attributed to the severe damage of cellular functions caused by anoxia and the consequent loss of energy stores. Patterson et al. [21], using a monkey model, have found the erosion of the Z-lines after tourniquet ischemia which may be responsible for reduced contractions.

CF was further reduced during reperfusion which indicates that muscle injury caused by one or two hours of I is aggravated by R. Reperfusion injury of fast and slow muscles was seen at 24 hours, and at 1 week of reperfusion.

Free radicals that are formed during reoxygenation might be the source of reperfusion injury [10, 11, 17, 22]. Free radicals can be produced by the ischemic activation of the enzyme xanthine oxidase (XO) [10, 11] and by the NADPH oxidase enzyme of the neutrophils (PMN) [2, 4]. The first process is mainly characteristic during the first hours of reperfusion, while the other can dominate only later. The functional loss of the two types of muscle seen after 2 h of I at 24 h of R suggests that free radicals produced by XO may have an important role during early reperfusion injury. This idea was supported by studies where inhibition or inactivation of the XO attenuated ischemia-reperfusion injury in other organs [8, 9]. This theory is also supported by other authors. They proved that radical scavengers have a protective effect in muscle ischemia-reperfusion [5, 16, 22, 23]. The decrease of CF to direct stimulation of both muscles following one hour of ischemia support the concept that the above-described role of radicals may be important in the pathophysiological processes. The other source of radicals along with other inflammatory mediators [10, 21] may play a further role at late reperfusion times. PMN activation and infiltration with the subsequent release of other mediators may be responsible for tissue damage [20, 22].

There are a few studies that investigated the nerve damage following tourniquet ischemia. Nitz et al. [18] in a hind limb tourniquet model found reduced electrical potentials following 1 hour of I. Nitz and Matulionis [19] also demonstrated ultrastructural changes, separation of myelin, periaxonal oedema, axonal degeneration, Schwann cell hypertrophy. They noted that these changes became severe with increasing durations of tourniquet compression. These changes may explain the differences between the results of direct and indirect stimulations seen after two hours of ischemia. The neural damage has recovered by the end of the second week of reperfusion.

The two types of muscle do not react to ischemia and reperfusion injury in the same way. CF is more heavily effected in the SOL muscle after 1 hour, and EDL has a reduced contraction force after 2 hours of I. However, regeneration in the SOL starts earlier. EDL has a higher glycolytic activity and a lower oxidative capacity in its

cytoplasm at rest [14]. Occlusion of the supplying arteries leads to lower ATP and PCR contents in the slow than in the fast muscle [15]. Because of the different metabolic status of the two muscles it is likely that the SOL could better compensate for one hour of ischemia.

Recovery of the muscles is different during prolonged reperfusion. There is no recovery of the EDL and very little in the SOL muscle after one hour of I. This is in agreement with the results of Fish et al. [6]. They have also measured significantly lower contractions after 1 hour of I and two weeks of R. Regeneration starts earlier in the SOL, after 1 w of R, following 2 h of tourniquet, which was observed in the EDL only during the second week of R. The later difference might be attributed to the different antioxidative capacity of the two types of muscle [22].

In conclusion, one or two hours of ischemia reduce the contraction force of the two types of muscle (fast and slow), and the rate of reduction depends on the duration of ischemia and the type of the muscle. The slow muscle is more severely damaged after one hour of tourniquet, but it can recover faster. The injury of the fast muscle was more severe after two hours of tourniquet. Reperfusion injury can also be seen mostly during the first week. There is also a recovery of muscle function at later reperfusion times, however, a complete recovery cannot be achieved within two weeks.

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Effects of midazolam on blood glucose fibrinolysis-serum lipids in normoglycemic–normolipidemic rats

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In normoglycemic and normolipidemic rats, the intraperitoneal administration of midazolam (1.25–2.5–5–10–15 mg/kg), a potent benzodiazepine derivative induced an enhancement of fibrinolysis, an augmentation of blood glucose level and a decrease of the serum lipids.

Keywords: rats, normolipidemy, normoglycemy, midazolam, fibrinolysis

In previous papers [2, 3], it was shown that midazolam a potent benzodiazepine had a quite potent lipid-lowering activity in Triton WR 1993 induced hyperlipidemia in rats as well as in diabetic rats. In this paper we will present the results obtained on the effects of this drugs on blood glucose level, serum lipids and fibrinolysis in normoglycemic and normolipidemic rats.

Materials and Methods

The experiments were carried out in male Wistar rats, weighing 150 to 200 g. The rats were fed a common rat chow, with water *ad libitum*. They were kept at constant temperature (20–22 °C) and humidity.

Midazolam (1.25–2.5–5–10–15 mg/kg) was injected intraperitoneally (ip.) four days from the vials. Each dose was administered in a different group of animals. Then,

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the food was withdrawn, water was allowed *ad libitum*. In the fifth day the blood was sampled from the retroobital sinus. The following determinations were performed: blood glucose level with the enzymatic procedure (Merck's test); total lipids (TL) by the method of Zöllner and Kirsch; total cholesterol (Tchol) with the enzymatic technique (Reanal's test); triglycerides (TG) with the enzymatic method (Merck's test) (all these procedures were described by [10]), the dilute blood-clot lysis time, using sodium acetate as a diluent [4].

The results obtained in various experimental groups were compared with those seen in a control normoglycemic and normolipidemic rats, which instead of midazolam received ip., the solution used for dissolution of midazolam (for composition, see our previous papers) a–b. The comparison was made by the unpaired "t"-test [11].

Results

Table I

The effects of midazolam on blood glucose and lipid levels and fibrinolysis in normoglycemic and normolipidemic rats

Group/Dose	n	Lysis time (minutes)	Blood glucose level (mmol/l)	Total lipids (mg%)	Tiglicerides (mmol/l)	Total cholesterol (mmol/l)
Control	10	42.88±1.019	2.97±1.18	396.54±11.84	0.748±0.12	1.88±0.094
Midazolam 15 mg/kg	10	40.22±1.1 p ns.	3.02±1.08 p ns.	400±12.24 p ns.	0.73±0.11 p ns.	1.92±0.101 p ns.
10 mg/kg	10	21.4±3.39 ($p<0.001$)↓	5.295±0.14 ($p<0.001$)↑	292.0±14.96 ($p<0.001$)↓	0.376±0.032 ($p<0.001$)↓	1.625±0.065 ($p<0.01$)↓
5 mg/kg	10	34.375±2.38 ($p<0.001$)↓	5.12±0.18 ($p<0.001$)↑	318.65±16.22 ($p<0.001$)↓	0.447±0.023 ($p<0.001$)↓	1.92±0.066 p ns.
2.5 mg/kg	10	26.66±3.34 ($p<0.001$)↓	2.99±0.123 p ns.	423.33±19.16 p ns.	0.715±0.062 p ns.	2.738±0.082 ($p<0.001$)↑
1.25 mg/kg	10	30.1±1.38 ($p<0.001$)↓	3.01±1.38 p ns.	410.33±20.2 p ns.	0.8±0.092 p ns.	2.11±0.10 p ns.

p: compared with diabetic control

ns.: nonsignificant

↓: decrease

↑: increase

The blood glucose level was not affected by the lowest doses of midazolam (1.25 and 2.5 mg). It was significantly raised by other doses. The clot lysis time was significantly reduced by all doses, i.e. the fibrinolysis was accelerated. TL were significantly reduced by the doses of 5, 10 and not changed by the other dose. Tchol was increased by the dose of 2.5 mg/kg, not modified by the doses of 1.25, 5 and 15 mg, significantly diminished by the dose of 10 mg. TG behaved like TL (Table I). It is noteworthy that doses larger than 15 mg/kg induced a very marked hypomotility and muscular relaxation; therefore, the highest dose applied was 15 mg/kg.

Discussion

Our experiments have shown that midazolam, administered ip. in normoglycemic and normolipidemic rats had induced a hyperglycemic reaction, which is contrary to the hypoglycemia observed in Triton treated rats as well as in diabetic ones [2, 3]. At the same time, serum lipids were reduced in all experimental situations. Thus, it seems that the effects on blood glucose level and on the serum lipids are independent phenomena. The cause(s) of this discrepancy is (are) not known.

Although the reduction of serum lipids proved to be statistically significant ($p<0.001$) it is too lower to be considerated as having biological significance.

It is noteworthy that our team has investigated the effects of the ip. injection of other BZD on blood glucose level, and serum lipids in normoglycemic and normolipidemic rats [7, 8]. The results of these studies indicate that diazepam and partly lorazepam, chlordiazepoxide, medazepam and oxazepam possessed a hypoglycemic activity, whereas dipotassium chlorazepate was hyperglycemic. At the same time, with the exception of diazepam, which had an obvious hypolipidemic activity, other BZD induced an increase of the serum lipids (at least for some doses). Dipotassium chlorazepate was completely inactive. So, no definite conclusion could be drawn from the above-mentioned data.

Thus, in normoglycemic and normolipidemic rats, in sharp contrast to the constant hypolipidemic activity seen with all investigated BZD (with the exception of tofisopam, which is an 1,2 BZD and not an 1,4–BZD [5] in hyperlipidemic animals, variable responses could be observed after BZD administration in normal ones. The same was valid for the blood glucose level.

However, some doses of midazolam given to normoglycemic and normolipidemic rats induced a clear cut reduction of the serum lipids accompanied by a hyperglicemic response. Concerning the mechanism of action of BZD on blood glucose level and serum lipids in normoglycemic and normolipidemic rats, our data do not provide any explanation. It is noteworthy that in hyperdyslipidemic rats, BZD act

probably by the stimulation of the peripheral type BZD receptors, they induce a decrease of serum lipids, the effects on blood glucose being variable [19]. However, this statement has not been confirmed in normoglycemic and normolipidemic animal so far.

Our previous investigations [1, 6, 7] have demonstrated that diazepam administered in normoglycemic and normolipidemic rats brought about an acceleration of the fibrinolysis. The same effects were observed in normal humans. At the same time, chlordiazepoxide and dipotassium chlorazepate were ineffective or less active (depending on the dose and the duration of the administrations), whereas lorazepam had an inhibitory action. An acceleration of the fibrinolysis was observed also with diazepam administered in margarine-induced hyperlipidemia as well as in hyperlipidemic humans [6]. Since fibrinolysis was enhanced both in normolipidemic and hyperlipidemic rats and humans, it concluded that the profibrinolytic activity was independent of the changes in serum lipids.

This study has shown that midazolam is akin to diazepam, since it also accelerated fibrinolysis. From these results, it may be inferred that BZD have marked effects on carbohydrate, protein and lipidic metabolism, when they were disturbed.

In conclusion, midazolam, given ip. in normoglycemic and normolipidemic rats induced an increase of the blood glucose level, an acceleration of the fibrinolysis and reductions of the serum lipids. All the changes, despite being significant from a statistical point of view, were too small to create concern.

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Ipriflavone metabolite-III inhibits LPS-induced nitric oxide release from RAW-264.7 cells

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Ipriflavone [CAS No.: 35212-22-7] is a novel drug used in the treatment of osteoporosis successfully. However, its mechanism of action has not been fully clarified yet. We investigated the effects of ipriflavone and its metabolites (I, II, III, V, VI, VII) on lipopolysaccharide (LPS)-induced nitric oxide (NO) release from RAW-264.7 mouse macrophage cells. Our data show that the LPS-induced NO release from RAW-264.7 cells was significantly inhibited by ipriflavone metabolite-III [7-isopropoxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one], [CAS No.: 97846-18-9] in a dose (3×10^{-8}–10^{-5} M)-dependent manner. Ipriflavone itself and its other metabolites had much lower inhibitory effect on the LPS-induced NO release from RAW-264.7 cells. The IC_{50}-value of ipriflavone metabolite-III (1.0×10^{-6} M) was between the IC_{50}-values of the two reference compounds dexamethasone (4.0×10^{-8} M) and N^G-Nitro-L-arginine (L-NNA, 7.5×10^{-5} M). Our finding suggests that some of the beneficial effects of ipriflavone in the treatment of osteoporosis might be mediated by its metabolite-III.

Keywords: osteoporosis, ipriflavone, nitric oxide, RAW-264.7 cell line

In postmenopausal women and ovariectomized animals the decrease in estrogen production is accompanied, partly, by an increased activity of osteoclast cells in the bone marrow [1, 2]. Only osteoclast cells are capable of resorbing the bone, and their enhanced activity plays a key role in several diseases, such as osteoporosis, rheumatoid arthritis, etc. It is well established that the bone marrow macrophages can stimulate osteoclast activity via several mediators, including IL-1, TNF-α, IL-6, GM-CSF and PGE_2 [1, 3, 4]. Recent findings suggest that the constitutive and inducible isoforms of nitric oxide synthase are present in osteoblasts and osteoclasts as well [5]. The nitric oxide (NO) produced by bone marrow macrophages, bone marrow endothelial cells, osteoblasts and osteoclasts can activate or inhibit the activity of osteoclasts depending

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on its concentration and the incubation time [6]. In addition to this, the fact that NO plays a key role in the progression of lesions [7] makes it important to find drugs that can inhibit the release of NO from activated bone marrow cells in case of osteoporosis.

In addition to hormonal and vitamin-D_3 preparations, ipriflavone [CAS No.: 35212-22-7] has recently been found to be effective in the treatment of postmenopausal osteoporosis (Osteochin® by CHINOIN Pharm. Chem. Works Ltd., Budapest, Hungary, and TC-80® by Takeda Chem. Ind., Ltd., Osaka, Japan) [8, 9]. In spite of the wide range of studies in this area, the mechanism of action of ipriflavone has not been clarified yet [8]. Therefore we studied the effect of ipriflavone and its six metabolites (I, II, III, V, VI, VII) on NO release.

The murine RAW-264.7 macrophage cell line served as a useful model system to investigate the effects of ipriflavone and its metabolites on the NO release in vitro. The RAW-264.7 cells secrete significant amount of NO upon stimulation by bacterial lipopolysaccharide (LPS) for 24 hours [10]. In the presence and absence of the drugs mentioned above, we measured the nitrite content of the cells' supernatants by Griess reaction [11]. In our experiments dexamethasone and N^G-Nitro-L-arginine (L-NNA) were used as reference compounds for the inhibition of NO release [12, 13,14].

Materials and Methods

Cell culture

Raw-264.7 mouse macrophage cells were maintained in RPMI-1640 (90%), FBS (10%) medium containing L-Glutamine (300 mg/L), HEPES (25 mM/L), $NaHCO_3$ (2 g/L), penicillin-G (10^5 U/L, all from Sigma) in humidified atmosphere at 37 °C and 5% CO_2 under pyrogen-free conditions as tested by LAL-test [Whittaker].

Test compounds

Dexamethasone (Serva) and L-NNA (Sigma) were used as reference compounds. Ipriflavone and ipriflavone metabolites were obtained as generous gifts from Dr. Péter Arányi (CHINOIN Pharmaceutical and Chemical Works, Ltd., Budapest, Hungary).

All test compounds and dexamethasone were dissolved in DMSO (Sigma) in a concentration of 10^{-1} M and further diluted to the appropriate concentrations by the culture medium (RPMI-1640 medium without fenol-red, 100%, containing L-Glutamine 300 mg/L HEPES, 25 mM/L, $NaHCO_3$, 2 g/L, penicillin-G, 10^5 U/L, all from Sigma).

L-NNA was dissolved directly in the culture medium in a concentration of 10^{-2} M and further diluted to the appropriate concentrations by the culture medium, as above.

Experimental procedure

The cells were seeded at a concentration of 5×0^5 cells/well in 96-well culture plates and were preincubated with the drugs (in triplicates at a final concentration of 10^{-5} M or in serial dilutions, as shown in Fig. 1) for 4 hours. After 4 hours LPS (*E. coli* 055; B5, Calbiochem) was added to the wells at a final concentration of 100 ng/ml. During the experiments the culture medium contained 1% FBS (Sigma). The total volume was 220 µl/well. After 24 hours incubation with LPS the supernatants were gently collected. NO synthase activity was measured as the accumulation of nitrite in the culture medium using the Griess reaction adapted for a 96-well plate reader [11]. Nitrite was measured by adding 100 µl of Griess reagent (1% w/v sulphanilamide and 0.1% w/v naphthylethylenediamine in 5% v/v phosphoric acid, all from Sigma) to 100 µl of sample culture medium. The optical density at 550 nm (OD_{550}) was measured using Easy Reader EAR-400 (SLT Labinstruments, Austria) after 10 minutes incubation at room temperature. The cell viability was checked after the experiments by tripan-blue exclusion method. In order to determine the direct effect of the compounds on the activity of NOS we used a 24-hour preincubation period with 100 ng/ml LPS. After that period of time the LPS-containing medium was removed and replaced by fresh medium containing the drugs in logarithmically scaled concentrations in triplicates for an additional 24-hour period.

Statistical analysis

Results are shown as mean inhibition percent ± S.E.M. from triplicate determinations (wells) from three separate experimental days (n=9). Student's *t*-test was used to determine the significance of differences between the means. The IC_{50} values were determined by log/logit transformation of inhibitory percent data from five separate experiments (n=15). Drug effects were expressed as percent inhibition where 100% means the nitrite content of LPS-treated cells' supernatants minus the nitrite content of LPS-untreated cells' supernatants.

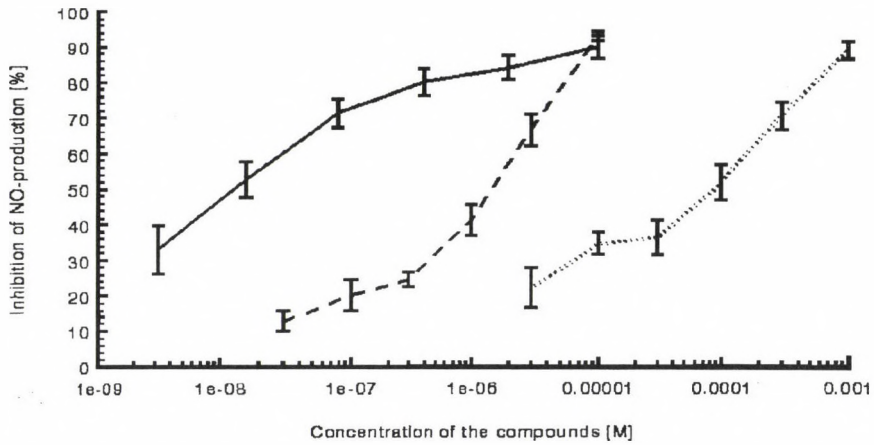

Fig. 1. Comparison of the effects of dexamethasone (————), N^G-Nitro-L-arginine (L-NNA, ...) and ipriflavone metabolite-III (- - - - -) on nitric oxide production from RAW-264.7 mouse macrophage cells (4 hours preincubation time with drugs followed by 24 hours incubation time with 100 ng/ml LPS). Data are represented as mean inhibition percent ± S.E.M. from triplicate determinations (wells) from five separate experimental days (n=15). Drug effects were expressed as percent inhibition where 100% means the nitrite content of LPS-treated cells' supernatants minus the nitrite content of LPS-untreated cells' supernatants

Results

At the screen concentration (10^{-5} M) only the ipriflavone metabolite-III [CAS No.: 97846-18-9] and the reference compound dexamethasone showed considerable NO release inhibitory activity (data not shown). None of the test compounds was found toxic up to 10^{-5} M. After the screening we determined the IC_{50} values of the most potent ipriflavone metabolite-III, and the control compounds dexamethasone and L-NNA in the same way as above (Table I). The IC_{50} of ipriflavone metabolite-III was found 1.0×10^{-6} M (the 95% confidence interval: 3.6×10^{-7}–2×10^{-6} M, n=15).The IC_{50} value of dexamethasone was 4.0×10^{-8} M (the 95% confidence interval: 7.5×10^{-9}–2.1×10^{-7} M, n=15). The IC_{50} value of L-NNA was 7.5×10^{-5} M (the 95% confidence interval: 2.1×10^{-5}–1.8×10^{-4} M, n=15). The ipriflavone metabolite-III had no direct effect on the NOS activity. In our experiments only L-NNA was able to inhibit the NOS activity in a dose-dependent manner after preincubating the cells with 100 ng/ml LPS.

Table I

Effects of dexamethasone, N^G-Nitro-L-arginine (L-NNA) and ipriflavone metabolite-III on LPS-induced nitric oxide (NO) release from RAW-264.7 mouse macrophages

Compound	IC_{50} (M)	95% conf. interval (M)
Dexamethasone	4.0×10^{-8}	7.5×10^{-9}–2.1×10^{-7}
L-NNA	7.5×10^{-5}	2.1×10^{-5}–1.8×10^{-4}
Ipriflavone metabolite-III	1.0×10^{-6}	3.6×10^{-7}–2.0×10^{-6}

Drugs were examined in 6 logarithmically spaced concentrations in triplicate in five independent experiment (n=15).

Discussion

In postmenopausal women and ovariectomized animals the decrease in estrogen production is accompanied, among others, by increased activity of osteoclast cells in the bone marrow. It is known that the activated bone marrow macrophages play a key role in the activation of osteoclasts and thus in the development of osteoporosis in postmenopausal women [1]. Several agents are released from bone marrow macrophages during their activation that can influence the osteoclasts' and osteoblasts' condition. Among others, nitric oxide (NO) is also released.

NO is known to be involved in neurotransmission, vasodilatation, serves as killing agent in inflammatory processes and plays a dominant role in the progression of lesions [7].

Recently, the constitutive and inducible nitric oxide synthases have been found in osteoblasts and osteoclasts. However, the role of NO in osteoclasts' activation is controversial [5, 6].

In the treatment of osteoporosis ipriflavone has been proved to be effective. However, the complete mechanism of action of ipriflavone and the role of ipriflavone metabolites is not yet known [8, 9].

We investigated the effects of ipriflavone and ipriflavone metabolites on NO production of activated macrophages. We used RAW-264.7 mouse macrophage cell line as a model for bone marrow macrophages as these cells produce high amount of NO after induction by bacterial lipopolisaccharide (LPS).

According to our experimental data the third metabolite of ipriflavone [7-isopropoxy-3-(hydroxyphenyl)-4H-1-benzopyran-4-one] had the highest inhibitory effect on NO production. Its IC_{50} value is in micromolar range (1.0×10^{-6} M), between

the IC_{50} values of dexamethasone (4.0×10^{-8} M) and N^G-Nitro-L-arginine (L-NNA, 7.5×10^{-5} M).

These results suggest that, in addition to the direct effects of ipriflavone, the ipriflavone metabolite-III might also contribute to the beneficial effect of ipriflavone on the osteoporosis by suppressing the NO release. The potential participation of ipriflavone metabolite-III in the effects of ipriflavone is supported by the observation that ipriflavone metabolite-III has a different pharmacokinetics compared to the other metabolites [15]. To measure the effect of ipriflavone metabolite-III on the human macrophages and remained to be seen.

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Regional heterogeneity and differential vulnerability of cerebral and spinal vascular CO_2-responsiveness during graded haemorrhagic hypotension

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Regional inhomogeneity of cerebrovascular CO_2-sensitivity as well as its changes at three different levels of standardized haemorrhagic hypotension were studied in ten distinct brain and spinal cord regions of anesthetized, ventilated cats. Regional cerebral blood flow was measured with radiolabelled microspheres in hypocapnic, normocapnic, and hypercapnic conditions, and CO_2-responsiveness was determined from the equation of the slopes of the best fit regression lines to the obtained flow values. It was concluded that in normotensive, normoxic cats response of the cerebral and spinal vessels to $PaCO_2$ alterations can be assigned to four major categories. The CO_2-responsiveness of a brain region is not solely determined by the rate of its basal steady state blood flow: CO_2-reactivity of the hypothalamus was significantly different from that of any other investigated regions with almost identical steady state flow values. Vulnerability of the cerebrovascular CO_2-sensitivity during hypotension was different from region to region, with the vessels of the pons-medulla oblongata region being the most sensitive to haemorrhage. Reduced regional cerebral and spinal CO_2-responsiveness during haemorrhage is not a consequence of a reduced L-arginine supply for nitric oxide generation since administration of an excess amount of the precursor L-arginine failed to restore the haemorrhage-induced reduction of regional CO_2-sensitivity at the 60 mm Hg mean arterial pressure level.

Keywords: CO_2-responsiveness, cerebral blood flow, spinal cord blood flow, haemorrhagic hypotension, nitric oxide

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Carbon dioxide has been shown to be one of the most potent endogenously produced physiologic vasoactive substances in respect of its effects on cerebral vascular smooth muscle. Since the early observations of Bronk and Gessell [7] and Schmidt [58] the relationship between arterial carbon dioxide tension ($PaCO_2$) and cerebral blood flow has been extensively investigated in experimental animals [23, 50] in man [1, 33, 38] as well as in mathematical models [20]. The increase in cerebral blood flow (CBF) under the influence of increased $PaCO_2$ can be characterized by a sigmoidal curve when viewed over a large range of $PaCO_2$ changes [50].

Alterations of cerebrovascular CO_2-reactivity has been shown to be influenced by a large variety of factors including hypoxia and cerebral oxygen consumption [19, 39, 49] ischaemia [34], cerebral vascular lesions [17, 43, 53, 57], aging [28], anesthesia [35, 39, 55], hypoglycemia [61], hyperammonaemia [64], hypothermia [10], carotid artery compression [13], autonomic sympathetic nerve activation and blockade [9, 30, 41] CNS (nucleus fastigii) stimulation [15], and, more recently, by the blockade of the NO-synthase enzyme [29, 42, 48, 54, 69]. Surprisingly, little effort has been made to obtain quantitative data on the regional heterogeneity of cerebral and spinal vascular CO_2-responsiveness either in steady-state conditions or at well-defined levels of standardized hypotension [26, 31, 36, 56, 66, 60].

With the recent recognition of neuropeptides and endothelium derived vasoactive substances as potentially important local regulatory factors of cerebral blood flow, it became evident that the local sensitivity of the cerebral vessels to CO_2 in different brain regions may be significantly modulated in both steady-state situations and in pathologic conditions by these agents that have short biological half lives and small diffusion distances [6, 54].

Impaired cerebrovascular response to CO_2 during decreased intraluminal pressure has been demonstrated in the dog [23], in the baboon [62], and in the human [1]. Data about reduced CO_2-sensitivity following both drug induced hypotension [2, 3, 4, 18, 21, 25, 44, 51] and haemorrhage induced hypotension [22, 23, 52] has been reported long ago. A careful survey of the existing data in the literature reveals, however, that the exact relationship of local, regional CO_2-reactivity of the cerebral and spinal cord vessels to standardized systemic arterial pressure reduction has still not been systematically investigated, although this data has both theoretical and clinical importance.

The purpose of the present study was threefold: (1) to evaluate the regional differences in CO_2-responsiveness of ten different brain and spinal cord regions of the normotensive cat, (2) to determine the regional differences in the vulnerability of the cerebrovascular CO_2-responsiveness of these same regions at three different levels of standardized haemorrhagic hypotension, and (3) to determine whether stimulation of the L-arginine-nitric oxide pathway by a massive dose of exogenous L-arginine

administration may counteract the haemorrhage-induced reduction of the regional CO_2-sensitivity. Studies in normotensive cats [54] and rats [29, 42, 48, 69] indicate the involvement of nitric oxide (NO) in the mechanism of CO_2-induced cerebral vasodilation.

Materials and Methods

Animal preparation

Twenty-nine male cats (2.0–3.8 kg) were utilized in this study. The animals were anesthetized with an initial dose of intraperitoneally injected chloralose (50 mg/kg) and urethane (300 mg/kg). The anesthesia was maintained with successive intravenous injections of these anesthetics in compliance with the recommendation of the American Heart Association Ethics Committee, and the Institutional Animal Care and Use Committee of the University of Pennsylvania. The animals were artificially ventilated by a respirator through an endotracheal cannula with room air, supplemented with oxygen. The temperature of the animals was kept constant at 37 °C by using a rectal thermometer (YSI, Model 73a, Yellow Springs, OH) and a servo-controlled heating lamp. Catheters were inserted into the right brachial and femoral arteries (for blood gas determination and for reference radioactive sampling), into the left brachial and femoral arteries (for continuous blood pressure monitoring on a polygraph (Grass, Model 7E, Quincy, MA) and blood withdrawal for arterial pressure reduction), into the left femoral vein (for drug injection) and into the left ventricle of the heart (for radiolabelled microsphere administration). Heparin was administered i.v. in a dose of 500 Units/kg.

Measurement of regional cerebral and spinal cord blood flow

Regional cerebral blood flow (rCBF) and regional spinal cord blood flow (rSBF) were determined simultaneously with radiolabelled microspheres using the reference sample method [37]. The 15±1.5 micrometer in diameter microspheres (DuPont, NEN-TRAC, Wilmington, DE), were suspended in 0.9% saline with 0.01% Tween-80, and were injected into the left ventricle of the heart, in a volume of 0.3–0.5 ml, with an activity of 40 μCi. The vials containing the microspheres were vigorously shaken by an electric shaker for at least 30 minutes before the injections to ensure even dispersion of the microspheres. 1.6×10^6 microspheres (^{57}Co, ^{85}Sr, ^{46}Sc) were injected over a 10 sec period for each measurement of flow, an amount which allowed to be delivered approximately 500 spheres to 1 gram cerebral tissue. The injection was followed by a

10 sec flush of 2.0 ml saline. Reference blood samples were collected via a femoral and brachial artery using a Harvard withdrawal syringe pump with a speed of 1.0 ml/min, beginning 30 sec prior to the injection and continuing for 2 min after the saline flush. The microsphere injection did not alter arterial blood pressure or heart rate. At the end of the experiments, the cats received an overdose of 150 mg/kg i.v. injected pentobarbital (Abbott Laboratories, Chicago, IL), and the brain was infused via the carotid artery with 10% formalin. After removing the brain from the skull, the following discrete areas of the brain were cut and placed in 5 ml vials: thalamus, hypothalamus (mediobasal area, including the median eminence), pituitary (neuro- and adenohypophysis together with the stalk), white matter (corpus callosum), fronto-parietal cortex (mid. suprasylvian and mid. ectosylvian gyri) cerebellum (vermis), pons-medulla oblongata (NTS region) and cervical, thoracic-, and lumbar spinal cord (coronal sections, gray and white matter together). The tissue samples were weighed and counted in a gamma scintillation spectrometer (Packard, Model MINAXI g, Auto-gamma 5000 series, Downers Grove, IL). The energy windows used for ^{57}Co, ^{85}Sr, and ^{46}Sc were 80–165 keV, 464–564 keV, and 700–1400 keV, respectively. Backscatter of lower energy emission from isotopes with higher energy windows was subtracted to obtain a corrected count value. Cerebral blood flow was calculated from the equation: $rCBF=C_b \times RBF/C_r$ where rCBF is regional cerebral blood flow in ml/g/min, C_b is counts per 1 g of brain tissue, RBF is reference arterial blood sample withdrawal rate in ml/min, and C_r is total counts in reference arterial blood sample.

Blood gas analysis

Arterial $PaCO_2$, PaO_2 and pH were determined from femoral arterial blood samples at 37 °C immediately after the samples were obtained, using a blood gas analyzer (Radiometer ABL-30, Copenhagen, Denmark). Arterial PaO_2 was kept in a normoxic range (above 90 mm Hg) throughout each experiment to avoid the interference of a hypoxic component with the results obtained. End-expiratory CO_2 was continuously monitored by using an end-tidal CO_2 analyzer (Beckman, LB-2, Fullerton, CA), and was recorded on a polygraph.

Standardization of systemic arterial hypotension

Standardized arterial hypotension was induced by haemorrhage using the modified method of Engelking and Willig [16]. A pressurized reservoir system was connected to the abdominal aorta via a femoral artery catheter. This method allowed us to keep the systemic mean arterial pressure (MAP) constantly at the desired level (80, 60, or at 40 mm Hg) during the entire time of the experiments.

Calculation of regional CO_2-responsiveness

Regional cerebral CO_2-responsiveness was tested by measuring the local CBF and SBF values of 10 distinct cerebral and spinal cord regions at normal, at decreased and at increased arterial $PaCO_2$ values. The increase of cerebral blood flow under the influence of elevated $PaCO_2$ can be best characterized by a sigmoidal curve when viewed over a large range of $PaCO_2$ changes [50]. One may, however, successfully fit a straight regression line to the experimental data when the $PaCO_2$-CBF relationship is studied in the range of 25–65 mm Hg $PaCO_2$ [11, 20, 55, 67]. In the present study this latter method was used since the normocapnic flow values (at 36–38 mm Hg $PaCO_2$) as well as the hypocapnic flow values (at 25–29 mm Hg $PaCO_2$) and the hypercapnic flow values (at 50–60 mm Hg $PaCO_2$) obtained in the study were measured in the "straight-line" range of the sigmoidal curve. Hypocapnia was obtained by increasing the rate of the respiration and hypercapnia was produced by 5% CO_2 gas inhalation. The rCBF and rSBF measurements at each $PaCO_2$ level were performed after a period of at least 10 min at the desired $PaCO_2$ level (continuously monitored with the end-tidal CO_2 recording) in order to ensure that after each $PaCO_2$ change a new steady state was attained, and there was an approximate period of 25–30 min between each consecutive $PaCO_2$ level alteration.

rCBF and rSBF values were plotted against the $PaCO_2$ values and the best fit regression lines to the hypo-, normo-, hypercapnic flow values as well as the 95% confidence limits were determined by a computer program using the least squares principle. CO_2 responsiveness (= ml blood flow change per 1 mm Hg $PaCO_2$ change) of each discrete brain and spinal cord region were calculated from the equation of the slope of the best fit regression line.

Experimental groups

The experiments were carried out in 5 groups. *Group 1*: normotensive, saline treated animals (n=6), *Group 2*: 80 mm Hg hypotensive, saline treated animals (n=6), *Group 3*: 60 mm Hg hypotensive, saline treated animals (n=6), *Group 4*: 40 mm Hg hypotensive, saline treated animals (n=6), *Group 5*: 60 mm Hg hypotensive, L-arginine treated animals (n=5). rCBF was determined three times in each cat after the desired arterial pressure level and normoxia were reached in (a) hypocapnia ($PaCO_2$=25–29 mm Hg), (b) normocapnia ($PaCO_2$=36–38 mm Hg), and (c) hypercapnia ($PaCO_2$=50–60 mm Hg).

Drugs

Anesthesia was obtained with 50 mg/kg i.p. injected chloralose (1,2-O-[2,2,2-Trichloroethylidene]-α-D-glucofuranose, Sigma, St. Louis, MO) and 300 mg/kg urethane (urethane ethyl carbamate, Sigma). Blood coagulation was prevented by intravenously administered heparin (heparin sodium, Elkins-Sinn, Inc. Cherry Hill, NJ).

Stimulation of the L-arginine-nitric oxide pathway was carried out by 30 mg/kg intravenously administered L-arginine, dissolved in 1 ml/kg saline, followed by a continuous 10 mg/kg/min i.v. infusion (L-arginine hydrochloride, Sigma, St. Louis, MO).

Statistical analysis

Regional CO_2 responsiveness of the cerebral and spinal vessels was calculated in each animal via linear regression analysis from three consecutive flow measurements at different $PaCO_2$ levels, as outlined above. The units of blood flow are ml/g/min per mm Hg change in $PaCO_2$. Responsiveness was evaluated in a total of 29 cats, six in each of the four blood pressure groups of 120, 80, 60, and 40 mm Hg (in which the cats were treated with saline) and there were 5 cats in a separate group of 60 mm Hg MAP level (in which the cats were treated with L-arginine). Individual regression coefficients were compared across all five experimental groups by using the two way analysis of variance (ANOVA), followed by the one way analysis of variance in the vehicle treated group of animals. As post hoc tests Scheffe's F test, Fischer's PLSD test, Dunett's t-test and Student's unpaired t-test were applied. Probabilities of 0.05 and less were considered significant.

Results

Regional differences in cerebrovascular CO_2-reactivity of the normotensive cat

The relationship between arterial $PaCO_2$ and local blood flow in distinct brain and spinal cord regions of the normotensive cat is shown in Fig. 1 and Table I. Although 10 regions were examined, only four characteristic types of reactions were obtained. Cerebral cortex, thalamus, cerebellum and pons-medulla oblongata showed an almost identical, high sensitivity to CO_2 (0.098 ml/g/min, 0.098 ml/g/min, 0.094 ml/g/min, 0.084 ml/g/min flow increase per 1 mm Hg $PaCO_2$ increase, respectively). Cervical-, thoracic- and lumbar spinal cord as well as the white matter showed equally low CO_2-responsiveness (0.030 ml/g/min, 0.021 ml/g/min, 0.030 ml/g/min, 0.016 ml/g/min flow increase per mm Hg $PaCO_2$ increase). CO_2

sensitivity of the hypothalamus (0.056 ml/g/min per mm Hg $PaCO_2$ increase) was significantly lower than that of the average sensitivity of the high sensitivity regions (0.094 ml/g/min flow increase per mm Hg $PaCO_2$ increase, $p<0.02$) and significantly higher than that of the average sensitivity of the low sensitivity areas (0.024 ml/g/min flow increase per mm Hg $PaCO_2$ increase, $p<0.004$). The vessels of the pituitary showed practically no response to CO_2.

CO_2-responsiveness of a brain region, at first glance, seems to be tightly coupled with the rate of the basal flow value. Regions with higher resting blood flow (Table II), in general, responded more intensely to CO_2 than regions with low resting flow values. This however, was not true for either the pituitary or the mediobasal hypothalamic area (Fig. 1, Table I). The resting blood flow of the pituitary was by far the highest among the regions involved in this study (3.43±1.69 ml/g/min), but pituitary vessels showed no sensitivity at all to CO_2. Since pituitary gland is part of the endocrine system rather than a "region" of the brain, this finding is neither new nor surprising. Hypothalamus, on the other hand, is a key region of the central nervous system in controlling autonomic functions. The resting blood flow of the hypothalamus (0.68±0.33 ml/g/min) was comparable to that of the sensory-motor cortex (0.66±0.19 ml/g/min), but in spite of the same rate of the basal flow in these two regions, the CO_2-sensitivity of the hypothalamic vessels was significantly lower than that of the cortical vessels ($p<0.02$).

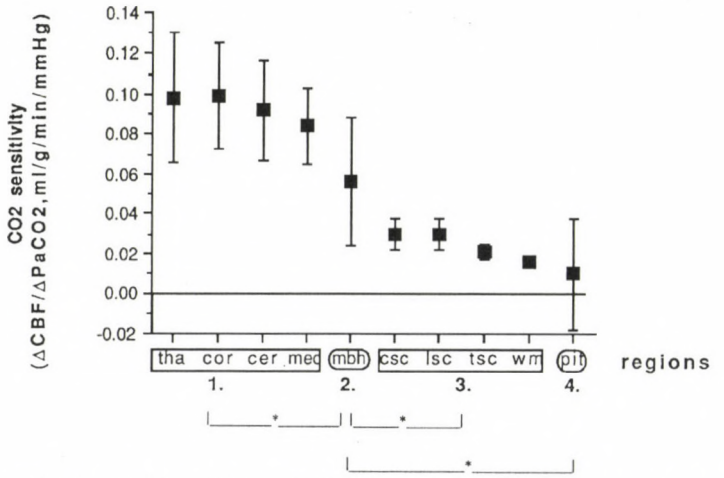

Fig. 1. Regional CO_2-sensitivity of the cerebral and spinal cord vessels in steady-state conditions. The results were obtained in 6 anesthetized, ventilated, normothermic, normoxic normotensive cats. each filled square represents the average mean CO_2-responsiveness of a region (ml/g/min flow increase per 1 mm Hg $PaCO_2$ increase, mean ± SEM). Regions: tha=thalamus, cor=sensorimotor cortex, cer=cerebellum, med=medulla oblongata, csc=cervical spinal cord, lsc=lumbar spinal cord, tsc=thoracic spinal cord, wm=white matter, pit=pituitary. Response of the cerebral and spinal vessels to $PaCO_2$ alterations can be assigned to four major groups: group 1 (tha, cor, cer, med), group 2 (mbh), group 3 (csc, lsc, tsc, wm) and group 4 (pit). CO_2-sensitivity of each group was significantly different (*=$p<0.05$) from that of any other investigated group.

Table I

Regional cerebrovascular CO_2-sensitivity in normotensive cats, in haemorrhagtic hypotensive cats (MAP = 60 mm Hg), and in haemorrhagic hypotensive cats (MAP = 60 mm Hg) pretreated with L-Arginine

Region	Normotensive controls (i.v. saline)		Hypotensive controls (i.v. saline)		Hypotensive L-arginine treated (30 mg/kg i.v. injection + 10 mg/kg/min i.v. infusion)	
	abs	%	abs	%	abs	%
Cortex	0.098±0.064	100	0.050±0.019	50.5±19.4	0.042±0.009	42.4±9.2
Cerebellum	0.094±0.062	100	0.039±0.022	41.5±23.4	0.034±0.029	36.2±30.8
Hypotalamus	0.056±0.037	100	0.022±0.01 a	39.3±17.9	0.025±0.008	44.6±14.3
Thalamus	0.098±0.079	100	0.046±0.023	46.9±23.5	0.044±0.023	44.9±23.5
Medulla oblongata	0.084±0.047	100	0.031±0.017 a	36.9±20.2	0.017±0.019 b	20.2±22.6
White matter	0.016±0.008	100	0.010±0.004	62.5±25.0	0.005±0.01 b	31.3±62.5
Cerv. spinal cord	0.030±0.018	100	0.013±0.006	43.3±20.0	0.014±0.009	46.6±30
Thor. spinal cord	0.021±0.009	100	0.014±0.006	66.6±28.6	0.018±0.008	85.7±38
Lumb. spinal cord	0.030±0.020	100	0.017±0.014	56.6±46.6	0.018±0.007	60.0±23.3
Pituitary	0.009±0.069	100	–0.019±0.023	–211±255	–0.006±0.028	66.6±311

Values are expressed as mean ± SD; n=6 in both control groups,m 5 in the L-arginine treated group; abs=ml/g/min blood flow change per 1 mm Hg change in $PaCO_2$; %=CO_2-sensitivity expressed in per cent of that of the saline treated normotensive controls as 100%. There was a significant reduction in CO_2-sensitivity in hypotensive controls compared to normotensive controls (a=$p<0.05$) and a significant reduction in CO_2-senstivity in L-arginine pretreated hypotensive animals compared to normotensive controls (b=$p<0.05$). However, the changes in CO_2-sensitivity following L-arginine treatment compared to the saline treated hypotensive controls did not reach the level of significance in any of the studied regions.

Effect of haemorrhagic hypotension on regional cerebrovascular CO_2-responsiveness

A previously unknown "autoregulatory" tendency was observed in the CO_2-sensitivity of the cerebral and spinal vessels during stepwise reduction of the systemic arterial pressure by blood letting. Hypotension generally attenuated hypercapnic flow increase (Figs 2, 3, 4). Gradual reduction of the arterial pressure, however, was not followed passively by a similar gradual reduction of the CO_2-responsiveness in the high CO_2-sensitivity areas. CO_2-sensitivity was almost equal at the 80 mm Hg and at 60 mm Hg arterial pressure levels, although considerably more blood had to be withdrawn in the 60 mm Hg group than in the 80 mm Hg group in order to reduce MAP to the desired pressure level. A statistically non-linear relationship was found between the arterial pressure reduction, and the CO_2-sensitivity reduction in the cortex, cerebellum, thalamus, pons-medulla oblongata and in the hypothalamus (Figs 2, 4) and the similar phenomenon was observed in the low CO_2-sensitivity areas: in the white matter, cervical, thoracic and lumbar spinal cord (Fig. 3). The vessels of the pituitary gland of the hypotensive cats (similarly to those of the normotensive cats) showed practically no sensitivity to CO_2 (Fig. 4).

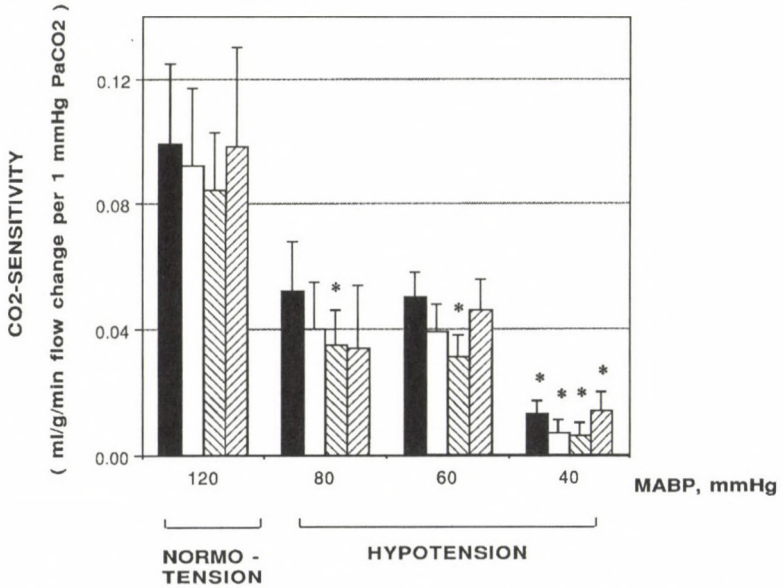

Fig. 2. Regional cerebrovascular CO_2-sensitivity of the ■ cerebral cortex, □ cerebellum, ▨ pons-medulla oblongata and ▨ thalamus in normotension, and at three different levels of hypotension. Results obtained in six normotensive, six 80 mm Hg hypotensive, six 60 mm Hg hypotensive and six 40 mm Hg hypotensive cats. Hypotension was produced by haemorrhage. Bars represent mean ± SEM. *=$p<0.05$ compared to the corresponding normotensive control CO_2-sensitivity value

Reduction in sensitivity to CO_2 due to the hypotension occurred at different blood pressure levels in different regions. In the pons-medulla oblongata reduction of the cerebrovascular CO_2-responsiveness was significant already at 80 mm Hg MAP (Fig. 2), in the mediobasal hypothalamus it became significant only at 60 mm Hg MAP level (Fig. 4), while in the cortex, cerebellum, thalamus as well as in the white matter, and in all three investigated segments of the spinal cord, CO_2 responsiveness was lost at the 40 mm Hg MAP level (Figs 2, 3).

Effect of exogenous L-arginine administration on regional cerebrovascular CO_2-responsiveness in haemorrhagic hypotension

The effect of intravenously administered exogenous L-arginine on the regional cerebral and spinal cord CO_2-responsiveness of the hypotensive cats is summarized on Table I. As shown in the Table, the excess amount of the nitric-oxide precursor L-arginine failed to restore the haemorrhage-induced reduction of CO_2-responsiveness at the 60 mm Hg arterial pressure level in any of the investigated brain and spinal cord regions.

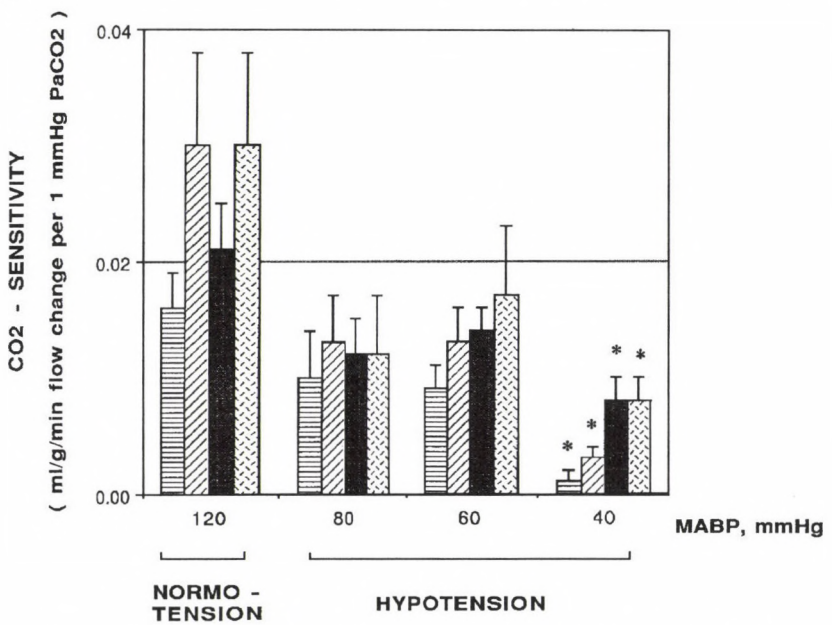

Fig. 3. Regional cerebrovascular CO_2-sensitivity of the ▤ white matter and the ▨ cervical, ■ thoracic and ▦ lumbar spinal cord in normotension and at three different levels of hypotension. Results obtained in six normotensive, six 80 mm Hg hypotensive, six 60 mm Hg hypotensive, and six 40 mm Hg hypotensive cats. Hypotension was produced by haemorrhage. Bars represent mean ± SEM. *=$p<0.05$ compared to the corresponding normotensive control CO_2-sensitivity value

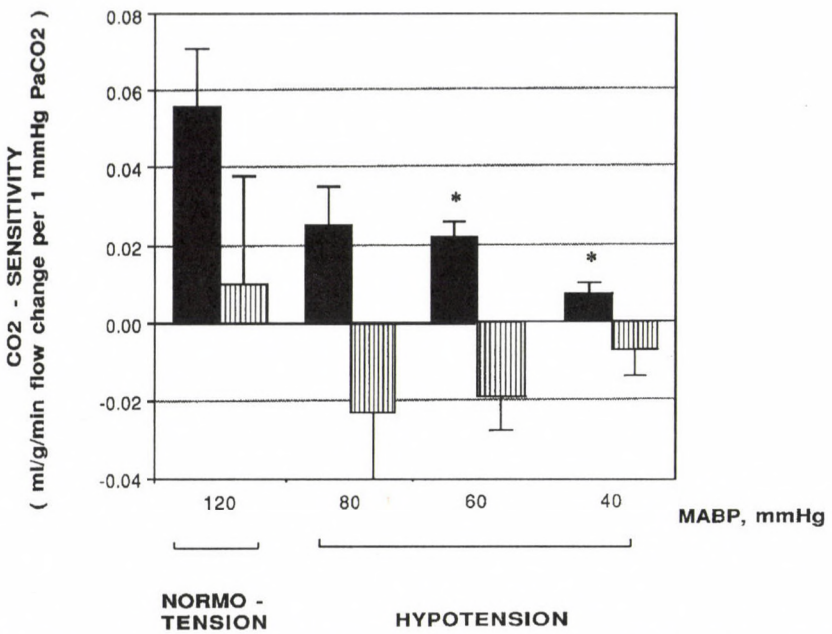

Fig. 4. Regional cerebrovascular CO_2-sensitivity of the ■ hypothalamus and ▨ pituitary in normotension and at three different levels of hypotension. Results obtained in six normotensive, six 80 mm Hg hypotensive, six 60 mm Hg hypotensive, and six 40 mm Hg hypotensive cats. Hypotension was produced by haemorrhage. Bars represent mean ± SEM. *=$p<0.05$ compared to the corresponding normotensive control CO_2-sensitivity

Arterial pressure and blood gas values

$PaCO_2$ changes minimally altered mean arterial pressure (MAP) in normotensive cats (Table III). MAP changes thus are not responsible for the observed regional CO_2 response characteristics. (The same applies for the hypotensive groups, where MAP was kept constant artificially at the 80, 60 and 40 mm Hg arterial pressure level by using a pressurized reservoir system during normo-, hypo- and hypercapnia). The normocapnic, hypocapnic and hypercapnic $PaCO_2$ values were similar in the normotensive and in the hypotensive groups. PaO_2 was kept in the normoxic range (i.e. above 90 mm Hg) in all groups in order to separate the $PaCO_2$-induced cerebrovascular effects from the effects of any simultaneous decrease in PaO_2. As shown in this Table, the alterations in arterial pH reflected changes induced by both altered $PaCO_2$ and by haemorrhage.

Table II

Resting regional cerebral blood flow (rCBF, ml/g/min) and cerebrovascular resistance (CVR, resistance units) values obtained at different mean arterial blood pressure levels (MAP, mmHg) in normoxic, normocapnic, saline treated cats

Regions	120 mm Hg		80 mm Hg		60 mm Hg		40 mm Hg	
	rCBF	CVR	rCBF	CVR	rCBF	CVR	rCBF	CVR
Thalamus	0.92±0.27	141±45	0.65±0.45	178±115	0.93±0.36	74±30*	0.67±0.19	68±32*
M.B. hypothalamus	0.68±0.33	269±265	0.48±0.12	176±49	0.49±0.26	156±80	0.37±0.08*	112±29
White matter	0.34±0.09	382±119	0.27±0.05	301±59	0.39±0.18	171±83*	0.33±0.10	129±30*
Cortex	0.66±0.19	189±42	0.61±0.25	144±39	0.86±0.34	83±40*	0.69±0.16	60±12*
Cerebellum	0.85±0.21	148±38	0.88±0.37	106±52	1.097±0.61	77±44*	0.92±0.31	49±6*
Medulla oblongata	1.03±0.14	118±14	0.87±0.24	99±30	1.025±0.44	69±30*	0.87±0.11*	46±6*
Pituitary	3.43±1.69	42±19	2.07±0.96	47±25	2.25±0.63	28±7	1.95±0.64	22±7*
Cerv. spinal cord	0.318±0.06	385±55	0.33±0.11	264±90*	0.47±0.23	161±82*	0.32±0.06	137±32*
Thor. spinal cord	0.24±0.05	525±113	0.27±0.09	330±50	0.35±0.14	198±85*	0.25±0.05	165±31*
Lumb. spinal cord	0.33±0.08	359±110	0.38±0.17	260±149	0.34±0.17	238±172	0.34±0.05	117±14*

Values are expressed in mean ± SD. n=6 in each group. CVR=MAP/rCBF, resistance units. * significant change ($p<0.05$) compared to the same parameter of the normotensive (MAP 120 mm Hg) group.

Table III

Arterial blood pressure and arterial blood gas values

Experimental groups	Hypocapnia				Normocapnia				Hypercapnia			
	MAP mm Hg	$PaCO_2$ mm Hg	pH	PaO_2 mm Hg	MAP mm Hg	$PaCO_2$ mm Hg	pH	PaO_2 mm Hg	MAP mm Hg	$PaCO_2$ mm Hg	pH	PaO_2 mm Hg
Normotensive	113±27	28±3*	7.38±0.04*	114±11	123±23	37±1	7.30±0.02	115±12	124±33	56±8*	7.17±0.06*	93±12*
Hypotensive (80 mm Hg)	80±1	28±2*	7.38±0.06*	114±16	80±2	37±2	7.31±0.06	121±29	80±2	56±5*	7.16±0.08*	109±32
Hypotensive (60 mm Hg)	60±2	27±2*	7.24±0.09	119±14*	60±1	37±2	7.22±0.11	129±20	60±2	52±5*	7.0±0.14*	113±11*
Hypotensive (40 mm Hg)	40±3	26±2*	7.17±0.12	114±24	40±2	37±2	7.15±0.15	120±17	40±1	65±13*	6.87±0.06*	99±18*
Hypotensive (60 mm Hg)+ L-Arginine treated	60±1	28±1*	7.24±0.09	167±21	60±1	38±1	7.06±0.14	149±10	60±2	56±3*	6.94±0.07*	158±31

Values are expressed as mean ± SD. n=6 in all groups except the L-arginine treated group where n=5. *$p<0.05$ compared to the normocapnic value of the same variable.

Discussion

There are several new findings in this study. The concept that there are striking differences in the cerebral and spinal vascular CO_2-responsiveness of the different regions of the normotensive cat is not new [31, 56, 60, 66]. It is a new observation, however, that all types of regional CO_2-responses may be arranged into four major categories. Second, the peculiar character of the pituitary vessels (i.e. being insensitive to CO_2) has been observed by others, and the fact that the pituitary fails to respond to a variety of other cerebral vasodilator stimuli was discussed in several papers in detail [12, 26]. What has not been previously reported, however, is that, in cats, the vessels of the mediobasal hypothalamus (which has a complex neurosecretory function but cannot be considered as an endocrine gland, as the pituitary) also respond in a significantly different manner to CO_2 than those of any other brain area with the same high steady-state blood flow. This observation may indicate a third conclusion: that the steady-state flow rate of a brain region may be a predominant factor but it is clearly not the only factor in determining regional CO_2-sensitivity. Fourth, during haemorrhagic hypotension there is an "autoregulatory range" of the CO_2-sensitivity in a number of cerebral and spinal cord regions since in spite of the reduction of the MAP from 80 mm Hg to 60 mm Hg the vasodilator response to hypercapnia was well preserved in this pressure range. Fifth, the different brain regions showed significant differences in the reduction of their CO_2-responsiveness during haemorrhagic hypotension: the vessels of the pons-medulla oblongata were the most vulnerable areas to haemorrhagic hypotension. Finally, stimulation of the L-arginine-nitric oxide pathway by an excess amount of exogenous L-arginine administration failed to restore the haemorrhage-induced depression of regional cerebrovascular CO_2-responsiveness. Therefore, the availability of L-arginine during haemorrhagic hypotension cannot be a limiting factor in nitric oxide generation, a process which has a strong influence on cerebrovascular CO_2-responsiveness in cats [54].

The regional differences of CO_2-reactivity obtained in normotensive cats are in reasonable agreement with those obtained by others in cats [56], monkeys [60], and humans [1, 27]. In the present study, four major categories of regional CO_2-responsiveness were clearly distinguishable. CO_2-sensitivity of the normotensive cats was almost uniformly high in the majority of the regions with high steady-state flow values (cerebral cortex, cerebellum, thalamus, pons-medulla oblongata; Category 1). The regions with low resting flow values showed almost equally low CO_2-sensitivity (white matter, cervical, thoracic, and lumbar spinal cord; Category 2). CO_2-sensitivity of the hypothalamic vessels was significantly lower than that of the high sensitivity regions but was significantly higher than that of the low sensitivity areas (Category 3). The vessels of the pituitary were practically insensitive to CO_2 (Category 4).

The anatomical peculiarities of the pituitary vasculature have been examined in detail [45, 46, 63, 65]. Involvement of peripheral baroreceptor and chemoreceptor mechanisms as well as arginine-vasopressin mechanisms in the regulation of pituitary blood flow have also been reported [8, 26, 63]. Our present findings in cats are in good agreement with the data of others who found that the vessels of the pituitary are insensitive to hypercapnia in sheep, dogs, and rats [8, 26, 68]. We found no data in the literature, however, on the peculiarities of the regional CO_2-responsiveness of the hypothalamic vessels in graded haemorrhagic hypotension. There is no clear explanation as to why the CO_2-sensitivity of this region is significantly different from that of either the high basal flow regions or the low basal flow areas. One may speculate, that the anatomical structure which is even more complex than that of the cerebral cortex, the unique architecture of the hypothalamic vessels, the high density of a number of peptide and hormone receptors, the high local concentration of vasoactive peptides, hormones, and neurotransmitters [47, 65, 68] may all contribute to the unique CO_2-response of these vessels. It is very likely that adrenergic mechanisms may also play a role in the observed phenomenon. In rat studies, blood flow in the median eminence and in the neural lobe of the pituitary increased significantly during hypercapnia when the rats were pretreated with the α-adrenergic blocking agent phentolamine [68].

It has been suggested that CO_2 reactivity over a wide range of cerebrovascular conductance values is proportional to the resting flow level and inversely related to the blood pressure [1, 56, 60]. The data of the present study also support this view in part since most regions with higher steady-state blood flow responded more intensely to CO_2 than those with low resting flow values. Some of our data, however, showed that the relationship between basal CBF and CO_2-sensitivity was more complex. The resting blood flow of the hypothalamus (0.68±0.33 ml/g/min) was very similar to that of the sensory motor cortex (0.66±0.19 ml/g/min) and was not significantly different from that of the cerebellum (0.85±0.21 ml/g/min), but the CO_2-sensitivity of the hypothalamic region was significantly lower than that of either the cortical or the cerebellar vessels. These findings indicate that the basal flow of a region can only be used as a good but rough guide to estimate the CO_2-responsiveness of that brain region, but the idea that the higher the basal flow the higher the CO_2-sensitivity cannot be applied as a rigid rule.

One of the interesting new findings of this study is the observation of a previously unknown "autoregulatory range" in cerebrovascular CO_2-sensitivity. Gradual stepwise reduction of the arterial pressure by haemorrhage was not followed by a similar, passive gradual reduction of the regional CO_2-sensitivity in the cortex, cerebellum, hypothalamus, pons-medulla oblongata, in the white matter and in the spinal cord. As shown in Figs 2, 3 and 4, reduction in arterial pressure to 80 mm Hg

resulted in a considerable decrease of the regional CO_2-sensitivity in all investigated regions. Further reduction of the MAP from 80 mm Hg to 60 mm Hg, however, was not followed by a comparable decrease in CO_2-sensitivity. The CO_2-responsiveness, in fact, remained unchange during this pressure reduction although a considerable amount of blood had to be withdrawn in order to produce the desired pressure decrease. Further reduction of the MAP to 40 mm Hg was followed by a significant decrease of the CO_2-sensitivity. Statistical analysis of the data revealed that the relationship between CO_2-responsiveness and systemic arterial pressure in all investigated areas was not linear. The mechanisms which help to maintain the ability of the vessels to respond to CO_2 at 60 mm Hg MAP with the same sensitivity as was observed at 80 mm Hg MAP is unknown. We may hypothesize, however, that at 40 mm Hg MAP cerebral vessels stop dilating with further reductions in blood pressure and vascular resistance stops going down but actually increases with further reductions in blood pressure. These findings are consistent with the interpretation that the vascular bed is maximally dilated at that point and one may not expect an increase in blood flow below this pressure level in response to increased $PaCO_2$ because the vessels cannot dilate anymore. Even though it may not be the sole determinant factor in determining CO_2-responsiveness, the resting level of blood flow is an important factor factor in determining the changes in CO_2-responsiveness at different mean arterial pressure levels. One may suppose, therefore, that the absence of change in CO_2-responsiveness between 80 mm Hg and 60 mm Hg MAP can be explained by the absence of a change in blood flow. Table II shows that no flow reduction was observed in any of the investigated regions when MAP was reduced from 80 mm Hg to 60 mm Hg.

Defensive mechanisms against reduced regional cerebral and spinal CO_2-responsiveness can be manyfold. Increased autonomic sympathetic activity following haemorrhage-induced hypotension may play a role in protecting the brain vessels from passive vasodilation, a condition when CO_2 is unable to induce a further increase in the vessel caliber [32]. The L-arginine-nitric oxide system may also be involved in the preservation of the CO_2-sensitivity of the cerebral vessels. In our previous studies, we observed a major role for nitric oxide in CO_2-sensitivity of cerebral vessels in normotensive cats [54]. This system is presumably more active when arterial pressure is lowered by haemorrhage since a progressive shift of arterial pH toward more acidic values (see Table III) is known to increase NO-synthase activity increasing the syntheses of NO from L-arginine [24]. An increase of plasma norepinephrine induced by haemorrhage may also contribute to the release of NO from the cerebral (pial) vessels [5, 59]. The data of the present study indicate, however, that the availability of L-arginine under our experimental conditions was not a limiting factor in nitric oxide generation, since administration of a massive dose of exogenous L-arginine did not

prevent the haemorrhage-induced reduction of the regional cerebrovascular CO_2-sensitivity.

It was interesting to observe in these studies that the vulnerability of the mechanisms enabling the cerebral vessels to dilate in response to increased $PaCO_2$ during haemorrhage differed from region to region. The vessels of the pons-medulla oblongata were the most sensitive to haemorrhage, since CO_2-responsiveness in this region was already reduced significantly at the 80 mm Hg MAP level. In the mediobasal hypothalamic area a significant reduction of CO_2-sensitivity was observed only at 60 mm Hg MAP, and all other investigated areas lost their CO_2-sensitivity only when MAP was reduced to 40 mm Hg.

The observation that 40 mm Hg MAP is the approximate lower pressure limit for cerebrovascular CO_2-sensitivity in cats following gradual haemorrhagic hypotension is in agreement with the data of others, obtained in other species. In the dog some residual CO_2-responsiveness was observed at 60 mm Hg haemorrhagic hypotension [22] which, according to the observation of others [23] completely disappeared at 50 mm Hg MAP. Rabbit pial arteries did not dilate with hypercapnia during haemorrhagic hypotension to 30–40 mm Hg [52]. The level of hypotension at which CO_2-responsiveness is completely blocked is less certain when arterial hypotension is produced by drugs [4, 21, 25, 44].

It is known that the effect of hypercapnia on the cerebral vasculature is affected by simultaneous hypoxia. Hypercapnia during moderate hypoxia increases CBF, whereas hypercapnia during severe hypoxia decreases CBF, both in comparison with normocarbic hypoxia [39]. To avoid this potential complication, arterial PaO_2 was maintained above 90 mm Hg throughout all experiments in the present studies (Table III).

In conclusion, in normotensive, normoxic cats response of the cerebral and spinal vessels to $PaCO_2$ alterations can be assigned to four major categories. The CO_2-responsiveness of a brain region is not solely determined by the rate of its basal steady-state blood flow: CO_2-reactivity of the feline mediobasal hypothalamic area was significantly different from that of other regions with almost identical steady-state flow values. A previously unknown phenomenon, preservation of the vasodilator response to hypercapnia was observed in the cortex, cerebellum, thalamus, hypothalamus, pons-medulla oblongata, white matter, cervical, thoracic and lumbar spinal cord in the mean arterial pressure range of 60–80 mm Hg during standardized haemorrhagic hypotension. Vulnerability of the cerebrovascular CO_2-sensitivity during hypotension was different from region to region, with the vessels from the pons-medulla oblongata region being the most sensitive to haemorrhage. Reduced regional cerebral and spinal CO_2-responsiveness during haemorrhage is not a consequence of a reduced L-arginine supply since administration of an excess amount of the NO-precursor L-arginine failed

to restore the haemorrhage-induced reduction of regional CO_2-responsiveness at a MAP of 60 mm Hg.

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Prevention of indomethacin-induced acute gastric mucosal injury by spermine in the rat

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The effect of spermine tetrahydrochloride was investigated on the indomethacin-induced acute mucosal injury and mucosal lipid peroxidation in rats. Spermine was given orally at doses of 25, 75 and 150 mg/kg and, intraperitoneally at 10, 25 and 50 mg/kg 1 hour before 25 mg/kg indomethacin administration. Macroscopic injury was measured by the use of a stereomicroscope. Mucosal malondialdehyde (MDA) levels were determined by an HPLC method. Oral spermine reduced dose-dependently the extent of macroscopic injury from 22.87±2.88 mm to 11.8±2.48 mm ($p<0.01$, 25 mg/kg), 6.53±4.19 mm ($p<0.0007$, 75 mg/kg) and 0.25±0.25 mm ($p<0.0001$,150 mg/kg). Intraperitoneally administered spermine prevented dose-dependently the indomethacin produced lesions from 19.25±4.31 mm to 12.63±3.18 mm ($p<0.01$, 25 mg/kg) and to 0.33±0.33 mm ($p<0.001$, 50 mg/kg). Doses necessary for intraperitoneal protection were lower than those achieving similar effect, orally suggesting different mechanisms of action. Neither oral or intraperitoneal spermine influenced the mucosal MDA levels.

Keywords: spermine, polyamine, indomethacin, malondialdehyde, free radicals, acute gastric mucosal injury

Normal epithelial growth of the gastrointestinal mucosa requires polyamines [1]. These polycations (spermine, spermidine, putresceine) have been proved to be efficient in the prevention and healing of acute gastric mucosal lesions induced by absolute ethanol [2, 3], acidified ethanol, histamine, pyloric ligation [4] and water-immersion stress [5].

Indomethacin typically produces round and linear ulcerations located mainly on the mucosal folds. Orally administered indomethacin induces acute lesions in a time-

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dependent manner, the lesions developing in 3–12 hours after administration [6–9]. The pathologic mechanisms leading to injury are complex and in many aspects different from other experimental ulcer models [6, 7]. Therefore, it was tempating to investigate the effect of spermine on the indomethacin induced acute gastric mucosal injury, which has not been studied. While free radicals are also involved in the pathogenesis of these lesions and certain polyamines also exhibit anti-oxidative effects [8], we have also investigated the effect of spermine on the indomethacin-caused lipid peroxidation.

Material and Methods

Materials

Spermine tetrahydrochloride and indomethacin were purchased from Sigma Chemical Company. Pentobarbital sodium (Nembutal) was a product of Ceva-Sanofi, Italy. All other reagents were of highest purity for laboratory use, produced in Hungary.

Experimental protocol

Wistar rats of either sex weighing 350–450 g were used. The animals were fasted for 48 h with free access to water, and kept in cages preventing coprophagia. Indomethacin (25 mg/kg) was dissolved in 5% sodium bicarbonate, and the pH was adjusted to 7.4. The drug was administered orally through a nasogastric plastic tube.

In order to assess local and also systemic effects of spermine on the indomethacin-induced acute gastric mucosal injury, the drug was administered to conscious rats in increasing doses either intragastrically or intraperitoneally in 8 groups of animals, each containing 6–8 rats. Spermine was dissolved in saline and the pH was adjusted to 7.4. It was administered orally (through a plastic nasogastric tube), or intraperitoneally, 1 hour before indomethacin. The oral doses were: 25, 75 and 150 mg/kg; 10, 25 and 50 mg/kg were given intraperitoneally. The animals were killed with a lethal dose of intraperitoneally administered pentobarbital sodium, 6 hours after indomethacin administration. Control animals received 1 ml saline orally or intraperitoneally instead of spermine.

Macroscopic study

After the sacrifice of the animals the abdomen was rapidly opened and the stomach removed. It was carefully rinsed in saline and cut along the greater curvature.

The glandular part of the stomach was excised and pinned to a plastic plate and the mucosal surface was examined under a dissecting microscope (K. Zeiss, Jena, Germany). The length of each lesion was measured in mm and the sum of the lengths was used as an ulcer index. The person measuring the lesion sizes was blind to the treatment of the given animal.

Determination of lipid peroxidation

Lipid peroxidation (LPO) of the gastric mucosa was measured by the determination of the concentration of thiobarbituric acid reactive substance (TBA) which is proportional to the level of malondialdehyde as an end product of LPO. After measurement of the size of the injury, a sample of 15×15 mm of the ulcerated mucosa was cut off, and it was chemically analyzed. Malondialdehyde (MDA) was determined by an HPLC method as described by Tatum et al. [10]. Protein content of the samples was measured by using Lowry's method [11]. The results were expressed as nM MDA/mg protein.

Statistics

Data are presented as means ±SEM of 6–8 rats in one group. Statistical evaluation was performed using the two-tailed Student's *t*-test and values $p<0.05$ were regarded as significant.

Results

Oral administration of spermine

Orally administered spermine prevented the indomethacin-induced acute gastric mucosal injury in a dose-dependent manner (Table IA, Fig. 1A). No significant changes of MDA values were observed in the groups receiving indomethacin + oral spermine as compared to the animals given indomethacin + saline (Table II, Fig. 2).

Intraperitoneal administration of spermine

Spermine reduced acute mucosal injury caused by indomethacin. The lower dose (10 mg/kg i.p.) was inefficient, but 25 mg/kg i.p. spermine prevented significantly the lesion formation while 50 mg/kg abolished them almost completely (Table IB, Fig. 1B). The protective effect of intraperitoneally administered spermine occurred at 3× lower doses as compared to the oral administration.

Table I

The effect of orally and intraperitoneally administered spermine on the macroscopic mucosal injury induced by indomethacin in rats

	Way of administration	Group No.	Treatment and dose of spermine (mg/kg)	Lesion index (mm) (mean+SEM)	p[x]
A.	Oral spermine	1	Saline	22.86±2.86	–
	+	2	25	11.83±2.48	0.01
	indomethacin	3	75	6.43±4.19	0.007
	(25 mg/kg)	4	150	0.25±0.25	0.0001
B.	Intraperitoneal	5	Saline	19.25±4.31	–
	spermine + oral	6	10	23.00±1.98	0.38
	indomethacin	7	25	12.63±3.18	0.014
	(25 mg/kg)	8	50	0.33±0.33	0.001

[x] The p value was calculated between the control groups (Nos 1 and 5) and orally (Nos 2–4) and intraperitoneally (Nos 6–8) treated groups, respectively.

MDA levels were assessed only in animals receiving the highest dose of spermine and no effect was observed (Table IIB, Fig. 2) as compared to the group receiving maximal oral dose of spermine (150 mg/kg).

Table II

Mucosal MDA concentration in rats receiving oral indomethacin, pretreated with oral or intraperitoneal spermine

	Way of administration	Group No.	Treatment and dose (mg/kg)	MDA concentration (nM/mg protein) (mean+SEM)	p
A.	Oral spermine	1	Saline	1.06±0.16	–
	+	2	25	0.95±0.13	0.53
	indomethacin	3	75	0.74±0.02	0.09
	(25 mg/kg)	4	150	0.91±0.20	0.28
B.	Intraperitoneal spermine + indomethacin (25 mg/kg)	8	50	0.95±0.06	0.71[x]

[x] Group 4 vs. 8.

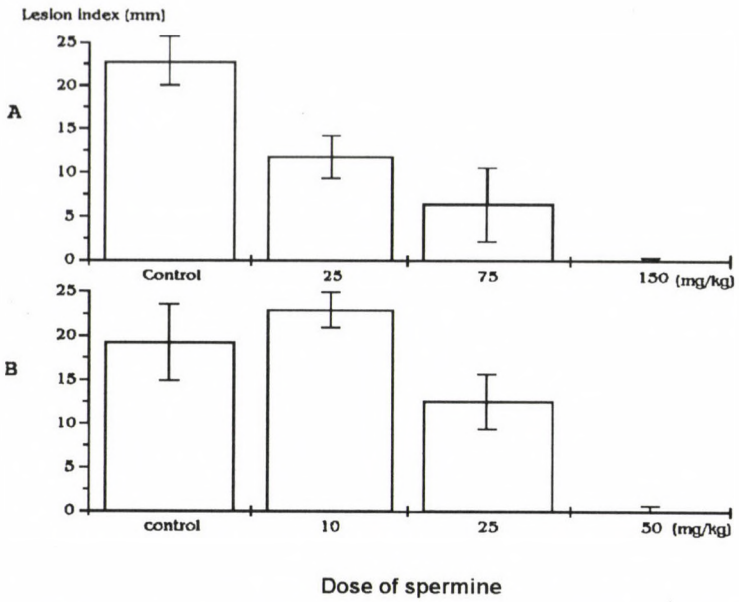

Fig. 1. The effect of oral (A) and intraperitoneal spermine (B) on the macroscopic mucosal injury induced by indomethacin in rats

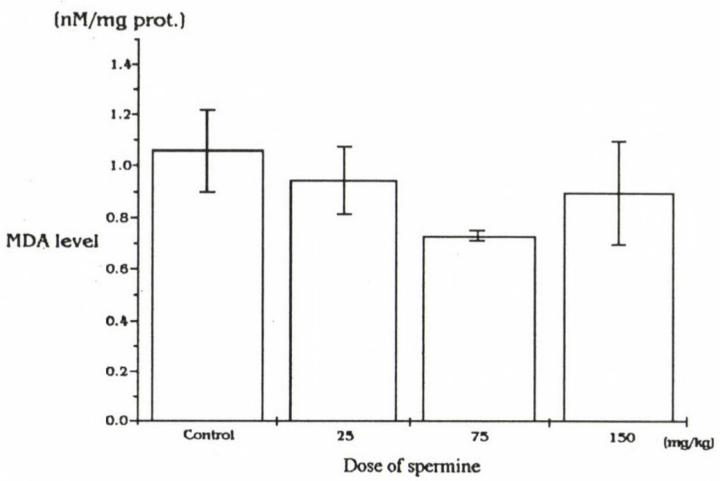

Fig. 2. The effect of oral spermine administration on the mucosal malondialdehyde (MDA) levels in rats receiving oral indomethacin

Discussion

The present study demonstrates that spermine administered orally or intraperitoneally, prevents dose-dependently the mucosal injury caused by indomethacin. This effect completes the spectrum of protective actions of this polyamine, proved thus far [2–5]. While it is active both after oral and intraperitoneal administration, local and systemic mechanisms of prevention would certainly be involved. These mechanisms are only incompletely understood. Earlier experiments showed that basal- and histamine-stimulated acid secretion was reduced in rats receiving spermine orally or subcutaneously [4]. Other studies support the anti-oxidative action of spermine [2, 8], however, this action could not been reproduced by others [3]. In case of ethanol and insulin-induced acute lesions, a vasoprotective action of spermine was proposed. In these studies there was a decrease of mucosal Evans blue extravasation [3, 12]. Preservation of tissue non-protein sulfhydryls might be an alternative pathway of this preventive action [13].

The pathogenesis of indomethacin-induced damage in the rat stomach involves, in addition to the well-known depression of endogenous prostaglandin synthesis [14] a shift to increased proulcerogenic leuktriene C_4 formation [9, 15], microvascular disturbances [16] and also free-radical-mediated injury [7]. The involvement of free radicals in the pathogenesis of these lesions have been repeatedly proved [18, 19]. While the xanthin oxidase inhibitor allopurinol does not prevent indomethacin-induced injury [19], the role of neutrophils as potential source of free radicals during their oxidative burst has recently been emphasized. Depletion of circulating polymorphonuclears by specific antiserum or methothexate prevents indomethacin-induced damage, without interfering with the prostaglandin synthesis [20]. A role of endothelial adhesion molecules in this process has also recently been suggested [21]. Gastric hypermotility caused by indomethacin could also lead to microcirculatory disturbances and release of free radicals [7], a process which occurs early, before the occurrence of mucosal lesions.

In our earlier experiments (unpublished results) we also found a threefold increase of mucosal MDA concentrations as a consequence of indomethacin administration. In this study, orally administered spermine, although prevented macroscopic lesion formation, it did not influence tissue levels of MDA, which remained unchanged after intraperitoneal administration of spermine, too. These results suggest that the local and systemic protective effect of spermine did not involve an antioxidative action.

The low intraperitoneal doses as compared to the oral doses of spermine achieving the same protective effect might suppose either differences of the local and systemic mechanisms of action or could be of pharmacokinetic origin. Polyamines are

readily taken up from the gut lumen in the rat [22] and enter the circulation quickly. Degradation of spermine by the intestinal wall and liver diamine oxidases [23], which occurs only after oral administration, could explain, at least in part, these differences. Further studies are necessary to elucidate this matter.

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ETB RECEPTOR MEDIATING PULMONARY HYPERTENSION AND BRONCHOCONSTRICTION INDUCED BY ENDOTHELIN-1 IN THE GUINEA PIG

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Abstract: Endothelin (ET-1) caused dose-related contraction of isolated superfused bronchus and pulmonary artery and bronchoconstriction and pulmonary vascular hypertension of the heart lung preparation (HLP) of guinea pig. The specific ETA receptor antagonist BQ 123 completely blocked the responses of the pulmonary artery, but failed to affect those of bronchus and of HLPs. The specific ETB receptor agonist Sarafotoxin S6c caused contractions of bronchus, but not of pulmonary artery, and bronchoconstriction and pulmonary hypertension in HLPs. It is concluded that non-ETA subtype receptors, perhaps ETB, appear to be the main responsible for the potent pulmonary hypertensive effects of ET-1.

Keywords: endothelin receptor subtypes – bronchoconstriction – pulmonary hypertension

Introduction

Endothelin-1 (ET-1) belongs to a family of endothelium-derived vasoactive peptides which possess potent and sustained contractile activity on a variety of blood vessels and non-vascular smooth muscle (5). Distinct subtypes of ET-1 receptors have been described, with a predominance of the ETA and non ETA, perhaps ETB, subtypes in guinea pig aorta and bronchus, respectively (2). The aims of our work were: 1) to characterize further the two subtypes of the ET-1 receptor in endothelin receptors mediating responses of isolated bronchial and pulmonary vascular tissues of guinea pig and 2) to explore the potential functional differences of the two receptor subtypes in a distinct experimental model, i.e. the heart-lung preparation (HLP), which allows the simultaneous assessment of both bronchial and pulmonary vascular parameters. The snake venom Sarafotoxin S6c, that exhibits selectivity for the ETB receptor (4) and the selective ETA receptor antagonist BQ123 (3) were used.

Methods

Male Dunkin Hartley guinea pigs anaesthetised with ethyl urethane were used. After exsanguination, the pulmonary artery and the primary bronchus were rapidly excised and mounted in a cascade. Isotonic responses of the isolated organs superfused (5 ml/min) with oxygenated Krebs solution at 37 °C were -recorded. HLPs were prepared in male guinea pigs, weighing 300–400 g, anesthetized with ethyl urethane, according to the previously described procedure (1). The recorded haemodinamic and bronchial parameters are reported in Figure 1. Endothelin-1 and Sarafotoxin S6c were given by bolus injection and BQ 123 (all drugs from Alexis) was added to the perfusing media.

Results

In isolated organs, administration of ET-1 (200–1600 ng) produced dose-dependent contractions of both pulmonary artery and bronchus. Accordingly, pulmonary vascular hypertension and bronchoconstriction were obtained by increasing doses of ET-1 (25–200 ng) in HLPs. The ETA receptor antagonist BQ-123 (1μM) completely blocked the ET-1 induced contractions of the superfused pulmonary arteries, but not those of the bronchi. In contrast, the presence of BQ 123

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in the perfusing blood failed to affect either the pulmonary hypertension or the bronchoconstriction produced by ET-1 in HLPs. The ETB-selective agonist Sarafotoxin S6c (200–1600 ng) was a potent contractile agonist of the isolated bronchus, whereas did not contract the pulmonary artery. Conversely, bolus injection of various doses of Sarafotoxin S6c (12,5–200 ng) produced strong pulmonary vascular hypertension and bronchoconstriction in HLPs (Figure 1).

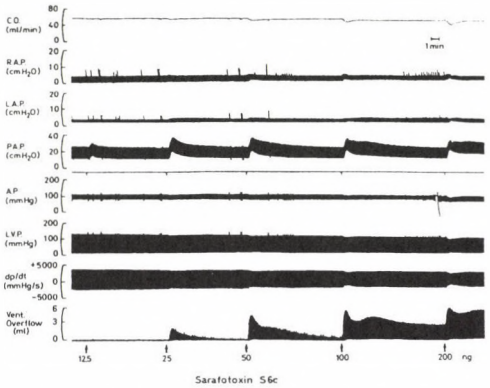

Figure 1. Representative original recordings of the effects of increasing doses of Sarafotoxin S6c on one heart lung preparation of guinea pig. C.O. = cardiac output; R.A.P. = right atrial pressure; L.A.P. = left atrial pressure; P.A.P. = pulmonary arterial pressure; A.P. = aortic pressure; L.V.P. = left ventricular pressure; dp/dt = first derivative of the L.V.P.; Vent. Overflow = resistance to air inflation

Discussion

The results of our study performed on isolated organs of guinea pigs indicate the predominance of ETA – in pulmonary artery and of ETB-subtype receptors in bronchus, respectively. However, the data obtained in the heart-lung preparation, which can be considered as intermediate between the *in vitro* isolated organs and the *in vivo* situation, demonstrate that the pulmonary hypertensive effects of ET-1 are, at least in part, dissociated from the vascular ETA subtype receptor activation.

Thus, our results strongly suggest that ETA subtype receptors may only partially account for the potent pulmonary hypertensive responses elicited by endothelin, whereas non-ETA subtype receptors, perhaps ETB, are the major responsible for the pulmonary hypertensive effects of the peptide.

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EFFECT OF GRANULOCYTE COLONY-STIMULATING FACTOR ON GRANULOPOIESIS OF CONGENITAL NEUTROPENIC CHILDREN

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Abstract: We report on two patients with congenital neutropenia, who were treated with filgrastim (recombinant human granulocyte-colony stimulating factor, G-CSF). A poor growth of bone marrow colonies and low sensitivity of colony forming units to colony stimulating factor *in vitro* before treatment seemed to be associated with a requirement for higher doses of G-CSF to achieve good clinical response *in vivo*.

Keywords: congenital neutropenia, CFU-GM, G-CSF

Introduction

Congenital neutropenia is a hereditary disorder associated with decreased granulopoiesis, the underlying disorders of which have only been partially characterized (4). Treatment of these patients relied on supportive care alone until the introduction of granulocyte colony-stimulating factor (G-CSF) therapy. Human G-CSF, a 174-amino acid glycosylated protein, preferentially stimulates the growth and differentiation of neutrophils and their precursors (1). Filgrastim is a non-glycosylated analogue with biological activities similar to that of the original human CSF molecule. This biotechnologically produced agent is available for clinical use. The beneficial effect of this therapy and the most effective time schedule remain to be evaluated. The examination of bone marrow for diagnostic purposes usually done before CSF treatment may be supplemented with estimation of the colony forming ability of bone marrow cells *in vitro*. This may contribute to the functional evaluation of the disorder of granulopoiesis and may even help to choose the right therapy, supposing that the colony forming capacity of granulocyte-macrophage progenitor cells (CFU-GM) in pretreatment bone marrow would predict the success of G-CSF therapy. We report on two patients with congenital neutropenia, who were treated with filgrastim.

Methods

Patients and treatment. A five years old boy (patient 1) and a 7 years old girl (patient 2) with congenital neutropenia had recurrent infections from early infancy. Their absolute neutrophil counts were permanently below 500 per mm^3, and bacterial sepsis, stomatogingivitis, bacterial infections of the skin, middle ear and lung were common in their histories. The daily dose of filgrastim (Neupogen, Hoffmann La Roche, Basle) was initially 5 μg/kgbw, and it was increased to 10 μg/kgbw if the initial lower dose did not produce an acceptable clinical response.

CFU-GM assay. CFU were determined in soft agar cultures of bone marrow samples aspirated for diagnostic purposes. Triplicate cultures of mononuclear bone marrow cells were grown in Mc Coy's 5A modified medium (GIBCO Grand Island, N.Y.) supplemented according to Pike and Robinson (3) as well as with 0.3% agar (Ionagar No2, Oxoid, London). Cultures of 10^5 cells per Petri dish (Greiner, Nürtingen, FRG) were incubated for 14 days in a humidified atmosphere containing 3% (v/v) CO_2. Colonies, defined as groups of at least 40 cells, were

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counted under a dissecting microscope. The source of colony stimulating activity was conditioned medium (PHA-LCM) of 4×10^6 human blood mononuclear cells stimulated by phytohemagglutinin and levamisol (2) or human recombinant granulocyte colony-stimulating factor (filgrastim, Neupogen, Hoffmann-La Roche).

Results and discussion

Before G-CSF treatment, the number of colonies developing in bone marrow cultures stimulated by 30 ng/ml of G-CSF was much lower in patient 1 than that in patient 2 (Table I). This almost fivefold difference was reduced substantially, when a more powerful stimulus of colony formation, namely PHA-LCM was applied. This indicates that, although a difference in the frequency of GM-colony forming units (CFU-GM) in the bone marrow samples of our patients cannot be excluded, the low colony numbers in G-CSF-stimulated bone marrow cultures of patient 1 were due mainly to a reduced sensitivity of his CFU-GM to stimuli. To see whether any factor inhibitory to granulopoiesis was present in the sera of the patients, the colony counts in cultures containing also autologous sera are shown in Table I. The differences were not significant.

Table I

Colony formation of granulocyte-macrophage progenitor cells in vitro *in two children with congenital neutropenia*

Patients	Colonies/10^5 nucleated bone marrow cells (mean ± SEM) stimulated by		
	G-CSF	PHA-LCM	PHA-LCM + autologous serum
Patient 1	4.5±2.0	16.5±2.47	11.3±1.25
Patient 2	22.1±1.45	23.8±1.96	21.9±0.71

Our *in vitro* data were compared with the *in vivo* response to G-CSF. In patient 2, whose CFU-GM's required less powerful stimuli to express their colony forming ability, the absolute neutrophil count (ANC) increased remarkably and clinical symptoms improved after the daily administration of 5 μg/kgbw of G-CSF. On the other hand, patient 1 with CFU-GM's less sensitive to stimuli did not respond well to the above dose and required 10 μg/kgbw of G-CSF for inducing an acceptable rise in ANC and clinical improvement.

These observations suggest that CFU-GM assay may be useful not only for estimating colony forming capacity of bone marrow cells *in vitro,* but also to predict the *in vivo* responsiveness of progenitor cells to G-CSF.

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CHANGES IN THE BILIARY EXCRETION OF EXOGENOUS ORGANIC ANIONS BY STREPTOZOTOCIN-INDUCED DIABETES

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Abstract: A transient depression of blood-glucose level was found after streptozotocin administration, which can be explained by insulin release due to the destroying effect of streptozotocin on beta-cells of pancreas, the biliary flow was elevated, when blood sugar level was low. After the transient decrease the blood glucose level was elevated and remained in a diabetic range (300–500 mg/100 ml). During the diabetic period a biphasic change was observed in the biliary flow: 23–48 hours after streptozotocin injection a depression, however, 5–10 days after streptozotocin administration an elevation was detected in the bile flow. Changes found in biliary excretion rate of exogenous organic anions were parallel with those of biliary flow.

Keywords: streptozotocin, diabetes, biliary excretion, rat

Introduction

It is known that diabetes causes alterations in the transport function and metabolism of various organs and tissues, little definitive data are available concerning the effects of diabetes on the hepatic drug metabolism and excretion [1, 2, 3]. In addition, the effect of experimental diabetes are equivocal, e.g. negative influence on the biliary flow was detected after short time diabetes produced by streptozotocin, however, significant increase was found in bile flow after long-term diabetes provoked by the same drug [4]. These experiments were designed to study the effect of both short and long term diabetes on the biliary flow and biliary excretion of drugs under the same experimental conditions, only one parameter was different: the time of investigation after streptozotocin administration.

Methods

Diabetes was induced in male Wistar rats (weighing 220–250 g) by i.v. administration of streptozotocin in a dose of 65 mg/kg. For the investigation of biliary excretion the bile duct was cannulated with a polyethylene (PE-10) tubing and bile was collected in anesthetized rats (urethane i.p., 1.2 g/kg). In order to eliminate complications caused by biotransformation, non-metabolized organic anions were used. Concentrations of organic anions in the bile were determined spectrophotometrically. Biliary excretion rate of drugs was calculated as a product of bile volume and biliary concentration of drugs, which were administered i.v. Data are expressed as the mean ± SEM of 4–6 rats.

Results

Effect of streptozotocin on the blood glucose level is shown in Figure 1. A transient depression of blood glucose level was found after streptozotocin administration which can be explained by insulin release due to the destroying effect of streptozotocin on beta-cells of pancreas, the biliary flow was elevated, when blood sugar level was low.

After the transient decrease the blood glucose level was elevated and remained in a diabetic range (300–500 mg/100 ml). The biliary flow was elevated during the transient

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depression of blood sugar level (Fig. 2). In the diabetic period a biphasic change was observed in the biliary flow: 24–48 hours after streptozotocin injection a depression, however, 5–10 days after streptozotocin administration an increase was detected in the bile flow. Changes found in biliary excretion of exogenous organic anions were parallel with those of biliary flow.

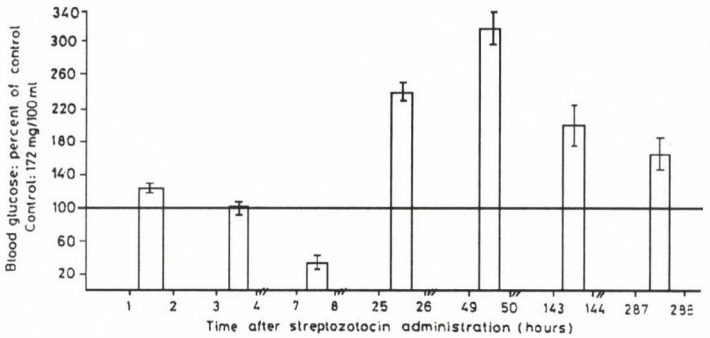

Fig. 1. Effect of streptozotocin on the blood glucose level

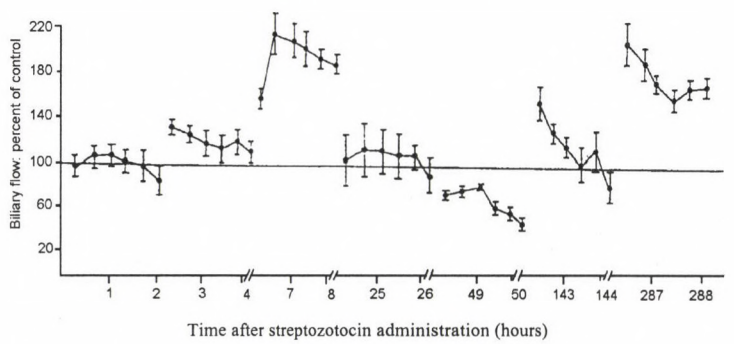

Fig. 2. Effect of streptozotocin on the biliary flow. Control biliary flow: 53.7±1.86 µl/kg/min=100%

Discussion

These results show that experimental diabetes produced by streptozotocin influences significantly both the biliary flow and the excretion rate of exogenous organic anions. These alterations are connected in part with changes of blood glucose level, however, other factors (e.g. lack of insulin, changes of metabolic processes and level of other hormones etc.) may also play a role in the mechanism of action of experimental diabetes [4].

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EFFECT OF GLIMEPIRIDE AND GLIBENCLAMIDE, INHIBITORS OF ATP-DEPENDENT K^+-CHANNEL, ON ISCHAEMIA-REPERFUSION INDUCED ARRHYTHMIAS IN RATS

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Abstract: Glibenclamide or glimepiride pretreatment (5 mg/kg ip. 30 min prior to coronary ligation) significantly improved the survival rate during reperfusion after 6 min myocardial ischaemia in rats (82% or 67% vs. 17% in controls). Smaller dose of glibenclamide (0.01 mg/kg) did not influence the survival rate (31%), while glimepiride still offered a significant protection (61%). These results suggest that different K_{ATP} inhibitors may increase the chance to survive life-threatening arrhythmias during myocardial ischaemia-reperfusion.

Keywords: glibenclamide, glimepiride – ischemia/reperfusion – arrhythmias – survival rate – rat

Introduction

Opening of ATP-dependent K^+-channels (K_{ATP}), as a consequence of the decrease in intracellular ATP concentration, may have a significant role in preserving ATP and thereby maintaining the function of the heart for a longer period during ischemia (2). On the other hand, shortening of the action potential duration by the same mechanism may result in the development of severe arrhythmias. Theoretically, on the basis of their cellular electrophysiological action, K_{ATP}-inhibitors would represent an ideal antiarrhythmic treatment that is more specific to the ischaemic myocardium. Ballagi-Pordany et al. (1) demonstrated that second generation sulphonylurea antidiabetic drugs inhibit the development of arrhythmias during the acute phase of experimental myocardial infarction in rats. In the present experiments the effect of two inhibitors of K_{ATP}, glibenclamide and glimepiride, were compared on the appearance of arrhythmias and sudden death during reperfusion after a brief myocardial ischaemia.

Methods

Animals: Male Sprague-Dawley CFY rats, weighing 300–350 g were used.

Coronary artery occlusion/reperfusion: During pentobarbitone anaesthesia and artificial ventilation the thorax was opened and coronary artery ligation was produced by tightening a loose atraumatic silk around the left main coronary artery for 6 min that was followed by reperfusion for 5 min. Changes in blood pressure and the ECG was registered continuously and the occurrence of arrhythmias and death was registered.

Drug pretreatments: Glibenclamide or glimepiride was applied in a dose of 0.01 or 5 mg/kg ip. 30 min prior to coronary ligation.

Results and discussion

Reperfusion after 6 mm myocardial ischemia resulted m the development of severe arrhythmias within 10–30 sec and death due to irreversible ventricular fibrillation in 83% of the control animals (Table 1). Pretreatment with glibenclamide or glimepiride in a dose of 5.0 mg/kg intraperitoneally significantly improved the survival rate during reperfusion (81% or 67%, respectively), while the incidence of severe arrhythmias (e.g. ventricular tachycardia, fibrillation)

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did not change. The protective effect was manifested in increasing the possibility of spontaneous termination of ventricular fibrillation (23% or 40% vs. 0% in controls).

Smaller dose of glibenclamide (0.01 mg/kg) did not influence the survival rate (31%), while the same dose of glimepiride still produced a significant increase in the survival (61%) and also offered a significant protection against the development of ventricular fibrillation (50% vs. 83% in controls).

Table 1

Effect of glibenclamide or glimepiride pretreatment on the survival rate and the incidence of arrhythmias during reperfusion after 6 min myocardial ischemia in anaesthetised rats

Group	Dose	N	Survived	Incidence of arrhythmias, n/%				
	mg/kg		n/%	None	VF	VT	Other	Brady
Control		23	4/17	0/0	19/83	23/100	11/48	0/0
Glibenclamide	0.01	13	4/31	0/0	10/77	13/100	8/62	1/8
	5.0	16	13/81*	0/0	13/81	16/100	15/94*	3/18
Glimepiride	0.01	18	11/61*	0/0	9/50*	18/100	16/89*	1/6
	5.0	18	12/67*	0/0	15/83	18/100	13/72	2/11

N = total number of animals, n = number of animals exhibiting the given response. None = no arrhythmia; VF = ventricular fibrillation; VT = ventricular tachycardia; Other = ventricular extrasystoles, bigeminia, salvos; Brady = bradycardia. Asterisk denotes statistically significant difference ($p < 0.05$) from the control group, calculated by the Chi-square method.

These results demonstrate that different K_{ATP} inhibitors may increase the chance to survive life-threatening arrhythmias during myocardial ischaemia-reperfusion. The protective action might be related to the inhibition of the potassium loss both during myocardial ischaemia and reperfusion, or to the prevention of the shortening of action potential duration and the refractory period in the damaged myocardium (3). Because of the significant dose-related differences between glibenclamide and glimepiride found in the present experiments, we suggest that such a protection may not necessarily be related to the antidiabetic action of these compounds, and the sensitivity of K_{ATP} channels/receptors to different inhibitors may not be the same in the Langerhans β-cells and in the myocardium.

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ρ-AMINOPHENYLALANINE^6 ANGIOTENSIN II (AII) IMPROVES LEARNING AND MEMORY IN RATS

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Abstract: AII and ρ-aminophenylalanine^6 angiotensin II, given icv (1 nmol) enhanced memory and stereotypic behaviour leaving gross motor activity unchanged. Both peptides showed also some anxiogenic action.

Keywords: ρ-Aminophenylalanine^6 angiotensin II, Behaviour, Memory, Rats

Introduction

We have recently shown that the inhibition of the AT_2 angiotensin receptors with the selective ligands CGP 42112A or PD 123319 markedly attenuates the cognition enhancing activity of AII in rats (1, 2). In the present study we addressed the question whether the stimulation of the AT_2 receptors with their selective agonist ρ-aminophenylalanine AII (ρNH_2Phe^6AII) might exert any cognitive effects. Cognition oriented (passive avoidance and object recognition) and auxiliary (open field, elevated "plus" maze and apomorphine stereotypy) behavioural procedures were used. Parallel experiments with AII were run for the reference purposes.

Methods

Male Wistar rats (160–180 g) were prepared according to the standard surgical procedure (2). After 48 h they were given intracerebroventricularly (icv) AII or ρNH_2Phe^6AII, each at the dose of 1 nmol, dissolved in 2 µl of saline. Control animals received the same amount of saline alone. Effects of these injections upon several aspects of behaviour were then tested according to the procedures described in detail earlier (2). Retention of the passive avoidance was measured in one trial learning, step-through situation in animals injected icv 15 min before the test trial. Recognition of objects was tested 1 h after their first exposition to animals followed by an icv injection. Locomotor and exploratory activity was measured in open field as number of crossings, rearings, and bar approaches made during 5 min starting 15 min after the icv injection. Anxiolytic action of the peptides was assessed 15 min after their icv injection in the elevated "plus" maze. Number of entries to, and the amount of time spent in the open vs. closed arms of the apparatus during the 5 min session was recorded. The effect of AII and ρNH_2Phe^6AII on apomorphine (1 mg/kg, intraperitoneally) induced stereotypy was taken as a measure of the peptides' influence on the dopaminergic system. The results of all experiments were evaluated statistically by analysis of variance (ANOVA) followed by Newman-Keuls or Bonferroni tests, except for the passive avoidance behaviour which was assessed with Mann-Whitney test.

Results

The rats injected with AII had significantly better retrieval of memory of passive avoidance behaviour. They also had better recognition memory for the previously seen objects (Table I). ρNH_2Phe^6AII caused similar though less pronounced changes.

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Specifically, it only slighty improved recall of the passive avoidance and almost as well as AII improved recognition. Auxiliary behavioural tests showed: a) negligible increase (crossings, rearings, and bar approaches) by AII, and decrease (bar approaches) by ρNH_2Phe^6AII, of the locomotor exploratory activity in the open field, b) some anxiogenic action of AII and ρNH_2Phe^6AII in the elevated "plus" maze – AII significantly decreased the time spent in open arms and the number of open arms entries. Similar effect, though weaker, was caused by ρNH_2Phe^6AII, c) significant enhancement of the stereotypic behaviour produced by apomorphine after the administration of either, AII or ρNH_2Phe^6AII.

Table I

Effect of AII and ρNH_2Phe^6 AII on object recognition

Variables	Treatment		
	Saline	AII	ρNH_2Phe^6AII
B – A'	–0.37	2.63***	2.09**
	(0.33)	(0.6)	(0.6)
A	20.45	24.54	15.9
	(3.32)	(3.04)	(2.14)
B + A'	8.54	10.45	9.36
	(1.6)	(1.81)	(1.89)
B–A'/B+A'	–0.1	0.31**	0.23*
	(0.1)	(0.09)	(0.07)

*p< 0.05, ** p< 0.01, *** p< 0.001 vs. NaCl group (For the details see text).

Discussion

These results obtained in the cognition oriented tests point to the clear-cut memory enhancing effect of ρNH_2Phe^6AII, a potent AT_2 angiotensin receptor agonist. Out of the 4 brain areas (locus corelueus, medial geniculate, hypoglossal nucleus, inferior olivary nucleus) containing high level of the AT_2 sites, locus corelueus seems to be most likely anatomical substrate of this activity. This structure integrates the central noradrenergic system and regulates the level of general arousal in mammals (3).

The results obtained in the auxiliary tests may be interpreted as follows: a) the unspecific motor effects of our treatments were negligible and could not contribute significantly to the results of the memory tests, b) anxiogenic activity of ρNH_2Phe^6AII is in line with the widely accepted view on the role of locus corelueus in anxiogenesis, c) dopaminergic stimulation by the peptides may be partly responsible for the overall cognition enhancing activity of AII and ρNH_2Phe^6AII.

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THE NMDA AND GABA-A RECEPTORS IN BEHAVIORAL ACTIVITY OF RATS

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Abstract: The effects of bicuculline (0.25 mg/kg ip) and AP-7 (5 nmols icv) on the processes of retrieval, consolidation of conditioned reflexes, object recognition and locomotor activity were tested in rats. AP-7, bicuculline and AP-7 with bicuculline increased (but not significantly) locomotor and exploratory activity in the open field test. Only coadministration of AP-7 with bicuculline facilitated retrieval of passive avoidance in rats, but was without effect on consolidation in this test. AP-7 or bicuculline also did not influence on consolidation, when they were given alone. Object recognition was impaired (but not significantly) in groups of rats treated with bicuculline and bicuculline with AP-7.

Keywords: bicuculline, AP-7, behavioral activity, rats

Introduction

Neuronal excitability, and probably synaptic plasticity, cognitive performance, learning and memory are frequently the results of the balance of GABAergic inhibitory and glutaminergic excitatory inputs. The several lines of evidence suggest an apparent metabolic interaction between GABA and glutamate, and the GABA-A receptor belongs to the group of ligand-gated receptors that include the glycine, and glutamate receptors, among others. The N-metyl-D-aspartate (NMDA) receptor subtype of excitatory the amino acids (EAA) receptor system has been proposed to play a role in synaptic plasticity, cognitive performance (5), and learning and memory (3). The evolution of the working hypothesis has paralleled the induction of selective glutamate receptor antagonists (7), and the development of our knowledge of long-term potentiation (LTP), which is believed to resemble some elementary features of memory formation at the neuronal level (3).

GABA is released in different brain areas during learning of different tasks and after the induction of LTP. GABA-A antagonist facilitates LTP (2).

The purpose of the present study was to investigate the effect of the antagonist of the GABA-A receptor and the antagonist of the NMDA receptor on behavioral activity in rats.

Methods and materials

Subjects were white, male Wistar rats weighing 160–180 g. AP-7 was administered into the lateral ventricle of the brain (icv), bicuculline – intraperitoneally (ip).

Behavioural testing: The procedure of object recognition was similar to that described by Ennaceur and Delacour (4). The passive avoidance response was induced using the one trial learning method of Ader (1). The open field test was used for estimation of locomotor and exploratory activity of rats.

The statistical comparison of the results was carried out by Kruskal-Wallis one-way analysis of variance followed by Mann Whitney U test and by analysis of variance (ANOVA) followed by modified t statistics and Benferroni's procedure (6) when multiple means were to be compared.

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Results and discussion

Our results provide a suggestion that GABAergic transmission contributes to the changing behavioral effects of the glutaminergic system.

In the present experiments, the down-regulation of the GABA-A receptor by bicuculline can provide to tendency to enhance locomotor activity in the open field test. The NMDA antagonist AP-7 also affects this activity, but without significance. When they were used in combination they induced locomotor activity in almost the same level as when they were given individually.

Our observations demonstrate that AP-7 and bicuculline given on the 3rd day before a single trial did not change retrieval of passive avoidance in rats. Of special relevance might be the fact that in the present study we obtained a facilitatory effect on retrieval of passive avoidance after coadministration of antagonists of receptors GABA-A with NMDA.

An unexpected divergence in the passive avoidance test, the facilitation of retrieval by AP-7 and bicuculline given together, was obtained.

Unlike the retrieval of passive avoidance, delayed recognition of objects examined were affected by bicuculline given alone or with combination with AP-7.

Pharmacological studies on the action of NMDA receptor antagonists on working memory are rather confusing. In most studies, the specific impairing effect of NMDA receptor antagonists on working memory is restricted to a narrow dose range and is easily confounded by non-associative factors.

To our knowledge there are no data on the presence of effects of the antagonist GABA receptors on working memory. In this study we obtained delayed recognition of objects by bicuculline and this effect was persistent with combined administration with AP-7. It is concluded that the NMDA receptor antagonist did not interact with the GABA-A antagonist in this behavioral procedure.

These results support our proposed hypothesis that there is an interaction between the GABAergic system and the glutamatergic system. The main conclusion that can be drawn from the present study is that glutamate acting on NMDA receptor modulates the behavioral effects of GABA-A receptors.

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LOSARTAN DOES NOT INFLUENCE THE BLOOD PLATELET AGGREGATION IN NORMOTENSIVE RATS

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Abstract: Platelet aggregation was studied in PRP upon stimulation with ADP and collagen in normotensive rats treated with losartan (10 mg/kg). The acute and subchronic (5 days) losartan administration did not change the aggregating response of rat platelets. Similarly, in vitro study aggregation of platelets remained unaltered following incubation with losartan and its active metabolite EXP3174. In this study we presented the lack of influence of losartan and its main metabolite on rat platelet aggregation in normotensive rats.

Keywords: losartan, EXP3174, platelet aggregation, rat

Introduction

There is considerable evidence from animal studies and from clinical observations that blood platelets play an important role in atherosclerotic and thrombotic complications associated with hypertension. The effect of various antihypertensive drugs on platelet function is of increasing interest. Losartan is a new antihypertensive drug, orally active, selective and competitive angiotensin II type 1 (AT_1) receptor antagonist (6). All these observations prompted us to investigate a possible losartan effects on platelet aggregation.

Methods

Male Wistar rats weighing 250–300 g were used in the experiments. Losartan (DuP 753, The Du Pont Merck Pharmaceuticals) at the dose 10 mg/kg p.o was administered 2 h and 4 h before aggregation or in the subchronic experiments once daily for 5 days. Blood samples were taken from the heart, and mixed with 3.13% sodium citrate in a volume ratio 9:1. The assay for blood platelet aggregation was carried out according to Born and Cross (1). Samples (250 µl) of platelet rich plasma (PRP) were pre-incubated at 37 °C for 1 min prior the addition of an aggregating agents: ADP (4 µM) and collagen (10 µg/ml). In in vitro study the PRP was pre-incubated with losartan or its metabolite EXP3174 (both at the concentration 10^{-8}–10^{-5} M) for 15 min at 37 °C. The aggregation is presented as a % of light transmission. Data were evaluated using Student's *t*-test. All data are expressed as the means ± standard error.

Results and discussion

In these study we observed, that losartan - AT_1 receptor antagonist did not influence rat platelet aggregation measured in PRP of normotensive rats after single dose and 5 days of treatment (Table 1). In vitro, losartan and its active metabolite EXP3174 (10^{-8}–10^{-5} M) failed to change platelet response to both stimulating agents (Table 2). To our knowledge, there are no information about the presence of Ang II receptor on rat platelets. We did some experiments in which it was observed that Ang II (10^{-7} M) had no direct effect on platelet aggregation and did not influence the aggregation induced by ADP or collagen. Moreover, after incubation with losartan (10^{-7} M) the aggregating response of platelet to ADP and collagen in the presence of Ang II remained unaltered (data not shown). The literature date allowed as to speculate that some

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effects of losartan may be unrelated to action of the compound at angiotensin receptor. The structure of losartan includes imidazole moiety which is known to be selective inhibitor of thromboxane synthetase in blood platelets (5).

Hypothesis that losartan in some way interferes with thromboxane is supported by observation that it is competitive antagonist to human platelet thromboxane A_2/prostaglandin H_2 receptors (4). It has been also demonstrated that losartan is a potent stimulus for PGI_2 and PGE_2 synthesis in cultured endothelial cells (3) and that both PGI_2 and nitric oxide participate in its antihypertensive effect in rats (2). In summary, we did not observed any influence of losartan or its metabolite on rat platelet aggregation in our experimental model. However, taking under consideration mentioned above findings, the possibility that this drug may alter the human platelet aggregation or aggregation the platelets of hypertensive rats should not be excluded.

Table 1

Effect of losartan on rat platelet aggregation ex vivo

	Acute experiments		Subchronic experiments
	2 h	4 h	
Control	59.0±2.9	58.8±3.8	46.1±5.2
Collagen	44.8±3.8	46.6±5.2	44.1±5.7
Control	48.1±8.6	46.1±5.7	39.0±4.3
ADP	47.6±2.9	49.0±3.3	33.3±0.9

Table 2

Effect of losartan and EXP3174 on platelet aggregation in vitro

	Drugs concentrations M				
	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}
Losartan					
ADP	45.8±1.6	45.3±2.1	43.7±2.1	50.2±1.6	42.6±4.2
Collagen	48.9±3.2	39.4±5.3	43.2±10.2	42.1±7.9	33.7±3.7
EXP3174					
ADP	38.9±3.2	33.7±4.7	34.2±4.7	36.8±1.6	37.4±3.2
Collagen	59.5±3.2	74.7±1.1	65.3±8.4	71.1±3.2	64.2±4.7

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THE NON-PEPTIDE TACHYKININ NK_1- AND NK_2-RECEPTOR ANTAGONISTS SR 140333 AND SR 48968 PREVENT CASTOR-OIL INDUCED DIARRHEA IN RATS

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Abstract: Castrol-oil induced diarrhea in rats was potently prevented by compounds SR 140333 and SR 48968, the first a tachykinin NK_1- and the second a NK_2- receptor antagonist. SR 48968 was more effective and also reduced fecal water content.

Keywords: tachykinin-antagonists, diarrhea, castor-oil, rat

Introduction

We investigated the non-peptide tachykinin NK_1-, SR 140333 (S)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl-4-phenyl-1-azoniabicyclo[2,2,2]octane chloride, and NK_2-, SR 48968 (S)-N-methyl-N(4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichloro-phenyl)butyl) benzamide hydrochloride, receptor antagonists [1] for their ability to prevent diarrhea induced by castor-oil. In rat and guinea-pig intestinal functional preparations [1], these compounds have been shown to be selective and potent NK_1 and NK_2 receptor antagonists, with affinity in the subnanomolar range (SR 140333, pK_B,10.5; SR 48968, pA_2, 9.5).

Methods

Male CD rats, 220±15 g, individually housed in grid-floor cages with water *ad libitum* but fasted 2 h, received either SR 140333 or SR 48968 dissolved in bidistilled water (2 ml/kg) 30 min before castor-oil (1 ml/100 g rat, by gavage) or a charcoal meal. The feces discharged during 210 min were weighed either immediately (wet weight) or after drying to constant weight (dry weight) [2]. The incidence of diarrhea was scored as the number of rats presenting unformed stools. Gastrointestinal transit (GIT, percentage of small intestine traversed by the charcoal meal in 15 min) was assessed in 24 h fasted rats. Means were compared by completely randomized analysis of variance (ANOVA) followed by Duncan's test [3] for multiple comparisons.

Results

Controls given only vehicle or the antagonists (data not shown) passed virtually no fecal pellets during the test; SR 48968 and SR 140333 had no effect on GIT (% GIT, mean ± SE, n=7: vehicle 60±4; SR 48968 and SR 140333 20 μg/kg sc, 60±3 and 57±2), but prevented castor-induced oil diarrhea; SR 48968 was more effective and at the highest dose tested also prevented the increase in fecal water content (Table 1).

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Table 1
Effect of SR 48968 and SR 140333 on castor-oil diarrhea in rats

		Castor-oil	Incidence of diarrhea	Dry weight[a] of feces (g)	Wet/dry weight[a] of feces
Vehicle		–	0/14	0.15±0.04	1.79±0.03[b]
		+	13/14	3.30±0.23**	3.04±0.16**
SR 48968	0.2 μg/kg, sc	+	4/7	1.81±0.24**°°	2.87±0.25**
	2	+	3/7	1.66±0.34**°°	2.71±0.15**
	20	+	0/7	0.59±0.05°°	2.39±0.16**°°
SR 140333	2 μg/kg, sc	+	3/7	1.29±0.06**°°	2.93±0.17**
	20	+	4/7	1.00±0.10**°°	2.66±0.17**

a Data are mean ± SE; b from feces collected in the 2 h before treatment; *, **$p<0.05$, 0.01 compared with vehicle alone; °°$p<0.01$ compared with castor-oil plus vehicle (Duncan's test)

Discussion

SR 8968 antagonism of diarrhea induced by castor-oil occurred at doses within the range of those preventing fecal excretion stimulated by the selective tachykinin NK_2-receptor agonist [β-Ala^8]NKA(4–10) [2]; this is consistent with a similarly tachykinin-mediated antidiarrheal action.

Our study suggests that NK_1- and NK_2-receptors are activated by endogenously released neurokinins in castor-oil diarrhea in rats and that SR 48968 and SR 140333 may have potential as new antidiarrheal agents, free from the constipating action of opioids.

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LONG-LASTING TREATMENT WITH ADENOSINE RECEPTOR ANTAGONISTS: EFFECTS ON HYPOXIC TOLERANCE AND VASCULAR RESPONSIVENESS

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Abstract: In electrically driven myocardial preparations obtained from chronically methylxanthine-[aminophylline (APH) and 8-phenyltheophylline (8-PT)] or solvent(DMSO)-treated guinea pigs no differences were found in alteration of mechanical activity under hypoxia and reoxygenation. The vasoconstrictor effects observed after *in vitro* exposure of pulmonary arterial preparations (excised from either methylxanthine- or solvent-treated guinea pigs) to both noradrenaline and $PGF_{2\alpha}$ were also similar. In methylxanthine-treated vascular tissues, however, nitroglycerin and NO exerted more pronounced vasorelaxant effect than in specimens prepared from solvent-treated guinea pigs.

Keywords: methylxanthines, hypoxia, myocardium, pulmonary artery, NO, noradrenaline

Introduction

An association between coffee consumption and the risk of coronary heart disease has long been suggested (1, 2). In addition, it was found that other methylxanthines (theophylline, 8-phenyltheophylline) antagonized the infarct size limiting effect of preconditioning (4), but the findings in the literature are contradictory. In this study we analyzed the action of long-lasting methylxanthine-treatment on the alteration in mechanical activity of myocardium under hypoxia and reoxygenation, as well as on responsiveness of vascular smooth muscle to different vasoconstrictor and vasodilator substances.

Methods

Female guinea pigs were treated once daily with APH (25 mg/kg i.p.) or 8-PT (2.5 mg/kg i.p.) and solvent (DMSO) for 10 consecutive days. Methylxanthine treatment were discontinued 48 h before the guinea pigs were killed and hearts, as well as pulmonary arteries were excised. Then left atria and circular segments from the proximal part of the main pulmonary arteries were prepared. The tissues were mounted in an organ chamber containing normal Krebs solution saturated with 95% O_2 and 5% CO_2 (under hypoxia: 95% N_2 and 5% CO_2). The atrial preparations were stimulated electrically by applying squarewave pulses of 1 ms duration. The frequency of stimulation was 3 Hz and its intensity corresponded to twice the excitation threshold. Mechanical responses were displayed on oscilloscope and recorded on polygraph or chart-recorder.

Results

In electrically driven left atrial myocardium, obtained from APH- or 8-PT-treated guinea pigs, the hypoxia-induced decrease of mechanical activity and the rate of recovery under reoxygenation did not change compared to those of controls (not shown).

In pulmonary arteries noradrenaline and $PGF_{2\alpha}$ caused a concentration-dependent contraction. The pA_{50}'s (negative log molar agonist concentration producing a half-maximal increase in tension) were 6.06±0.06 (n=6) and 5.11±0.10 (n=4), respectively (solvent-treated

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controls). No significant differences ($p>0.05$) were found in mechanical responses to noradrenaline in pulmonary arteries removed from APH-treated (pA_{50}: 6.35±0.12; n=6) and 8-PT-treated animals (pA_{50}: 6.20±0.08; n=6). Similar findings were found for $PGF_{2\alpha}$ induced contraction (pA_{50}'s 5.08±0.05 (n=4) and 5.19±0.08 (n=4) for APH and 8-PT-treated specimens, respectively).

In vascular tissues (precontracted with 1 μM noradrenaline) prepared from control guinea pigs nitroglycerin (an exogenous NO donor; 1 pM – 1 μM; n=16) and NO (1 nM – 1 μM; n=15) exerted a concentration-related relaxation with $-\log EC_{50}$'s (EC_{50}: a concentration inducing a 50% decrease of noradrenaline-induced precontraction) of 9.43±0.09 and 6.63±0.10, respectively. Chronic treatment by APH of guinea pigs significantly ($p<0.01$) increased the sensitivity of pulmonary arteries to NO ($-\log EC_{50}$: 7.58±0.26) without influencing the effectiveness of nitroglycerin ($-\log EC_{50}$: 9.45±0.23). In the case of specimens removed from 8-PT-treated animals the $-\log EC_{50}$'s were 9.81±0.04 for nitroglycerin and 7.49±0.14 for NO ($p<0.01$ for solvent- vs. 8-PT-treated tissues).

Discussion

The results presented here clearly indicate that in vivo pretreatment of guinea pigs has no detrimental action either on the susceptibility of myocardium to oxygen deprivation or reoxygenation or on the vascular responsiveness to endogenous vasoactive substances. It was previously observed that APH is capable of significantly preventing the hypoxia-induced suppression of functional activity of the electrically driven myocardium (3). This effect of APH can be due to its ability to antagonize the cardiodepressive action of endogenous adenosine released under hypoxic circumstances. Because A_1 adenosine receptors have usually been upregulated under long-lasting methylxanthine treatment (5), in our experiments on atrial myocardium, theoretically, a more pronounced decrease of contractile tension would have been expected during hypoxia. The mechanism of this contradictory finding remains to be elucidated.

In addition, methylxanthine treatment does not increase the vasopressor responses to noradrenaline or $PGF_{2\alpha}$. Chronic exposure to both APH and 8-PT, however, enhances the susceptibility of vascular tissue to NO displaying a more prominent vasorelaxation. These experimental data do not support the theory according of which chronic methylxanthine treatment aggravates the ischemic damage of the heart.

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LONGEVITY TREATMENT WITH (–)DEPRENYL IN FEMALE RATS: EFFECT ON COPULATORY ACTIVITY AND LIFESPAN

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Abstract: Six months old ovariectomized female rats (n=9) were treated with (–)deprenyl in a dose of 0.25 mg/kg s.c. three times a week, and (n=9) with physiologic saline (0.1 ml/100 g) till decay. It was found that control females (n=9) decayed within the age of fifteen months while the members of the (–)deprenyl treated group were all alive at that age. Moreover three (–)deprenyl treated female rats reached 36 months of age. Sexual activity was quite absent in both groups. The data suggests that (–)deprenyl extended the lifespan of female rats only in total absence of gonadal hormones and sexual activity.

Keywords: (–)deprenyl, female rat, lifespan, copulatory activity

Introduction

Previously it was found that a close correlationship exists between copulatory activity and lifespan in male rats. Longevity treatment with (–)deprenyl in sexually inactive male rats extended their lifespan and also increased their copulatory activity (1, 2). Experiments were conducted whether this observation could be detected in female rats as well.

Methods

A group of 18 female Wistar rats was made sexually inactive by ovariectomy without gonadal hormone substitution. Ovariectomy was made at five months of age. At the age of six months nine females were treated with (–)deprenyl in a dose of 0.25 mg/kg s.c. three times a week and the other nine females with physiologic saline (0.1 ml/100 g) till death. Sexual activity in both groups was checked once a week with a sexually vigorous male rat.

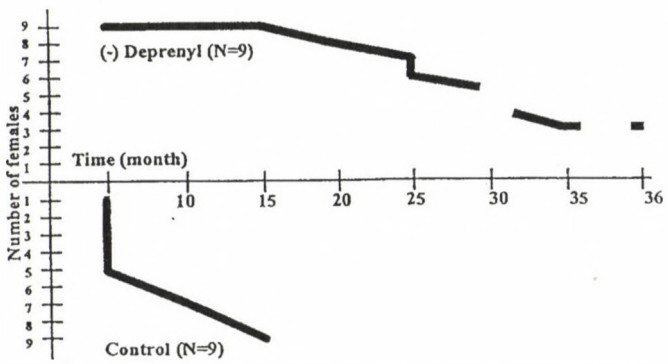

Fig. 1. Effect of (–)deprenyl treatment on lifespan in ovariectomized sexually inactive female rats (n=9) in comparison to the saline treated ones (n=9)

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Result and discussion

Figure 1 shows the death-rate of both groups. Saline treated females decayed much more rapidly than (–)deprenyl treated ones. Each control female died before fifteen months of age, while the first (–)deprenyl treated female decayed at 20 months of age and three females were still alive at the age of 36 months. The copulatory activity was quite absent in both groups. In conclusion (–)deprenyl extended the lifespan also in female rats in the complete absence of ovarian hormones and sexual activity. These data support the inhibitory theory of the female rat's sexual behavior (3) and suggests the elementary role of the ovarian steroids in the observed effect.

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Z1046: BIOCHEMICAL CHARACTERIZATION OF ITS DOPAMINERGIC ACTIVITY

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Abstract: Affinity of Z1046 for dopamine receptor subtypes, its ability to modulate D1- and D5-mediated AC stimulation and D1-induced cAMP accumulation were evaluated. On D1-like receptors Z1046 and fenoldopam (fen) showed a similar high affinity, being more potent than DP-5,6-ADTN and 5,6-ADTN. For the D2-like receptors, the affinity rank orders were: D2: Z1046 ≥DP-5,6-ADTN>fen=5,6-ADTN; D3: Z1046>DP-5,6-ADTN>fen=5,6-ADTN; D4: Z1046=DP-5,6-ADTN>fen=5,6-ADTN. In AC studies the rank order was: Z1046=fen>DP-5,6-ADTN>5,6-ADTN. Z1046 was more efficient than fen in stimulating cAMP accumulation. These results make Z1046 an innovative agent combining D1-like and D2-like activities.

Keywords: Z1046, dopamine receptors affinity, adenylyl cyclase, cAMP accumulation

Introduction

Peripheral dopamine (DA) receptor agonists have been shown to possess beneficial cardiovascular and renal effects (4). Molecular biology techniques have defined five DA receptors. D1 and D5 receptors form the D1-like family and are linked to the stimulation of adenylyl cyclase (AC) activity (3). D2, D3 and D4 receptors belong to the D2-like family. Z1046 is a new potent and specific peripheral dopaminergic agent discovered in our laboratories (5). The affinity of Z1046, in comparison with fen, 5,6-ADTN and DP-5,6- ADTN, for the five DA receptors was determined using membranes from Chinese hamster ovary (CHO)·transfected cell lines. The ability of Z1046 to stimulate D1-like receptor-mediated AC activity in CHO cells was also evaluated and compared to its ability to induce cAMP accumulation in a porcine kidney cell line (LLC-PK1), a more physiological model of peripheral D1-like receptors (1).

Methods

BINDING ASSAY: For saturation experiments ^{3}[H]-SCH23390 was used as radioligand for D1-like receptors and ^{3}[H]-Spiperone for D2-like. Bmax and Kd were calculated using "LIGAND" program. *cAMP ACCUMULATION (IN LLC-PK1 CELLS):* Whole cell cAMP assay was performed in the presence of 500 μM IBMX. Cell monolayers grown at confluency in 24-well plates (8E5 cells/well) were incubated for 20 min at 37 °C. cAMP levels were quantified using a commercially available EIA kit. *ADENYLYL CYCLASE ASSAY:* The conversion of ^{14}[C]-ATP to ^{14}[C]-cAMP was determined as described by Johnson and Salomon (2).

Results and discussion

Z1046 and fen show a similar high affinity for D1-like receptors (Table 1) and they both are more potent than the two 5,6-OH-aminotetralines. Among tested compounds Z1046 possesses the highest affinities for the D2-like receptors.

Considering the D2-like receptors, Z1046 shows the highest affinity for D3 subtype. Our results allow few structure-activity considerations: N,N-di-n-propyl substitution of 5,6-ADTN, which is itself inactive, results in a dramatic increment in D1-like and D2-like activity. The substitution of a propyl group of DP-5,6-ADTN with a longer arilalchil chain, to originate Z1046, confers a higher D1-like and D2-like affinity and potency. Z1046 potently stimulates AC

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activity in membranes from D1 and D5 transfected CHO cells (Table 2). Z1046 is also able to induce cAMP accumulation in LLC-PK1 cells with a potency greater than that of fen (data not shown).

Table 1

Inhibition constants (Ki, nM) for ligands binding to DA receptors expressed in transfected CHO cells

Compounds	D1h	D5h	D2h	D3h	D4h
Dopamine	1199±137	312±37	4970±870	58±2.8	123±31
Z1046	25±9.3	12±3.6	9.9±3.9	0.7±0.1	1.9±0.6
5,6-ADTN	9796±2796	2152±345	3357±396	344±57	4404±407
DP-5,6-ADTN	170±22	37±6.2	27±1.4	3.1±1.4	1.1±0.6
Fenoldopam	27±2.3	13±1.0	1971±500	311±73	591±125

Values are mean±S.E. of 3–5 separate experiments carried out in triplicate

Table 2

Effect of dopaminergic agents on AC activity in membranes from D1h and D5h transfected CHO cells

Compounds	D1h receptor			D5h receptor		
	EC50±S.E.	ECR	Efficacy	EC50±S.E.	ECR	Efficacy
Dopamine	855 ± 89	1.00	1.00	350 ± 64	1.00	1.00
Z1046	64.7 ± 16	0.09	0.96	66.8 ± 38	0.20	0.79
5,6-ADTN	3337 ± 150	5.67	1.21	4041 ± 121	2.81	0.68
DP-5,6-ADTN	104 ± 13	0.19	0.94	196 ± 58	0.12	0.74
Fenoldopam	52 ± 23	0.07	0.78	15.3 ± 39	0.04	0.65

Values are mean±S.E. of 3–5 separate experiments carried out in triplicate ECR=Equipotent concentration ratio vs. Dopamine

In conclusion our results demonstrate that Z1046 is a dopaminergic compound endowed with both D1-like and D2-like high affinity. Both in membranes from transfected CHO cells and in whole LLC-PK1 cells Z1046 possesses a high potency in stimulating either AC activity or cAMP accumulation.

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EFFECT OF ALMOKALANT A SPECIFIC INHIBITOR OF I_{Kr} ON MYOCARDIAL ISCHAEMIA-REPERFUSION INDUCED ARRHYTMIAS IN RABBITS

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Abstract: The antiarrhythmic effect of almokalant, a new type III antiarrhythmic agent, was examined by occluding and releasing the left circumflex coronary artery for 10 min, respectively, in open-chest, pentobarbital-anaesthetized albino rabbits. Almokalant pretreatment increased the number of animals developing no arrhythmias (5/9 vs. 1/12 in controls), and decreased the incidence of ventricular fibrillation (1/9 vs. 9/12) during reperfusion. According to our results almokalant can protect the heart against arrhythmias induced by ischaemia and reperfusion.

Keywords: almokalant, antiarrhythmic effect, ischaemia-reperfusion, rabbit

Introduction

Almokalant, a new type III antiarrhythmic compound (3), blocks mainly the rapid component of the voltage dependent delayed rectifier potassium current (I_{Kr}). Although this agent has undergone extensive observation, there are relatively few data gained from in vivo experiments about its antiarrhythmic effectiveness. Thus the aim of present experiments was to examine the effect of almokalant on arrhythmias induced by coronary artery occlusion and reperfusion in rabbits.

Methods

New Zealand White rabbits (n=24) weighing 1.5 to 2.9 kg from either sex were anaesthetized with pentobarbital sodium (30 mg/kg, i.v.). Catheters were implanted into the right carotid artery and into the marginal vein of the left ear for recording arterial blood pressure and infusion of drugs. After tracheotomy, the animals were mechanically ventilated with a Harvard respirator. Having performed left thoracotomy and pericardiotomy, the first branch of the left circumflex coronary artery was ligated just under its origin. Saline or almokalant (250 nmol/kg) was administered intravenously for 10 min in continuous infusion (infusion volume 2 ml) right before the occlusion. Coronary artery was occluded and released for 10 min, respectively. ECG was registered during the experiments. At the end of reperfusion (or after three min ventricular fibrillation) heparin sodium (500 U.I./kg, i.v.) was administered and the rabbits were killed with an overdose of pentobarbital. The heart was cut out from the chest in order to determine the size of the ischaemic zone. After occluding the coronary branch, the heart was perfused retrogradely with saline and ethanol. The non-denatured area (area at risk) was excised and its extent was expressed in percentage of the weight of ventricles. If the area at risk was less than 16% or was bigger than 32%, then the animal was excluded from the final evaluation. Data are presented as mean±SEM and *n* indicates the number of observations. Student's paired *t* test and χ^2 test with Yates' correction were applied for statistical analysis. Differences were considered statistically significant when $p<0.05$.

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Results

Almokalant pretreatment in a dose of 250 nmol/kg modestly reduced the heart rate (278±7 vs. 269±9 min^{-1}) and lengthened both the QT interval (153±3 vs. 170±7 ms) and the rate corrected QT interval (329±4 vs. 357±12 ms). This dose of almokalant significantly increased the proportion of animals developing no arrhythmias during reperfusion (5/9 vs. 1/12 in control group), and significantly decreased the incidence of ventricular fibrillation (1/9 vs. 9/12). Table 1 shows the incidence of arrhythmias in the control group and in the treated group during occlusion and reperfusion.

Table 1

Effect of almokalant (250 nmol/kg, i.v.) on the incidence of arrhythmias during coronary artery occlusion and reperfusion in anaesthetised rabbits

	Occlusion		Reperfusion	
	Control	Almokalant	Control	Almokalant
n	15	9	12	9
no arrhythmia	47%	67%	8%	56%*
ES	47%	33%	58%	44%
VT	0%	0%	42%	0%
VF	27%	0%	75%	11%*
died	20%	0%	58%	11%

n = number of animals; no arrhythmia = animals developing no arrhythmias; ES = ventricular extrasystoles; VT = ventricular tachycardia; VF = ventricular fibrillation; died = died because of VF; * $p<0.05$ vs. control

Discussion

As a result of delayed rectifier potassium channel blockade, almokalant lengthens the action potential duration and the refractory period of atrial and ventricular muscle cells (2). Previous clinical trials (1, 4) showed that almokalant lengthened the QT (and QTc) interval dose dependently and exhibited dose dependent antiarrhythmic effect in patients with ventricular premature contractions or with supraventricular reciprocating tachycardias. In the present study, almokalant also widened the QT and QTc interval on the ECG and parallel to this action, protected the heart against arrhythmias induced by coronary artery occlusion and reperfusion in anaesthetized rabbits.

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Z1046: A NEW SPECIFIC PERIPHERAL DOPAMINERGIC COMPOUND

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Abstract: It is well established that peripheral dopamine receptors activation evokes vasodilation and neurohormonal modulation. Z1046 is a potent mixed dopaminergic agonist and is highly selective over adrenergic and serotoninergic (5-HT_2) activities. In contrast, dopamine had agonist activities on all adrenergic receptors while it is known that dopexamine has a β_2 agonist activity and is an inhibitor of the neuronal uptake. As far as fenoldopam is concerned, selective stimulation of D_1-like receptors leads to an increase of renin release.

In conclusion, Z1046 is a potent and specific drug for dopamine receptors. This profile makes Z1046 different from other dopaminergic agents.

Keywords: Z1046, dopamine receptors, isolated organs

Introduction

It is well established that peripheral dopamine receptors evoke different cardiovascular effects. These effects occur through selective activation of D_1-like and D_2-like receptors. The D_1-like receptors mediate direct peripheral vasodilatation, besides diuresis and natriuresis. The D_2-like receptors mediate indirect peripheral vasodilatation by inhibition of norepinephrine release. Moreover, D_2-like-mediated inhibition of aldosterone release, could contribute to enhance natriuretic effect. Until now however, no specific mixed peripheral dopaminergic compound is available. In fact dopamine acts also on adrenergic receptors, dopexamine has a β_2 agonist activity and is an inhibitor of neuronal uptake (3) and fenoldopam, due to its selective D_1-like receptors activation, leads to the increase of renin release (2). Aim of the study was then to compare the activity of Z1046, a new dopaminergic drug (1), to that of dopamine on different peripheral receptors in isolated organs.

Methods

New Zealand male rabbits were sacrificed and the splenic artery, the central ear artery and the thoracic aorta were removed. Hartley male guinea-pigs were killed and the right atrium and the trachea were removed. The tissues were suspended in isolated organ baths containing heated and oxygenated Krebs-Henseleit solution added with EDTA (10 μM) in order to prevent catecholamine oxidation. The vasorelaxant D_1-like receptor activity was studied in superfused splenic arteries which were previously medicated with indomethacin (5 μM), 1-propranolol (1 μM) and phenoxybenzamine (3 μM) in order to prevent prostanoids formation and to block β and α receptors, respectively. Splenic artery rings were precontracted with U46619 (0.03 μM), a TxA_2 mimetic, and added with a PDE inhibitor, IBMX (3 μM), in order to amplify the relaxant response D_1-like receptor mediated by the inhibition of cAMP breakdown. Then, we examined the vasorelaxant effect of the compounds. The D_2-like receptor mediated inhibition of the electrically-induced norepinephrine release was studied in superfused ear artery. The superfusion fluid was added with desipramine (0.1 μM) and corticosterone (30 μM) in order to prevent neuronal and extraneuronal catecholamine uptake. Field square wave pulses of 10 Hz, 100 mA, 1 ms every 5 minutes, evoked norepinephrine mediated rings contractions. Then, we examined the inhibitory effect of the compounds.

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The α_1 and the 5-HT_2-like receptor activities were performed in rabbit aorta testing concentration-response curves of phenylephrine or serotonin (5-HT) before and after Z1046. The chronotropic β_1 receptor activity was performed in the guinea pig right atrium testing isoprenaline, Z1046 and dopamine. The α_2 receptor mediated inhibition of the electrically-induced heart rate increase was evaluated in the guinea pig right atrium. The inhibitory activity of clonidine, Z1046 and dopamine was tested on field square wave pulses of 2 Hz, 40 V, 1.5 ms every 6 minutes. The bronchorelaxant β_2 receptor activity was performed in guinea pig trachea precontracted with methacholine (0.1 μM). Isoprenaline, dopamine and Z1046 were cumulatively administered and their relaxant effect was recorded.

Results

The results are summarized in Table 1.

Z1046 is a potent mixed D_1-like/D_2-like receptors agonist on both rabbit splenic artery and ear artery, respectively. As a D_1-like agonist it is slightly more potent than dopamine and dopexamine; fenoldopam was about 40-fold more potent than Z1046. As a D_2-like agonist Z1046 is significantly more potent than either dopamine or dopexamine. The effects of the compounds were all significantly shifted by the selective antagonists (SCH23390 and domperidone, respectively).

Furthermore, Z1046, differently from dopamine, is a mild α_1 antagonist (pA_2=6.6±0.11) and, like dopamine, has an α_2 agonist activity. Finally, Z1046 lacks of any β_1, β_2 and 5-HT_2 activities up to 30 μM.

Table 1

Z1046 activity on different receptors in comparison with reference compounds

Compounds	D_1-like	D_2-like	α_1-adren.	α_2-adren.	β_1-adren	β_2-adren	5-HT_2
Z1046	6.8±0.08	9.8±0.07	antagonist	6.7±0.14	n.a.	n.a.	n.a.
Dopamine	6.4±0.06	7.8±0.04	4.3±0.05	6.4±0.17	4.8±0.04	4.5±0.07	–
Fenoldopam	8.4±0.12	n.a.	–	–	–	–	–
Dopexamine	6.1±0.09	6.4±0.11	–	–	–	–	–
Phenylephrine	–	–	6.5±0.12	–	–	–	–
Clonidine	–	–	–	7.6±0.15	–	–	–
Isoprenaline	–	–	–	–	8.5±0.07	8.1±0.05	–
5-HT	–	–	–	–	–	–	6.9±0.04

Values are pD_2 ($-Log\ EC_{50}$) ± S.E. of 3–5 separate experiments, n.a. = not active

Discussion

Z1046 is a potent and specific dopaminergic receptor agonist lacking significant adrenergic and serotoninergic (5-HT_2) agonist activities. This profile makes Z1046 different from other dopaminergic agents and useful for the treatment of cardiovascular diseases such as congestive heart failure and hypertension.

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PERIPHERAL SEROTONERGIC MECHANISMS IN THE CARDIOVASCULAR SYSTEM OF EPIDERMOID LUNG CANCER PATIENTS

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Abstract: The aim of the work was to evaluate the peripheral serotonergic mechanisms in patients with epidermoid lung cancer. The study was performed on lung cancer patients diagnosed on the basis of histopathological findings. The subjects were treated by radiotherapy. Whole blood 5HT in patients with epidermoid lung cancer was increased, whereas uptake of ^{3}H-5HT by platelets was diminished in these patients when compared to control group. Radiotherapy caused a lowering of serotonin in blood to levels observed in healthy subjects. Platelet aggregation induced by ADP was diminished in patients with epidermoid lung cancer, however radiotherapy resulted in an increased sensitivity of platelets to ADP (an enhanced aggregation). On the other hand, serotonergic amplification of ADP-induced platelet aggregation was higher in these patients. This effect was inhibited by radiotherapy. On the basis of our results, we may suggest that peripheral serotonergic mechanisms play an important role in pathogenesis and course of epidermoid lung cancer in humans.

Keywords: serotonin, epidermoid lung cancer, platelets

Introduction

Practically, since their discovery, the role of biogenic amines like noradrenaline or serotonin is in the center of interest of the pathogenesis and monitoring of cancer. Increased blood serotonin levels were reported for the first time in carcinoid. In the last years increased levels of this amine were found in melanoma and ovary tumors. It has been demonstrated that cancer tissue can synthesize 5HT, however its role in the course of cancer remains still unclear (1). The aim of the study was to evaluate peripheral serotonergic mechanisms in patients with epidermoid lung cancer.

Materials and methods

Patients with histopatologically proven epidermoid lung cancer were included in the study. Diagnosis was performed using specimens obtained during bronchoscopy. The studies were performed on 23 patients aged 45–68 years. The patients were treated by means of megavolt therapy, 60Co and X rays 9 MeV from linear accelerator Neptun 100. Radiotherapy was delivered as a conventional fractionation 2 Gy/g/die. Blood was drawn from the antecubital vein into 3.8% sodium citrate (volume ratio 9:1). Whole blood serotonin was estimated as described by Drumond and Gordon (2). ^{3}H-5HT uptake was measured according to the method of Gordon and Olverman (3). Platelet aggregation was studied according to Born (4). Results were expressed as means ± SEM and were evaluated using Student's *t*-test.

Results and discussion

In our study we demonstrated a significantly higher whole blood serotonin in patients with epidermoid lung cancer when compared to the control group (Table 1). Tumor through its microcirculation network, secretes many biologically active substances, i.e. serotonin synthesized in its cells, into the bloodstream. Radiotherapy (50 Gy) resulted in a lowering of whole blood serotonin to the levels comparable to those of the healthy volunteers.

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Physiologically, SHT is taken up and stored in platelets. It has been observed that ^{3}H-5HT uptake by platelets obtained from patients was significantly decreased and remained unaltered during radiotherapy. Platelet aggregation induced by ADP was significantly diminished in patients with epidermoid lung cancer. Moreover, radiotherapy resulted in an enhancement in platelet sensitivity to ADP (increased aggregation). Some authors have suggested that this phenomenon may contribute to metastasis formation (5). It should be stressed that serotonergic amplification of ADP-induced platelet aggregation was significantly increased in our patients (165+64.6%) and subsequent radiotherapy inhibited this interesting effect. The result of our studies may suggest that peripheral serotonergic mechanisms play a role not only in the pathogenesis and course of epidermoid lung cancer in humans, but also may contribute to the drawbacks of radiotherapy.

Table 1

Serotonin of whole blood, uptake of 3H-SHT by trombocytes and platelets aggregation in patients with epidermoid lung cancer

	n	Serotonin in whole blood (ng/ml)	Uptake of serotonin (pmol/10^9 platelets /min)	Platelet aggregation (cm)		Percentage of intensification of aggregation by serotonin
				ADP (2 μM)	ADP (2 μM) 5-HT (0.1μM)	
Control	6	136.7±7.9	73.8±10.2	6.4±5.2	8.5±7.4	32.8±34.3
Patients before radiotherapy	15	201.5±43.4 *	59.0±10.3 *	4.1±2.2 *	10.9±4.7	165.9±64.6 ***
10 Gy	8	150.0±20.0	57.7±11.8	10.2±6.5 ##	15.6±8.1	52.9±15.7 ###
20 Gy	8	151.5±23.8	53.2±5.7	7.4±5.4 #	11.1±6.1	50.0±9.4 ###
50 Gy	8	135.0±19.1 #	57.4±6.6	8.6±5.9 #	11.9±8.0	38.4±21.4 ###

Each value represents the mean ± SD. Significance of the difference from the control: *$p<0.05$, ***$p<0.001$, in comparison with the group of patients before radiotherapy: # $p<0.05$, ## $p<0.01$, ### $p<0.001$

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EFFECT OF HYPERGLYCEMIA ON THE INTESTINAL ELIMINATION OF p-NITROPHENOL IN THE RAT

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Abstract: Effect of hyperglycemia on the intestinal elimination of p-nitrophenol has been investigated in rats. Hyperglycemia was produced by a continous i.v. infusion of glucose, p-nitrophenol was used as a model compound for the investigation of intestinal metabolism and excretion of drugs. Intestinal conjugation of p-nitrophenol with glucuronic acid did not change significantly in hyperglycemic rats, however, formation of sulfoconjugate of p-nitrophenol was enhanced by hyperglycemia. Sum of metabolites (p-nitrophenol glucuronide and sulfate) appeared in the intestinal lumen in hyperglycemic rats was similar to the total luminal appearance of these metabolites of control rats.

Keywords: hyperglycemia, intestinal elimination, p-nitrophenol

Introduction

The importance of liver in biotransformation and excretion of drugs is well known, however, newer date show that intestinal tract may also influence the elimination of xenobiotics [2, 3, 4]. Experimental diabetes and hyperglycemia can alter the hepatic metabolism and biliary excretion of drugs [1, 5], however, the influence of hyperglycemia on the intestinal elimination of xenobiotics is not known. The aim of present experiments was to study the effect of hyperglycemia on the intestinal metabolism and excretion of p-nitrophenol.

Methods

Hyperglycemia was maintained by a continuous i.v. infusion of glucose after the administration of a priming dose of glucose enabling a high blood glucose level (20–40 mM) to be reached right at the start of glucose infusion. Rats were unesthetized with urethane (1.2 g/kg i.p.), a jejunal loop was cannulated. Perfusion through the lumen of small intestine with isotonic medium containing 100 μM concentration of p-nitrophenol (PNP) was carried out at a rate of 13 ml/min in a recirculation mode. Samples were obtained from the perfusion medium coming out from the jejunal loop. PNP and its metabolites (PNP-glucuronide: PNP-G, PNP-sulfate: PNP-S) were separated and quantitated by reverse-phase HPLC with absorbance detection at 280 and 305 nm, concentrations were determined from the standard calibration curve using external standards. Data are expressed as the mean ± SEM of 4–6 rats. Significant differences were calculated by Student's *t*-test (*:$p<0.05$).

Results

Luminal appearance of PNP-G and PNP-S in control and hyperglycemic rats is shown in Figure 1. No significant differences were found in luminal appearances of PNP-G of control and hyperglycemic, rats (except the values measured at 15 min perfusion time). Luminal appearance of PNP-S was definitely lower than that of PNP-G, however it was increased significantly by hyperglycemia (Fig. 1). The sum of metabolites (PNP-G + PNP-S) measured in the jejunal loop was practically unchanged in hyperglycemic rats (Fig. 2).

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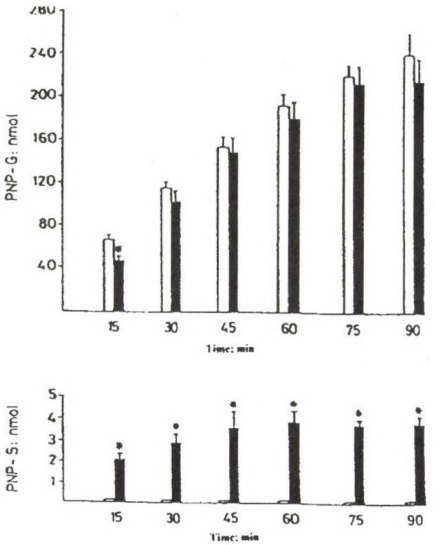

Fig. 1. Luminal appearance of PNP-G and PNP-S in control (□) and hyper-glycemic (■) rats

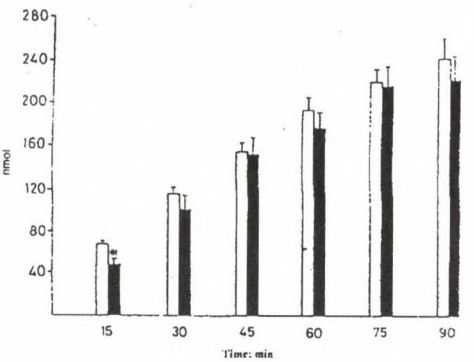

Fig. 2. Sum of metabolites (PNP-G + PNP-S) in the jejunal loop in control (□) and hyperglycemic (■) rats

Discussion

Formation and excretion of the predominant metabolite of PNP (PNP-G) in the jejunal loop was not influenced by hyperglycemia, however, the minor metabolite (PNP-S) was significantly enhanced in hyperglycemic rats. On the basis of these changes, the total amount of metabolites showed a decreasing tendency, but the change did not reach the significant level. Hepatic conjugation and excretion of drugs can be depressed by hyperglycemia and diabetes (1, 5), which shows sharp contrast between the ability of the small intestine and liver to metabolize xenobiotics.

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DO SODIUM NITROPRUSSIDE AND L-NAME AFFECT PYROGEN FEVER IN RABBITS?

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Abstract: Thermoregulatory responses after treatment with nitric oxide (NO) donor, sodium nitroprusside (SNP-3 mg/kg/h), or NO synthase inhibitor, N^G-nitro-L-arginine methylester (L-NAME-100 mg/kg) were investigated in febrile rabbits (lipopolysaccharide *E. coli*-1 mcg/kg). Pretreatment with SNP attenuated pyrogen fever as well as metabolic rate. L-NAME also inhibited postpyrogen increases in metabolism; however, this effect did not lead to antipyresis.

Keywords: sodium nitroprusside, L-NAME, pyrogen fever, rabbit

Introduction

There is evidence that NO is involved in the control of thermoregulatory processes in normothermia and fever. In normal animals it has been demonstrated that NO donors increase prostanoids production (3). On the other hand, it may act as one of the endogenous antipyretics in pyrogen fever (2). With respect to the above-mentioned data the purpose of this study was to investigate whether changes in the endogenous NO system – mimicked by systemic application of NO donor, SNP, or HO synthase inhibitor, L-NAME – affect the effector thermoregulatory mechanisms in pyrogen fever.

Methods

The experiments were performed on mixed breed rabbits of both sexes (2.5–3.0 kg) at an ambient temperature of 20.0±1.0 °C. The effects of SNP (3 mg/kg/h) or L-NAME (100 mg/kg, i.v.) were tested on the model of pyrogen fever (1 mcg/kg of *E. coli* lipopolysaccharide, LPS, i.v.). The infusion of SNP or L-NAME started 15 min before LPS treatment and lasted 2 or 1.25 hours, respectively. The following measurements and calculations were made: rectal (Tre) and ear skin (Te) temperatures, metabolic rate (M), respiratory evaporative heat loss (Eres).

Results

Sodium nitroprusside reduced significantly pyrogen fever during the time course of infusion (Fig. 1A). This antipyretic effect was accompanied by inhibition of metabolic rate and reduction of postpyrogen falls in Te (Figs 1C and 1B). On the other hand, L-NAME did not affect pyrogen fever (Fig. 1A). However, this compound inhibited significantly the metabolic rate (Fig. 1C).

Discussion

This study showed that SNP produced antipyresis, when used in febrile rabbits. This kind of action was also noticed after treatment with other NO donors (2). On the other hand, inhibition of NO synthesis by L-NAME did not affect pyrogen fever. Surprisingly, there was a significant inhibition of postpyrogen metabolic rate. With regard to the above-presented data it may be concluded that general systemic NO synthase manipulation produces thermoregulatory responses, but in a non-coordinated manner. Similar observations were made when NO donors or NO synthase inhibitors were examined in normothermic rabbits (1).

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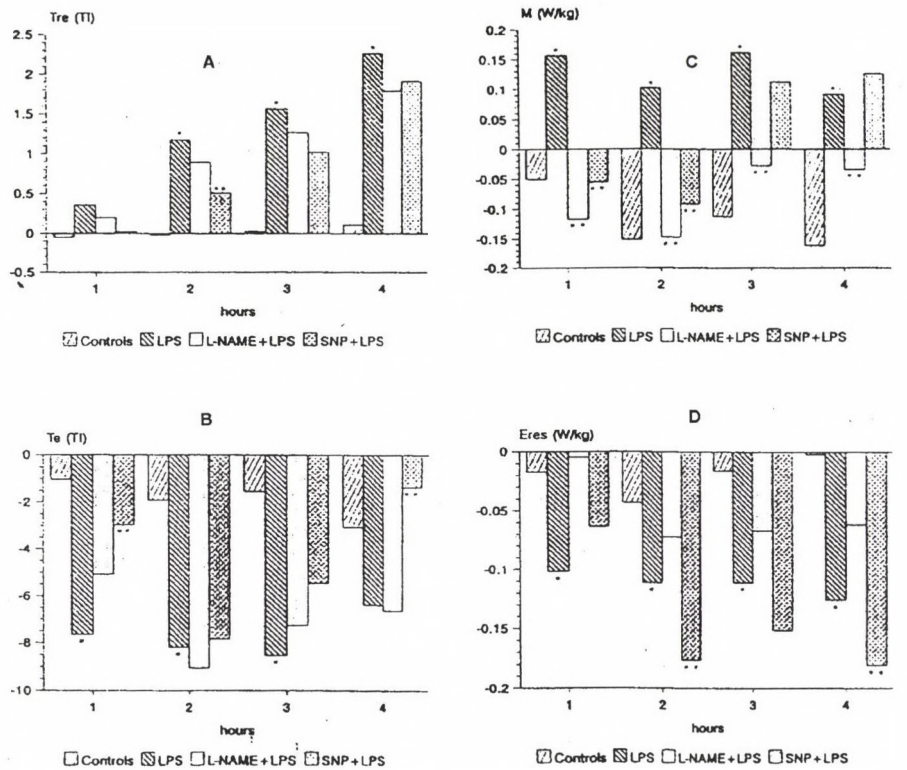

Fig. 1. The effect of L-NAME and SNP on body (A) or ear skin (B) temperatures, metabolic rate (C) and evaporative heat loss (D) in febrile rabbits. $p \leq 0.05$ as compared with saline control (▨) or LPS group (▧)

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PHARMACOLOGICAL ANALYSIS OF NERVES SUPPLYING THE OESOPHAGUS

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Abstract: Morphological studies indicate that cholinergic, peptidergic and "nitrergic" (nitric oxide releasing) nerves supply the oesophageal muscle. In the present in vitro study, intramural nerves of guinea-pig and human oesophagus were activated by means of the ganglion stimulating drug nicotine and electrical field stimulation (EFS) in organ bath experiments.

In general, stimulation-induced primary contractions were diminished by atropine, while non-adrenergic, non-cholinergic (NANC) relaxations and after-contractions were inhibited by the NO-synthase inhibitor N^G-nitro-L-arginine (L-NNA). Thus we obtained pharmacological evidence for cholinergic muscarinic, as well as nitric oxide mediated mechanisms in the functional innervation of the oesophageal muscle.

Keywords: human oesophagus, guinea-pig oesophagus, cholinergic nerves, nitrergic innervation

Introduction

Ample morphological data indicate the presence of cholinergic, peptidergic (4) and NO-releasing nerves in the regulation of the motility of human and guinea-pig oesophageal body (1). Much less functional data are available concerning the types of nerve supplying the oesophageal muscle; most studies available used the opossum oesophagus as test object (3, 5). In rodents, striated muscle predominates in the whole length of the oesophagus, whereas in man and opossum, the lower part is composed mainly of smooth muscle (2). In the present study, intramural nerves of the guinea-pig and human oesophagus (the latter obtained from uninvolved surgically resected tissues from patients who underwent resections) were stimulated electrically or by means of nicotine and an attempt has been made to characterize the types of nerve mediating the motor responses by selective acting drugs.

Methods

Specimens were obtained from guinea-pigs of 400–1000 g, killed by a blow to the head and bled. Segments of human oesophagus derived from operation specimens. Longitudinally-oriented segments of whole oesophagus of the guinea-pig, as well as mucosa-free longitudinal and circular muscle strips of human oesophagus were suspended in oxygenated Krebs solution at 37 °C. Electrical field stimulation (EFS; 80 V, 0.2 ms impulse width) was delivered through platinum electrodes. Motor responses were recorded on compensographic ink writers either isometrically (field stimulated guinea-pig oesophagus) or isotonically (load, 6 mN).

Results

Guinea-pig preparations. Single shocks of EFS caused a rapid twitch followed by a slower tonic contraction. Trains of stimuli (1–5 Hz for 20 s) caused a tonic contraction with superimposed twitches. The anticholinergic drug (muscarine receptor blocker) atropine (10^{-6} M) strongly reduced (Fig. 1), whereas the cholinesterase inhibitor physostigmine (5×10^{-8} M) enhanced the

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tonic, but not the phasic contractions. The neuromuscular blocking drug pipecuronium (10^{-6} M) failed to have any conclusive effect. Nicotine (10^{-5} M) caused a biphasic response composed of a phasic and a subsequent tonic component (similar to the pattern observed to single impulses), both phases were inhibited by atropine (10^{-6} M).

Human preparations. EFS at various frequencies caused primary contractions followed by after-contraction either in the circular or in the longitudinal muscle. The contractions were comparable in time course to the tonic component of the responses seen in the guinea-pig. All primary contractions, but not after-contractions were abolished by atropine (10^{-6} M) (Fig. 2). Atropine unmasked a primary relaxation in some preparations (Fig. 2). This and the after-contraction were suppressed by L-NNA (3×10^{-5} M) (Fig. 2). Nicotine (2×10^{-5} M) caused relaxation even in the absence of atropine; this was blocked by L-NNA (3×10^{-5} M).

Discussion

These data show the presence of cholinergic muscarinic (guinea-pig), and cholinergic muscarinic excitatory as well as "nitrergic" inhibitory mechanisms (human) in the oesophageal muscle. Both mechanisms have also been detected in the opossum oesophagus (2, 3). Surprisingly, we were unable to demonstrate any significant cholinergic nicotinic receptor-mediated mechanism, which would be expected at least in the striated muscle of the oesophagus (1).

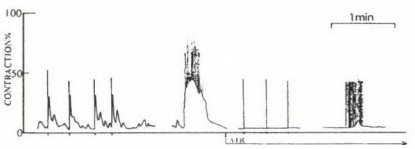

Fig. 1. Responses of the guinea-pig oesophageal longitudinal muscle to EFS; arrowheads, single shocks; horizontal line, 2 Hz for 20 s. ATR-atropine (10^{-6} M)

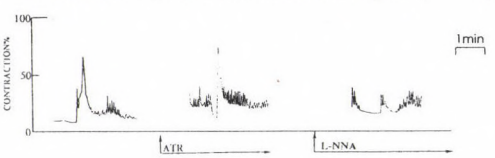

Fig. 2. Responses of the human oesophagus circular muscle to EFS (1 Hz for 10 s, horizontal bar), in the absence and in the presence of atropine (ATR, 10^{-6} M) or atropine plus N^G-nitro-L-arginine (L-NNA, 3×10^{-5} M). Note that both the NANC relaxation and after-contraction was abolished by L-NNA

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NITRIC OXIDE ACTIVATES AN IBERIOTOXIN-SENSITIVE POTASSIUM CHANNEL IN HUMAN SAPHENOUS VEIN

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Abstract: Synthetic iberiotoxin (IBTX), an inhibitor of large conductance calcium-activated potassium channel (BKCa), was used to study the possible involvement of a specific hyperpolarizing ion channel in nitric oxide (NO)-induced venodilation. Serotonin (0.125 μM)-induced contraction of isolated human saphenous vein was dose-dependently relaxed with 50–1550 nanoM exogenous NO. 30 min preincubation of venous preparations with 90 nanoM IBTX decreased vasorelaxation induced by NO. In conclusion, a hyperpolarizing potassium channel, BK_{Ca}, may be involved in the venodilator effect of endogenous NO.

Keywords: nitric oxide, iberiotoxin, saphenous vein, human

Introduction

The precise mechanism by which nitric oxide (NO) dilates venous blood vessels is still not known. Recent investigations have presented evidences at cyclic GMP elevating drugs are able to activate BK_{Ca}, a hyperpolarizing potassium channel (1, 2). Synthetic IBTX (3), the known most selective inhibitor of large conductance calcium activated potassium channel (BK_{Ca}), has been shown to decrease acute venodilatation induced by nitroglycerine (4). In the present study, the possible involvement of BK_{Ca} channels in venous dilatation by NO was investigated using isolated human saphenous veins.

Methods

Saphenous veins were prepared from patients suffered from varicose vein disease and put into ice-cold Krebs-Henseleit solution (KHS). The veins were cleared of connective tissues and cut into 5 mm rings. Then, rings were put into KHS bubbled with 95% O_2 and 5% CO_2 gas mixture; pH 7.4 at 37 °C. Nitric oxide was obtained by reducing $NaNO_2$ in the following medium: 140 mM Na_2SO_4, 100 mM NaI, and 270 μl concentrated H_2SO_4 in 50 ml double distilled water. This solution was then bubbled with 95% N_2 and 5% CO_2 gas mixture for 45 min in an air-tight vacutainer tube. $NaNO_2$ was injected into this O_2 free solution to result in 100 μM stock solution. Rings were suspended on a force transducer to measure the isometric contractions. In parallel measurements, two rings prepared from the same vein were streched up with 10 mN in 2 ml recording chambers containing KHS. Equilibration of the rings lasted for 60 min. Mechanical responses of venous preparations were displayed on a pen recorder. After 30 min equilibration of the two venous preparations with 90 nM IBTX and with the corresponding volumes of solvent contractions were induced with serotonin (0.125 μM). At the steady state of contractions, cumulative concentrations of NO (50–1550 nM) were applied. For statistics one-way analysis of variance (ANOVA) was used. ED_{50} values were calculated by fitting the equation of A/(1+exp(b*(x–c))) to the mean values.

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Results

NO in a concentration range of 50–1550 nanoM dose-dependently relaxed both IBTX pretreated and control saphenous rings. Significant differences have been found between the corresponding values of NO and IBTX+NO (Table 1). The calculated IC_{50} values of NO were 1471.9 nanoM (with IBTX) and 952.7 nanoM (at the control), respectively.

Table 1

Effect of iberiotoxin on nitric oxide-induced relaxation of human saphenous vein

	Relaxation (%)				
	50	150	350	750	1550
			nitric oxide (nanoM)		
Serotonin	1.0	5.5	16.8	29.9	65.5
	±1.0	±1.2	±4.2	±3.1	±6.9
Serotonin	0.0	0.0	4.5	15.4	34.8
+IBTX	±0.0	±0.0*	±1.7*	±3.6*	±7.3*

Number of preparations=6. Data are mean ± S.E.M., *$p<0.05$ between the corresponding values of serotonin and serotonin + IBTX

Conclusion

In the light of these observations, we suppose that BK_{Ca} channels exist in the smooth muscle membrane of human saphenous vein. From these and from our previous findings (4, 5) we can conclude that vasodilation by nitroglycerine in some blood vessels may be mediated through NO-induced opening of BK_{Ca} channels.

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ELECTROPHYSIOLOGICAL CHARACTERISTICS OF VENTRICULAR MUSCLES FROM DIABETIC HEARTS

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Keywords: diabetic heart, cardiac electrophysiology

Introduction

Diabetes mellitus is associated with cardiovascular complications including alterations in myocardial mechanical and electrical activity (2, 3). Changes in both the action potential configuration (3, 7, 8) and the ECG of diabetic patients (1) have been described and may be responsible for the increased incidence of cardiac arrhythmias. Several investigators found a significant increase in the action potential duration of diabetic rat ventricular muscles (2, 7, 8) and a decrease of the K^+ currents of rat ventricular myocytes (4, 6, 9).

In spite of the abundant electrophysiological data of diabetic rat heart we have no information about those of the diabetic rabbit heart. The aim of this study was to compare the electrophysiological properties of diabetic rabbit ventricle with those of diabetic rat ventricle.

Materials and methods

New Zealand rabbits were used for control and diabetic groups. Rabbits were made diabetic by alloxan tetrahydrate (560 μmol/kg). Manifest diabetes without ketosis was induced in 8 rabbits. Up to the end of the 8 weeks of diabetes duration the fasting blood glucose increased from 5.19± 0.43 to 14.6±2.5 mmol/l ($p<0.001$) and considerable amount of glucose (25.19±11.1 mmol/l) was excreted. The control rabbits (n=6) were maintained on the same diet until they were used 8 weeks later. Wistar rats (200–250 g, n=10) were given i.v. streptozotocin (60 mg/kg) and became diabetic for 9 weeks (blood glucose level 23.3±0.23 mmol/l). The control rats (n=12) were given vehicle only (blood glucose level was 5.3±0.2 mmol/l–7.0±0.1 mmol/l) Animals were killed by a blow on the head. The hearts were rapidly removed and the right ventricular papillary muscles and right atrium were isolated.

The preparation was placed on organ bath perfused with oxygenated (95% O_2–5% CO_2) Tyrode solution (its composition(mM/l):137 NaCl, 4.0 KCl, 1.8 $CaCl_2$, 1.5 $MgCl_2$, 11.9 $NaHCO_3$, 0.42 NaH_2PO_4, 5.5 glucose) (34 C, pH 7.4). The preparation was stimulated by rectangular (duration 0.5 ms) pulses, frequency of 1.0–2.0 Hz, voltage of twice the stimulus threshold. To record AP glass capillary microelectrodes filled with 3 M KCl, having resistance of 5–25 megohmes were used, APs were stored and calculated by IBM computer and Intrasys (Experimetria) system.

Drugs: 4-aminopyridine (Sigma); d-sotalol (Bristol-Myers Squibb)

Results and discussion

Figure 1 shows the most important parameters of the action potential (AP) of control and diabetic rabbit ventricular papillary muscle. No significant difference was established in the resting membrane potential (RP), in the amplitude of action potential (APA) in the maximum rate of

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depolarization phase (V_{max}) the action potential duration (APD), however, was markedly and significantly longer in muscles from the diabetic rabbit than from the control. The highest prolongation (65%) was observed in the early repolarization phase at 20% of repolarization. In the presence of 4-aminopyridine(4-AP) (1 mmol/l), a potent blocker of K^+ channels the control APD became similar to the diabetic ones (Fig. 1). Figures 2 illustrates the AP characteristics of control and diabetic rat ventricular muscles. In diabetic rat ventricle a significant decrease of V_{max} and significant prolongation of repolarization phase (76 and 40% at 20% and 50% of repolarization, respectively) were observed (Fig. 2). d-sotalol (0.1 mmol/l) in diabetic ventricle shortened the APD. Our results show that diabetes mellitus induced a marked lengthening of the action potential in rabbit ventricular muscles similarly to that of diabetic rats.

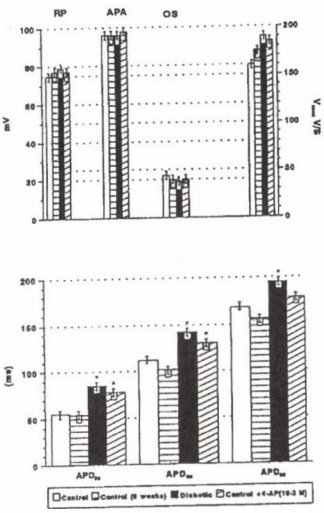

Fig. 1. Rabbit ventricular muscles

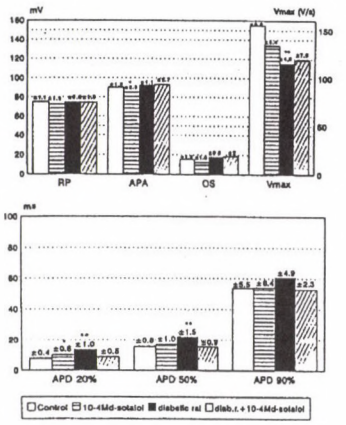

Fig. 2. Rat ventricular muscles

These results that the prolongation of AP in diabetic rabbit ventricle is due to be the decrease in underlying K^+ currents similarly to the previous data (4, 6, 9) obtained in diabetic rat ventricles however, the implication of other ionic currents (Ca^{2+}) cannot be excluded, either. The final conclusion about the ionic mechanisms for the prolongation of diabetic rabbit ventricular muscle must await further experiments using ionic currents measurements. The ineffectiveness of d-sotalol on diabetic rat ventricle suggests that the response of diabetic heart to some antiarrhythmic drugs should change.

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LONG-TERM ISCHAEMIC PRECONDITIONING OF THE HEART INDUCED BY REPEATED β-ADRENERGIC STRESS

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Abstract: In the present study we tested the preconditioning effect of repeated beta adrenergic stress induced by Isoproterenol in chronically instrumented, conscious rabbits. We have found that at least 5 intravenous administrations of Isoproterenol, repeated at 10 min intervals, were necessary to induce a long-term cardiac adaptation manifested by a significant reduction of the harmful ischaemic changes due to cardiac stress 24 and 48 hours after preconditioning. These results suggest that a well-defined threshold level of the preconditioning stress is needed to trigger induction of metabolic changes leading to development of delayed and long-term cardiac adaptation.

Keywords: long-term ischaemic preconditioning, heart, repeated β-adrenergic stress

Introduction

A delayed form of cardiac adaptation to stress (DCA) protecting the heart from harmful effects of a severe stress, days after an inducing minor stress was described by us in 1983 and studies on its mechanism recently summarized (1, 2). DCA protecting from consequences of severe ischaemia, such as extension of ischaemia, early and late postocclusion and reperfusion arrhythmias, disturbance of transmembrane ion-transport and early structural changes could be evoked by interventions stimulating the adenylate-cyclase/cAMP system. Our earlier studies with prostacyclin-analogues suggested that induction of DCA is dose-dependent. Use of a sufficiently high dose or intensity to induce DCA may involve the hazard of unwanted side effects. A fractionated application of the DCA-inducing stress, such as repeated brief periods of ischaemic stress seemed to be more expedient. In the present experiments we studied the effect of fractionated application of isoproterenol (Isuprel) on development of DCA.

Methods

Male New Zealand white rabbits weighing 2.5–3 kg were anaesthetized with 15 mg/kg diazepam given into the ear vein. A bipolar electrode catheter was inserted under aseptic conditions via the right jugular vein into the apex of the right ventricle. The correct position was determined by the intracavital electrogram recorded from implanted electrode catheter. In addition, a polyethylene catheter was introduced via the left carotid artery into the left ventricle to measure the intraventricular pressure. It was connected to a Statham P23Db transducer and a multichannel recorder. Another polyethylene catheter was inserted into the left jugular vein in order to administer the drugs. At least a 4 day recovery period was allowed before the experiments. We used a combination of an α- and a β-adrenoceptor agonist (2 μg/kg isoproterenol + 16 μg/kg phenylephrine i.v.) as a test stress. The first control stress was followed after 30 min by a second one to check its reproducibility. Then ten times repeated administrations of isoproterenol (2 μg/kg) at 10 min intervals were performed. The test stress was repeated 6, 24, 48, 72 hours after preconditioning. To determine the threshold for the stress just evoking DCA and consecutive delayed and prolonged cardioprotection, the number of isoproterenol administrations used was reduced (from 10 to 5 then to 3) in subsequent experiments. The endocardial ST

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segment elevation was considered as an indicator of myocardial ischaemia. Furthermore, the increase in LVEDP was the sign of the deterioration of the left ventricular pump function.

Results and discussion

Repeated administration of isoproterenol in a dose of 2 μg/kg significantly reduced the stress induced elevation in the ST segment of the endocardial ECG 6, 24 and 48 h after treatment, in the LVESP 24, 48 and 72 h after treatment and in the LVEDP 48 h after treatment. However, for sake of a safe induction of the DCA-process higher number of isoproterenol administrations were used. We found that 5 repeated injections of isoproterenol, given in 10 min intervals, were sufficient to induced DCA, while 3 or less administrations failed to do so. In our chronically instrumented conscious rabbit model we were able to show that repeated low dose isoproterenol treatment could induce DCA protecting against the consequences of a global myocardial ischaemia.

We found that at least 5 repeated isoproterenol administrations (2 μg/kg i.v.) were necessary to induce DCA. It remains to be determined to what extent the elevation of the myocardial cAMP content is to be expected after 5 isoproterenol administrations. This would give the threshold concentration of cAMP needed to trigger metabolic changes leading to the development of delayed and prolonged cardiac adaptation to stress. Timing of optimal protection suggests that stress protein (SP) expression could be also involved in DCA. Further studies are needed to clarify this question.

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EFFECT OF RESTACORIN ON THE EARLY AND DELAYED AFTER DEPOLARIZATION IN DOG PURKINJE FIBRES

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Abstract: The effect of restacorin and flecainide on the early afterdepolarization (EAD) and on the delayed afterdepolarization (DAD) was studied in dog cardiac Purkinje fibers by applying the conventional microelectrode technique. Restacorin and flecainide at 5 μM concentration partially abolished EAD. Further increase of the concentrations of the drugs to 10 μM EADs were completely abolished. The amplitude of DADs were significantly decreased by both restacorin (9.9±2.1 mV v.s. 2.9±1.8 mV, $p<0.01$, $n=5$) and flecainide (11.1±1.3 mV v.s. 0.6±0.6 mV, $p<0.01$, $n=7$). These results suggest that the well-established antiarrhythmic effect of restacorin and flecainide at least partially may be explained by their beneficial action against triggered abnormal automaticity.

Keywords: afterdepolarization, Purkinje fibre, restacorin, flecainide

Introduction

Arrhythmias may be generated due to disturbances of impulse propagation or impulse formation. Triggered activity is a special form of abnormal impulse initiation which includes both EAD and DAD. Antiarrhythmic drugs could abolish triggered activity which may be an important mechanism in their antiarrhythmic actions. In the present study the effect of a new antiarrhythmic drug, restacorin was studied on the triggered activity and compared to that of flecainide.

Methods

To study the effect of the drugs on triggered activity in dog Purkinje fibers the conventional microelectrode technique was applied which was described earlier (2). EADs were evoked by low stimulation rate (0.1–0.5 Hz) and application of 1 μM dofetilide +20 μM $BaCl_2$ + 2 mM CsCl. DAD was elicited by 0.2 μM acetylstrophantidin. The preparations were stimulated at 3 Hz frequency and in the presence of acetylstrophantidin. When the stimulation was interrupted by a 10 s resting period DAD and/or one or two spontaneous action potentials developed.

Results and discussion

Both restacorin and flecainide – used as a control drug – were applied in 5 and/or 10 μM concentrations. In 1 out of 6 fibres 5 μM restacorin completely abolished the EAD (Figure 1) and decreased its severity in 3 other ones. In two fibres 5 μM restacorin did not influence the formation of EADs. By increasing the concentration of restacorin to 10 μM, the EAD was abolished in two additional fibres. Flecainide applied in 5 μM concentration abolished EAD formation (Figure 1) in 3 out of 6 fibers and did not change it in 3 other ones. When the concentration of flecainide was increased to 10 μM, the drug completely abolished EAD in 1 fibre and decreased the severity of EADs in two other ones. The effect of restacorin and flecainide on Purkinje fibres was also associated with a marked shortening of the repolarization which is most likely due to the known inhibition of the inward sodium current by these drugs (1, 3). Another form of triggered activity, namely DAD, was studied (Figure 2) in fibers treated with

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acetylstrophantidin (see Method). Administration of 5 μM restacorin eliminated or decreased the amplitude of the DAD from 9.9±2.1 mV to 2.9±1.8 mV ($p<0.01$, $n=5$).

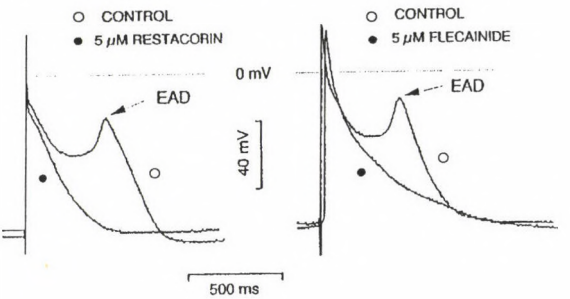

Fig. 1

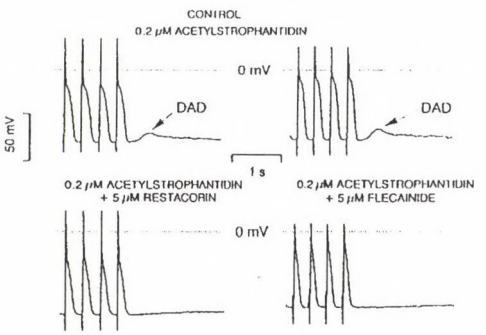

Fig. 2

In addition, the compound eliminated the spontaneous beats observed in one fibre in the presence of acetylstrophantidin. The amplitude of DAD before administration of flecainide (applied as a control drug) was 11.1±1.3 mV which was decreased to 0.6±0.6 mV ($p<0.01$, $n=7$). In 3 fibres 5 μM flecainide abolished the spontaneous beats elicited by acetylstrophantidin. From these findings it might be concluded that restacorin, like flecainide, would not increase the risk of the bradycardia related torsade de pointes arrhythmias and the compound would be effective against DAD related arrhythmias often associated with digitalis toxicity and myocardial ischemia.

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THE ENHANCEMENT AND THE INHIBITION OF NORADRENALINE-INDUCED CYCLIC AMP ACCUMULATION IN RAT BRAIN BY STIMULATION OF METABOTROPIC GLUTAMATE RECEPTORS

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Abstract: The actions of several metabotropic glutamate receptor agonists and antagonists on noradrenaline (NA)-stimulated [^{3}H]-cyclic AMP accumulation were investigated in rat cerebral cortical slices. Quisqualate (QUIS), L-2-amino-3-phosphonopropionic acid (L-AP3) and glutamate (GLU) elicited concentration-dependent inhibition of (NA)-stimulated [^{3}H]-cyclic AMP accumulation. In contrast (2S,3S,4S)-α-(Carboxy-cyclopropyl)glycine (L-CCGI), 1-Aminocyclo-pentane-1S,3R-dicarboxylate (1S,3R-ACPD), ibotenate (IBO) and (RS)-4-carboxy-3-hydroxy-phenylglycine (CHPG) elicited a concentration-dependent enhancement of NA-stimulated [^{3}H)-cyclic AMP accumulation. A putative mGluR antagonist – L-AP3, inhibited the 1S,3R-ACPD-induced enhancement of the action of NA on [^{3}H]-cyclic AMP accumulation in a biphasic manner with an IC_{50} of 4.5 μM for the high affinity site, which represented 65% of the total and an IC_{50} of 283 μM for the low affinity site.

Keywords: cyclic AMP; noradrenaline; excitatory amino acids; metabotropic glutamate receptors

Introduction

Glutamate (GLU) acts not only as a major excitatory neurotransmitter, but also plays an important role in neuronal plasticity and neurotoxicity in the central nervous system (CNS). The functional diversity of GLU is reflected by the fact that this EAA elicits its effects through diverse GLU receptors. They can be categorized into two distinct groups termed ionotropic and metabotropic receptors. The metabotropic glutamate receptors (mGluR) are G-protein-coupled receptors that modulate various second messenger systems (7). Recently cloned mGluR can be divided into at least 8 subtypes described as mGluR1 to mGluR8 (6). One of the interesting features of EAA, recently described, is their ability to modify the effect of other substances which are directly coupled to adenylyl cyclase (8). It has been proposed that an mGluR exists that belongs to a growing class of receptors (such as α-adrenoceptors, $GABA_B$, H1-histaminergic) that potentiate cAMP responses to activation of Gs-coupled receptors (2, 5). Here we decided to investigate the interaction between the endogenous neurotransmitter NA and EAA on cyclic AMP accumulation in the brain.

Methods

Cerebral cortical cross-chopped slices (300 μm), from Wistar rats (150–200 g) prepared with McIlwain tissue chopper, were suspended in oxygenated Krebs medium pH 7.4. The formation of [^{3}H]-cyclic AMP in [^{3}H]-adenine-prelabelled slices was assayed as described previously (5). Briefly, after 15 min equilibration at 37 °C in the gassed (O_2/CO_2, 95:5) Krebs medium, pH 7.4, slices were further incubated with the [^{3}H]-adenine (25 μCi/20 ml) for 45 min. After washing with Krebs buffer, the gravity-packed slices (50 μl) were pipetted into plastic tubes containing Krebs buffer and incubated for 10 min. Agonists were then added in 10 μl of medium, to give a total volume of 500 μl. The incubation was terminated after 10 min with 550 μl of a cool 10% trichloroacetic acid; then the slices were homogenized and centrifuged (10,000 g, 10 min). [^{3}H]-cyclic AMP was extracted from the supernatant by a sequential Dowex-alumina chromatography.

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Prior to extraction, samples were spiked with [^{14}C]-cyclic AMP to allow for a percentage recovery correction. The accumulation of cyclic AMP was assessed as a per cent of the conversion of [^{3}H]-adenine into [^{3}H]-cyclic AMP.

Results and discussion

The NA (100 μM) induced cyclic AMP accumulation was reduced in a concentration dependent manner by QUIS and L-AP3 with IC_{50} values of 105±29 and 275±36 μM, respectively, GLU was 10 times less potent with an IC_{50} of 944±150 μM, L-AP4 was not effective up to concentration of 0.5 mM. The involvement of ionotropic glutamate receptors in the inhibitory effect is excluded since these effects were neither antagonized by CNQX or MK-801 nor followed by NMDA or AMPA. QUIS is a potent agonist for mGluR1 and mGluR5 receptor subtypes (6), but as DHPG which selectively activates mGluR1 and mGluR5 (3) was without effect on NA-induced cyclic AMP accumulation, the involvement of these subtypes is rather equivocal. The involvement of mGluR2 and mGluR3 is also not feasible as 1S,3R-ACPD and L-CCG-I which activate those receptor subtypes (4, 6, 8) did not inhibit NA-stimulated cAMP accumulation. Several data indicate also against the involvement of mGluR4, mGluR6 and mGluR7 subtypes: 1) L-AP4 an agonist of those receptors group was not effective; 2) these receptors are very sensitive to GLU and less sensitive to QUIS (6, 7), while in our experiments an opposite order of potency was observed, one must be aware, however, that it is problematic to correlate the potency of glutamate between cloned mGluRs and cortical slices because glutamate uptake will affect the results obtained differentially; 3) these receptor subtypes are insensitive to L-AP3 (4, 6) and it was quite a potent inhibitor in our experiments (IC_{50} 275 μM). Therefore it seems that the QUIS and L-AP3-sensitive subtype of mGluR exist which is negatively coupled to adenylyl cyclase. The fact, that the endogenous transmitter GLU was able to inhibit the effect of NA may indicate that this is a predominant interaction between these two neurotransmitter systems in an adult rat brain.

The second major interaction between EAAs and NA in cortical cerebral slices was the enhancement of the effect of NA on cyclic AMP accumulation by several EAAs. 1S,3R-ACPD, an agonist of mGluR, markedly (about 5-fold) enhanced the stimulatory action of NA (100 μM) on cyclic AMP accumulation with an EC_{50} of 42±1.3 μM. The response to NA was enhanced in a concentration-dependent manner not only by 1S,3R-ACPD but also by IBO (EC_{50} of 97.8± 2.1 μM), which was twice less potent and slightly less effective than 1S,3R-ACPD. CHPG enhanced the stimulatory action of NA (100 μM) approximately twofold with an EC_{50} of 157± 13.4 μM (Fig. 2). The most potent, but less effective was L-CCG-I (EC_{50} 2.5±0.11 μM). At 250 μM L-CCG-I enhanced approximately 3 fold the effect of NA, while at 1 mM concentration the enhancement was about 2-fold. The ionotropic EAA receptor antagonists CNQX (300 μM) and MK-801 (10 μM) were without effect on the interaction between NA (100 μM) and 1S,3R-ACPD (100 μM) on cyclic AMP accumulation again excluding the involvement of ionotropic GLU receptors. To test further if mGluR are involved in the interaction between NA (100 μM) and 1S,3R-ACPD (100 μM), different concentrations of a putative metabotropic receptor antagonist L-AP3 were used. L-AP3 produced a biphasic, concentration-dependent inhibition of the interaction. The IC_{50} for the high affinity site, which represented 65% of the total, was 4.5 μM. The IC_{50} for the low affinity site was 283 μM. The high affinity portion represented the effect of L-AP3 on the potentiation of NA-induced cAMP response evoked by 1S,3R-ACPD whereas low affinity component has an IC_{50} identical to that for the L-AP3-induced inhibition of NA-stimulated cyclic AMP accumulation. The results indicate that mGluR responsible for the synergistic interaction with NA are blocked with high affinity by L-AP3.

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INTRACELLULAR Ca^{2+} IMAGING OF CULTURED CHICKEN TELENCEPHALIC CELLS: CHARACTERIZATION OF IONOTROPIC GLUTAMATE RECEPTOR ACTIVATION

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Abstract: In the present study we investigated the intracellular $[Ca^{2+}]$ increasing effect of the excitatory amino acid agonists kainate, AMPA and NMDA on fura-2/AM loaded chicken telencephalic cells in various conditions. Kainate (100 μM) increased $[Ca^{2+}]_i$ to 256±23% of the basal level (n=7). In Ca^{2+}-free medium the effect of kainate on intracellular Ca^{2+} was completely abolished indicating that the primary source of the Ca^{2+} signal was the extracellular pool. Voltage dependent Ca^{2+} channel antagonsim by Cd^{2+} decreased the intracellular Ca^{2+} elevation caused by 100 μM kainate indicating the involvement of voltage dependent Ca^{2+} channels (VDCC).

Keywords: glutamate receptors, intracellular calcium, chick neurons

Introduction

Based on selectivity of agonists the ionotropic glutamate receptors have been classified into three distinct groups termed NMDA, AMPA and kainate receptors. Glutamate receptors are thought to play a role in neurological disorders such as stroke, ischemia or slowly progressing neurodegenerative diseases. These receptor ion channels may allow the entry of Ca^{2+} into the cell but the VDCCs in the plasmamembrane also contribute the increase in the $[Ca^{2+}]_i$ because of the depolarization caused by kainate receptor activation (1).

Methods

Chick cortical neurons are obtained from embryos on the 7th embryonic day, mechanically dissociated, and plated onto plastic NUNC dishes. The culture medium is Parker TC 199 medium with 20% foetal calf serum and 20% glucose. The $[Ca^{2+}]_i$ is monitored using Fura-2 with an intensified charge coupled device (ICCD) camera (PTI). The cells are excited via a 40x fluorine objective using a 75 W Xenon light. The cells are studied in an inverted phase-contrast microscope (Nikon). Changes in $[Ca^{2+}]_i$ are expressed as a ratio of the fluorescence at 510 nm due to excitation of fura-2/AM at 340 and 380 nm produced by two monochromators (PTI).

Results and Discussion

100 μM kainate induced a reproducible increase in the intracellular Ca^{2+} level of chicken telencephalic cells applied by pressure ejection to the vicinity of the chosen cells. Application of NMDA and AMPA at 100 μM concentration also caused an increase in the intracellular Ca^{2+} level. The Ca^{2+} signals by AMPA was considerably smaller in these cells than that of caused by kainate. Our data have demonstrated that in chicken telencephalic cells kainate can produce Ca^{2+} signals in all neurons investigated in our culture. The effect of NMDA was not homogenous in the cell culture; it is possible that this receptor is not expressed on all the cell types.

It is demonstrated that kainate selective glutamate receptors have a considerable Ca^{2+} permeability (2). When KA was administered in Ca^{2+}-free medium no response was observed to KA indicating that the response to KA needs Ca^{2+} entry from the extracellular space. When the first KA stimulus was given by pipette filled with Ca^{2+}-free medium and the second KA dose by

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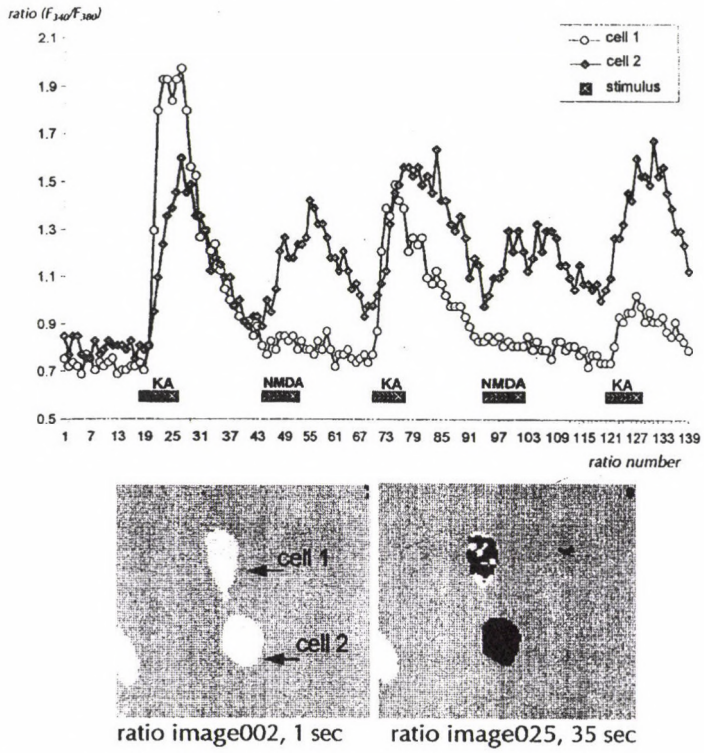

Fig. 1. Effect of local application of kainate and NMDA on chicken telencephalic cells. Kainate at 100 μM concentration increased ic. Ca^{2+} level (ratio image 025 shows the effect on the images of the chosen telencephalic cells, both cells were able to respond to KA). However, the effect of NMDA was different at the two cells, only cell 2 showed clear response to NMDA. At the other cell no increase in ic Ca^{2+} by NMDA application was observed

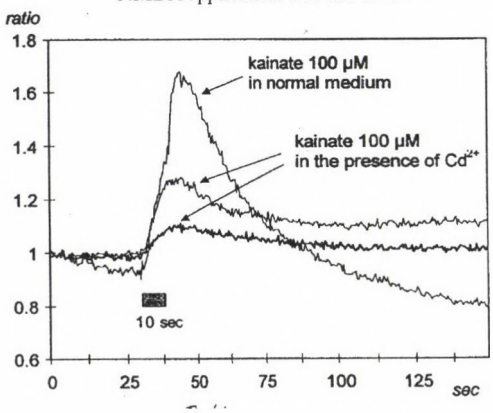

Fig. 2. Effect of voltage dependent Ca^{2+} channels in the action of kainate. In the presence of Cd^{2+}, kainate is not able to increase intracellular Ca^{2+} in the same extend

pipette with Ca^{2+} containing medium (and the extracellular space around the cells was Ca^{2+}-free), the later response was even higher than the effect of KA in the normal medium indicating the intracellular Ca^{2+} concentration increasing effect of KA depends even on a small amount of Ca^{2+} present in the pipette.

The question arises whether the Ca^{2+} entry by KA stimulus utilizes voltage dependent Ca^{2+} channels. In a series of experiments after a Ca^{2+} response to KA was recorded, the medium over the culture was exchanged in the microscope stage to Cd^{2+} (100 μM) containing solution. Five minutes after the exchange of the solution Cd^{2+} could inhibit the effect of KA to increase $[Ca^{2+}]_i$ so we can conclude that the Ca^{2+} entry by KA application, at least in a large part, was mediated via VDCCs.

Since patch clamp experiments on the same cell type have revealed that the intracellular Ca^{2+} signals produced by KA can influence potassium channels and ionomycin a Ca^{2+} ionophore is able to elicit a similar response we investigated the effect of local application of ionomycin on the $[Ca^{2+}]_i$. Two kind of response to 10 μM ionomycin can be distinguished in chicken telencephalic cell culture: a persistent elevation in intracellular Ca^{2+} and a transient Ca^{2+} peak in some cases. Ionomycin at 1 μM concentration is still able to elicit increase in $[Ca^{2+}]_i$ but the amplitude is lower than that of observed at 10 μM ionomycin.

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EFFECT OF ETHYLDEOXYURIDINE ON 5-FLUOROURACIL-INDUCED NEUTROPENIA

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Abstract: It is supposed that the toxic effect of 5-fluorouracil (5FU) on tumour cells may be increased by pretreatment with ethyldeoxyuridine (EDU). We studied the effect of this combination on neutrophil count in mice. Our present studies demonstrated that the neutropenia induced by 5×20 mg/kg 5FU became more severe when each dose of 5FU was preceded by 200 mg/kg EDU.

Keywords: 5-fluorouracil, ethyldeoxyuridine, neutropenia, mouse

Introduction

The fluoropyrimidines, in particular 5FU, are widely used drugs for patients with colorectal or breast cancer (4). To improve the efficacy or reduce the adverse effects of 5FU, investigations have recently focused on agents influencing the activity of 5FU through the modification of its metabolism (5). It was shown that the cytotoxic effect of 5FU to human tumour xenografts *in vivo* was increased by pretreatment with EDU (2, 3). However, this interaction would be really beneficial if EDU did not induce a similar increase in the toxicity of 5FU to normal tissues. The toxic effect of most cytostatic drugs affect primarily the so-called cell renewal systems, *e.g.* the bone marrow, resulting in neutropenia. We studied the effects of combined administration of 5FU and EDU on the absolute neutrophil count in the peripheral blood of mice.

Methods and materials

Experimental animals: (BALB/c × CBA)F_1 mice of both sexes at least 8 weeks old.

Materials: 5-fluorouracil (Fluoro-uracil Roche, Hoffmann-La Roche, Basle) and 5-ethyl-2'-deoxyuridine (EDU, synthesised at the Central Research Institute for Chemistry, Hungarian Academy of Sciences) were used to treat animals.

Statistical evaluation: Data were analyzed with Kruskal-Wallis test and non-parametric multiple comparisons according to Conover (1).

Treatment: Both drugs were given intraperitoneally at a volume of 10 ml/kg. 5FU was given in five equal doses of 20 mg/kg each in 24 hour intervals. EDU (200 mg/kg) was administered 60 minutes before each dose of 5FU. Four treatment groups were formed, including the control one (Table I).

Table I. *Treatment groups*

Groups				
Control	S	+	S	S : 5×10 ml/kg 0.9% NaCl
5FU alone	S	+	5FU	5FU: 5×20 mg/kg 5-fluorouracil
EDU alone	EDU	+	S	EDU: 5×200 mg/kg ethyldeoxyuridine
EDU+5FU	EDU	+	5FU	

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The time of the last dose of 5FU or – in those groups in which mice were not given 5FU – the substituting NaCl was considered as starting (0) time. Four hours, 1, 2, 3, 4 and 5 days after this time neutrophil granulocytes in the peripheral blood were counted.

Results and discussion

The absolute neutrophil count in the '5FU alone' group was significantly lower than the control until 4 days after the last dose of 5FU. The nadir was reached at day 2 (12% of control), then the neutrophil count increased continuously. In mice treated with 'EDU alone', the absolute neutrophil count was lower than the control during the whole observation period, but the difference was statistically significant only 2 days after the last dose of 5FU. The neutropenia was always significantly ($p<0.01$) more severe in the 'EDU+5FU' group than in any other group. The lowest absolute neutrophil count in this group was observed on day 5 (0.4% of control). Although the neutrophil count did not begin to recover till the end of the observation period (5 days), the number of progenitor cells in the bone marrow (to be published elsewhere) showed signs of regeneration.

Our experiments demonstrated that the neutropenia caused by 5×20 mg/kg 5FU given in 24 hour intervals was significantly increased by 200 mg/kg EDU given 60 minutes before each dose of 5FU, although EDU given alone at the same dose did not show a significant neutropenic effect.

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KAINATE-INDUCED INHIBITION OF VOLTAGE-ACTIVATED POTASSIUM CURRENTS IN CULTURED CHICK TELENCEPHALIC NEURONS

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Abstract: Kainate indirectly produces an inhibition of voltage-activated outward potassium currents on cultured chick telencephalic neurons, besides directly evoking activation of AMPA/kainate receptors (current with linear I–V curve, reversal potential near 0 mV). The former effect is also mediated by AMPA/kainate receptors. The role of $[Ca^{2+}]_i$ in the inhibition was investigated by whole cell- and nystatin-perforated patch-clamp technique. Elevation of $[Ca^{2+}]_i$ evoked by the Ca^{2+} ionophores ionomycin and A23187 caused an inhibition of outward currents as well. When the elevation of $[Ca^{2+}]_i$ is prevented, the inhibition is decreased. These results suggest that $[Ca^{2+}]_i$ elevation may be involved in the process.

Keywords: potassium currents, chick, glutamate receptors, intracellular Ca^{2+}

Introduction

The excitatory neurotransmitter, glutamate is a key factor in acute neurodegenerative disorders (e.g. cerebral ischemia, trauma, epilepsy), being present in high extracellular concentrations during these processes, and causing excitotoxicity. The susceptibility of neurons to excitotoxicity is greatly influenced by the functional state of their voltage-gated ion channels. Glutamate is known to alter transmembrane conductances of neurons by two main ways: the activation of ionotropic receptors, and the modulation of voltage-gated ion channels via metabotropic receptor activation. In a few cases, however, the modulation of voltage-gated ion channels was reported to be mediated by the activation of ionotropic receptors (see e.g. in Ref. 1). Since activation of both ionotropic and metabotropic receptors results in an increase in intracellular calcium concentration ($[Ca^{2+}]_i$), we investigated if the elevation of $[Ca^{2+}]_i$ evoked by the selective ionotropic glutamate receptor agonist kainate might mediate the modulation of voltage-gated ion channels.

Methods

Chick telencephalic neuronal cultures were prepared as previously reported (3). Briefly, cells of the telencephalon from embryos on the 7th embryonic day were mechanically dissociated, and plated onto polylysine-coated plastic NUNC dishes. The culture medium was Parker TC 199 medium with 20% foetal calf serum and 20% glucose. 5–15 day old cultures were used for experiments.

Membrane currents were measured with the patch-clamp method, using the conventional whole cell, and the nystatin-perforated patch configurations. Pipettes of 5–10 MOhm contained (in mM): KCl-140; NaCl-4; $CaCl_2$-0.5; HEPES-10; EGTA-5, adjusted to pH 7.3 with KOH; and in the case of nystatin-perforated patch recording: KCl-55; K_2SO_4-75; $MgCl_2$-7; HEPES-10; glucose-10, adjusted to pH 7.3 with KOH. Nystatin was added right before the experiment, at a concentration of 120 μg/ml. The external recording solution contained (in mM): NaCl-140; KCl-5; $CaCl_2$-2; $MgCl_2$-2; HEPES-10; glucose-10, adjusted to pH 7.3 with NaOH. Recordings were made with an Axopatch-1D. The pClamp software package (Axon) was used for data acquisition and analysis.

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Results and discussion

Kainate (100 μM) evoked an inward current in the cells. At holding potential (–70 mV) the amplitude of the current was 216.2±113.6 pA, the current developed fast (Within 200 ms) and did not show desensitisation. When the voltage-activated conductances were blocked (by replacing intracellular potassium ions by cesium ions and applying 1 μM tetrodotoxin extracellularly) the current-voltage curve was close to linear, with a reversal potential near 0 mV. The EC 50 value of kainate was found to be 120±40 μM in our previous study (3). In the absence of the block, we observed an additional effect of kainate: The amplitude of outward currents slowly began to decrease after the start of kainate application, and it reached a maximal decrease of 54.2±26.6% of the control in 1–2 minutes. The inhibition was partially reversible upon washout. The relative inhibition of outward currents showed correlation with the amplitude of the directly evoked current: the correlation coefficient was –0.66 (n=32). Using cesium as intracellular cation prevented the decrease of voltage-activated outward currents evoked by kainate application, 30 mM tetraethylammonium extracellularly prevented the decrease of the sustained component of the currents. These results suggest that the main current component that is inhibited by kainate is mediated by voltage-activated potassium channels. The inhibition is mediated by non-NMDA ionotropic receptors, since it can be dose dependently blocked by the non-NMDA receptor antagonists CNQX and GYKI 53784 (a more potent analog of GYKI 52466 (4)).

Although kainate was shown to evoke an elevation of $[Ca^{2+}]_i$ via different routes (2), the role of $[Ca^{2+}]_i$ in the inhibition of potassium currents has not been addressed in neurons. In our experiments the elevation of $[Ca^{2+}]_i$, independent from glutamate receptor activation – evoked by releasing calcium from intracellular stores using the calcium ionophores ionomycin (1 and 10 μM) and A23187 (5 μM) – produced decrease in the amplitude of outward potassium currents, which did not recover during washout. The inhibition evoked by kainate was decreased (but it was still present) when the extracellular fluid was changed to a nominally calcium-free solution, or in the presence of 300 μM Cd^{2+} in the extracellular medium.

These results show that the inhibition of potassium currents caused by activation of AMPA/kainate receptors is enhanced by the elevation of $[Ca^{2+}]_i$ but the elevation is not essential for the effect.

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DEVELOPMENT OF SOMATOSTATIN RADIOIMMUNOASSAY FOR THE MEASUREMENT OF PLASMA AND TISSUE CONTENTS OF HORMONE

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Abstract: A specific and sensitive radioimmunoassay was developed in our laboratory for measuring plasma and tissue somatostatin levels. The hormone content of arterial blood and skin samples of untreated and mustard oil (a specific agent causing neurogenic inflammation) treated animals was detected by this method. The somatostatin level of the inflamed tissue was significantly higher, but no difference was found between the plasma concentrations.

Keywords: somatostatin (SOM), radioimmunoassay (RIA), neurogenic inflammation

Introduction

Somatostatin was discovered in 1973 by Brazeau et al. (1) as a peptide which potently inhibited growth hormon release from the anterior pituitary. Since then SOM has been found in neurons of the central and peripheral nervous system and in the D-cells of the gastrointestinal tract. The first somatostatin radioimmunoassays were developed at the end of the seventies by different groups of scientists (2, 3). In this study we report the development of our somatostatin RIA method and its application in experiments on neurogenic inflammation. The mechanism of neurogenic inflammation is that different neurotransmitters are released from the activated primary sensory nerve endings (tachykinins, CGRP). These mediators act on the blood vessels, cause vasodilatation and plasma extravasation. It has recently been revealed that local neurogenic inflammation has a systemic anti-inflammatory effect (4). Participation of SOM in this action has also been assumed since this peptide is present in the sensory nerve endings.

Materials and methods

Antiserum: Somatostatin antiserum was raised against SOM-14-bovine thyroglobulin antigen in sheep. Antiserum "775/7" was the most successful for the development of RIA at 1:600,000 dilution. *Tracer:* $Tyr^{(1)}$-somatostatin-14 (Sigma) was labelled with ^{125}I isotope by Iodogen and the mono-iodinated peptide was separated from the other fragments on reverse phase HPLC column. *Standard:* Somatostatin-14 (Sigma) was used as RIA standard in range 0–1000 fmol/ml. *RIA buffer:* Assays were prepared in 1 ml 0.02 mol/l phosphate buffer (pH: 7.4) containing 0.75% v/v bovine albumin from a 22% solution (Ortho), 0.1 mol/l NaCl, 0.1% EDTA and 0.05% NaN_3. *Immunoassay procedure:* 100 µl SOM-14 standard or unknown sample, 100 µl antiserum "775/7" (working dilution 1:60,000) and 100 µl tracer (5000 cpm) were measured into the RIA tubes. After 48–72 hours of incubation at 4 °C the antibody-bound label was separated from the free labelled peptide by addition of 100 µl separating solution (100 g charcoal, 1 g dextran, 0.2 g fat free milk powder in 100 ml distilled water). After measuring radioactivity, SOM concentrations of the unknown samples were read from a calibration curve.

Sample preparation: a) Tissue samples were boiled for 15 minutes in 0.2 mol/l acetic acid, homogenised and centrifuged at 10000 g for 10 minutes (3). b) Blood samples were taken

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into ice-cold glass tubes containing EDTA and Trasylol. Following centrifugation at 4 °C the hormone from the plasma was extracted by addition of three volumes of absolute alcohol (2). After precipitation and second centrifugation the samples were dried down under nitrogen flow. The samples were resolved in assay buffer before RIA determinations.

Experiments on neurogenic inflammation: 30 minutes after acute bilateral denervation of the hindlegs of anaesthetised female Wistar rats (weight 200–220 g) mustard oil in 1% concentration was smeared on the left paw. This chemical induces neurogenic inflammation by selective stimulation of the primary sensory nerve endings and releasing their neurotransmitter content. Blood samples and skin specimens of the treated and untreated paws were taken 20 minutes after the mustard oil application. Student's t-test was used for statistical evaluation and the results were expressed as mean ± SEM (n=6).

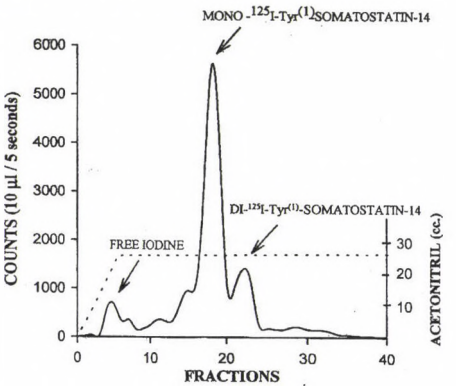

Fig. 1. Purification of ^{125}I-Tyr$^{(1)}$-somatostatin-14 on reverse phase HPLC column

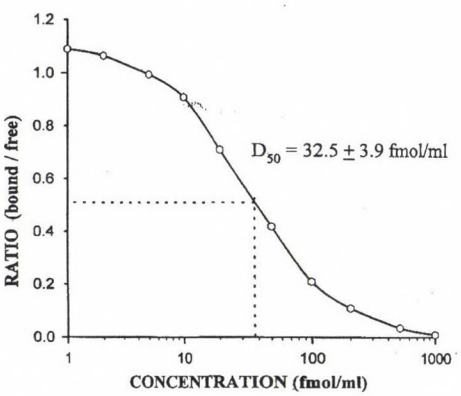

Fig. 2. Calibration curve of the somatostatin radioimmunoassay

Results and discussion

The elution pattern of the radio-labelled tracer is shown in Fig. 1. Mono-iodinated $Tyr^{(1)}$-SOM-14 was separated in a very sharp peak from the other major components of the labelling reaction. Somatostatin radioimmunoassay developed in our laboratory has a high sensitivity and specificity. In ten consecutive assays the concentrations of standard required for 50% inhibition of label to antibody was 32.5±3.9 fmol/ml (Fig. 2). During the application of our RIA in practice, the difference of SOM plasma levels between control- and mustard oil treated animals was not significant (8.87±1.43 vs. 10.12±3.8 fmol/ml); but SOM content of the mustard oil treated skin samples was significantly higher than in cases of untreated paws (6.54±0.32 vs. 7.41±0.24; $p<0.05$). As SOM level was significantly increased in the inflamed tissues, this peptide is likely to be released from the activated nerve endings in higher quantity. The explanation for not finding significant difference between the plasma levels can be the very short half life-time (<3 min) of SOM.

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COMPARISON OF THE EFFECTS OF RESTACORIN AND FLECAINIDE ON VARIOUS CARDIAC TRANSMEMBRANE POTASSIUM CURRENTS

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Abstract: The effect of restacorin (5 μM) and flecainide (5 μM) on the transmembrane ionic currents was compared in dog and rabbit ventricular muscle by applying the conventional microelectrode and patch-clamp techniques. Neither restacorin nor flecainide influenced significantly the ATP-sensitive and inward rectifier potassium currents. Flecainide moderately decreased the amplitude of the transient outward current while restacorin did not change this current. The delayed rectifier potassium current was depressed markedly by flecainide and moderately by restacorin. These results suggest that although flecainide and restacorin cause similar changes in the various transmembrane ionic currents, there are some differences between the effects of the two antiarrhythmic compounds.

Keywords: potassium currents, antiarrhythmic drugs, flecainide, restacorin

Introduction

Restacorin a new antiarrhythmic compound was found to be highly effective in various in vivo experimental arrhythmia models (3). The restacorin induced changes on the action potential parameters is very similar to that of flecainide (2, 4). The effect of restacorin on the transmembrane ionic currents underlying the cardiac action potential has however not yet been extensively studied.

Methods

The experimental methods were described earlier in detail (4, 5). To study the effect of the drugs on the ATP-sensitive potassium current (I_{KATP}) the conventional microelectrode technique was used. In these experiments, performed in dog right papillary muscle, 10 μM pinacidil was applied to activate I_{KATP} which significantly shortened action potential duration. Prevention or attenuation of the pinacidil evoked action potential duration shortening was considered as inhibition of I_{KATP}. To investigate the possible effect of restacorin and flecainide on the inward rectifier, on the transient outward and delayed rectifier potassium currents in single rabbit ventricular myocytes the patch-clamp technique was applied in the presence of 250 μM $CdCl_2$ to block the inward calcium current.

Results

Restacorin (4 μM), like flecainide (4 μM) failed to prevent the 10 μM pinacidil evoked action potential shortening in dog right papillary muscle, suggesting that at therapeutically relevant concentration the drug, as flecainide, does not inhibit I_{KATP}. In rabbit ventricular myocytes neither restacorin (5 μM) nor flecainide (5 μM) changed significantly the inward rectifier potassium current (n=5–6 cells) in the voltage range of –140 to 0 mV measured as steady-state current at the end of 400 ms voltage pulses. The transient outward potassium current (I_{to}) was evoked by 400 ms voltage pulses from –90 mV holding potential to test potentials ranging from 0 to +60 mV. Flecainide (5 μM) moderately but significantly decreased the amplitude of I_{to} (at +60 mV from 1412±222 pA to 1153±151 pA, n=5, $p < 0.05$), while restacorin (5 μM) did not

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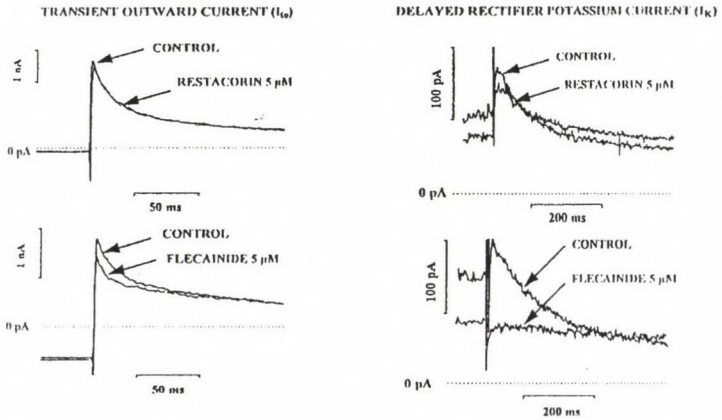
significantly affect I_{to} (at +60 mV control = 1357±276 pA vs restacorin = 1248±253 pA, n=6, N.S.). The delayed rectifier potassium current (I_K) was determined as tail current at –40 mV after successive 3 s test voltage pulses between –10 to +30 mV. Flecainide (5 μM) depressed markedly (from 100.0±18.1 pA to 22.1±2.8 pA, n=5, $p<0.01$) while restacorin decreased moderately (from 108.0±15.0 pA to 84.1±8.5 pA, n=6, $p<0.05$) the amplitude of the I_K tail current.



Discussion

The above results are consistent with earlier findings that the well-established antiarrhythmic effect of both restacorin and flecainide is most likely attributed to their previously described effect on the inward sodium current (2, 4). It is however worth to note that both restacorin and flecainide was able to depress I_K, which effect in certain situations may result in Class III antiarrhythmic effect i.e. lengthening of repolarization. Flecainide but not restacorin depressed I_{to} which may be related to the beneficial effect of flecainide in atrial fibrillation (1).

In summary, these results suggest that although flecainide and restacorin cause similar changes in the various transmembrane ionic currents, there are some differences between the effects of the two antiarrhythmic compounds.

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EFFECT OF GLUTATHIONE DEPLETION ON ELECTROPHYSIOLOGICAL CHARACTERISTICS OF GUINEA-PIG VENTRICLES

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Abstract: In this study the effect of acute and chronic glutathione depletion with L-buthionine-S,R-sulphoximine (BSO) on action potential characteristics of guinea-pig left ventricle papillary muscles were investigated. BSO caused significant decrease of maximum rate in rise of depolarization phase (V_{max}) and duration of AP (APD) at 25%, 50%, 90% of repolarization in both cases of depletion and a slight but not significant decrease in the action potential amplitude, but did not modify the resting membrane potential. Pretreatment with bisaramil prevented the effect of BSO on APD in both cases of depletion.

Keywords: glutathione, depletion, BSO, action potential

Introduction

Reactive oxygen metabolites such as superoxide anion(O^{2-}), hydrogen peroxide (H_2O_2) and the hydroxyl radical(-OH) have been implicated as mediators of cellular injury in ischemic/reperfused hearts (2, 8). The reduction of cellular defense mechanisms (glutathione, glutathione peroxidase, superoxide dismutase and catalase) against reactive oxygen metabolites during ischemia and hypoxia has been suggested as evidence that the heart is more sensitive to these metabolites on reperfusion or reoxygenation (4, 5). Because protection against oxidant injury from various sources correlates with levels of glutathione, glutathione peroxidase,. and glutathione reductase in many noncardiac tissues, it is likely that glutathione might be important in the heart as well. This is supported by the findings that glutathion-depleted hearts exhibit evidence of increased lipid peroxidation during reperfusion (1) and by findings reporting that depletion of myocardial glutathione can interfere with its contractile ability (3, 4). In the present study the effect of acute and chronic glutathione depletion with L-buthionine-S,R-sulphoximine (BSO), an inhibitor of gamma-glutamylcysteine synthetase on action potential characteristics of guinea-pig left ventricle papillary muscles were investigated.

Materials and methods

Guinea pig's cardiac glutathione were depleted by intraperitoneal injections. For acute depletion 3 mmol/kg BSO was used once, 3 hours before reperfusion and for chronic depletion 2 mmol/kg BSO was used during 4 days. Hearts were quickly removed and retrogradly perfused with oxigenated (95% O_2+5% CO_2) Tyrode solution for 30 min at 37 °C through the aorta. After 30 min of normoxia the hearts were subjected to 30 min of ischemia by using anerobic solution (90% N_2+10% Co_2). This was followed by 30 min reoxygenation and reperfusion. The hearts were allowed to beat spontaneously. After that left ventricle papillary muscles were isolated and transmembrane action potentials (APs) were recorded by Intrasys computer-analysing microelectrode system (Experimetria).

Results

In guinea pig's left ventricular papillary muscles BSO caused significant decrease of maximum rate in rise of depolarization phase (V_{max}) (Figure 1) and duration of AP (APD) at 25%, 50%, 90%

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of repolarization in both cases (acute, chronic) (Figure 1) of depletion and a slight but not significant decrease in the action potential amplitude, but did not modify the resting membrane potential. Pretreatment with bisaramil (PO:10 mg/kg) an antiarrhythmic drug (7) with possibly antioxidant properties (6) prevented the effect of BSO on APD in both cases of depletion (Fig. 1).

Discussion

The protocol used here to deplete the glutathione of the heart is similar to that used by Chatham et al. (4) for rat hearts. In the present work we have studied the effect of glutathione depletion by BSO on action potential of ventricular papillary muscles of guinea pigs. The results show that the predominant and most consequent, significant electrophysiological effect of acute and chronic glutathion depletion by BSO on ventricular muscles is the decrease of the duration of AP (APD). BSO also caused significant decrease of maximum rate in rise of depolarization phase (V_{max}) in both cases of depletion (Figure 1).

A slight, but not significant decrease in action potencial amplitude was also caused by BSO which may explaine the data reporting that depletion of cardiac glutathione led to a decline in function compared to untreated hearts (3, 4). As APA and one part of the plateau phase of cardiac AP can generally be considered as indirect indicator of the activity of slow L-type Ca^{2+} channels, and as V_{max} can be regarded as indirect indicator of the fast Na^+ channel activity and as the duration of cardiac AP is determined by a fine balance of different inward and outward currents the present data suggest that BSO may influence the Ca^{2+}, the Na^+ and K^+ channel activities in cardiac preparations. The fact that bisaramil prevented the BSO-induced APD shortening (Figure 1) seems to be interesting and may be important for further investigations into this drug. Summarising our study we can come to the conclusion that glutathione plays an important role in cardiac metabolism and function and its depletion may cause changes in electrophysiologic parameters and function of the heart.

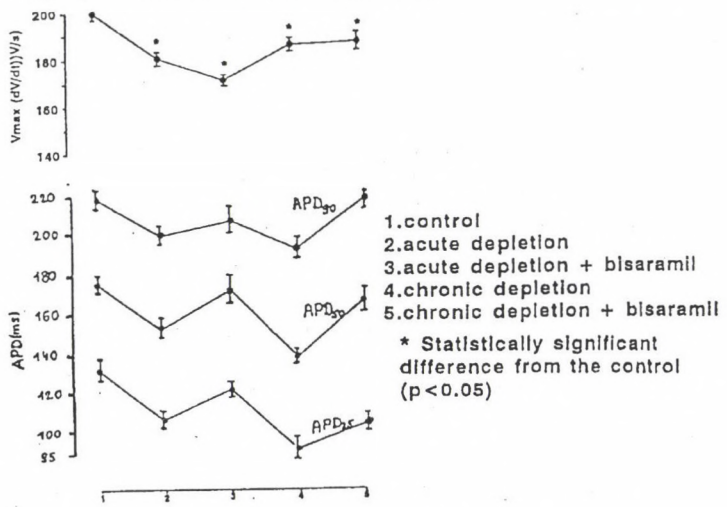

Fig. 1. Effect of glutathione depletion on electrophysiological characteristics of guinea pig ventricles

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INFLUENCE OF MCI-9042, A NOVEL 5-HT$_2$ RECEPTOR BLOCKER ON BLOOD VESSELS OF THE RAT

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Abstract: We demonstrated that MCI-9042 potently inhibited the vasoconstrictory effects of serotonin in the isolated perfused hindlegs of the rat. Concentrations of MCI-9042 in the range of 0.01–1 μmol/l caused a dose-dependent inhibition of the vasoconstrictory effect of serotonin (1–30 μg/0.1 ml). The maximal inhibitory effect of MCI-9042 was about 97% (1 μmol/l). MCI-9042 caused a concentration-dependent shift to the right of concentration-response curve to serotonin in the rat tail artery. The present data demonstrate MCI-9042 as a potent 5-HT$_2$ receptor antagonist.

Keywords: MCI-9042, serotonin, vessel system.

Introduction

It is obvious that serotonin can be an important mediator in the cardiovascular system. The normal subject efficiently removes serotonin from the circulation. In certain pathological conditions, however, the mechanisms of uptake, release, and metabolism of serotonin may be disturbed. Moreover, blood vessels may become hyperactive to the vasoconstrictor effects of serotonin, e.g., in hypertension, old age, and atherosclerosis (2). These observations provide a rationale to explore a highly selective 5-HT$_2$ receptor antagonist for therapy of cardiovascular disease. MCI-9042, (+)-1-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]-3-(dimethylamino)-2-propyl hydrogen succinate hydrochloride, is a newly synthesized compound which exhibits specific anti-5-HT2 receptor characteristics (1). The aim of the present study was to examine the influence of MCI-9042 on the vasoconstrictory response of rat vessels to serotonin.

Materials and methods

The experiments were performed on male Wistar rats (200–250 g). The preparation of the rat tail artery has been described in full elsewhere (3). Cumulative concentration-response curves were constructed by increasing the concentration of 5-HT in geometrical sequence. When a single response to a 5-HT was observed, and maximal effect reached, the bath solution was replaced by Krebs solution. The artery was then perfused with Krebs solutions containing MCI-9042 (Mitsubishi Kasei Corp.) for 30 min and the contractile effects of the agonist were again determined. Isolated perfused hindlegs of the rat was carried out according to Smid et al (4). *Statistical analysis.* Results were expressed as mean ± SEM. The data were evaluated using Student's t-test, and "p" values of less 0.05 were regarded as significant.

Results and discussion

Most smooth muscle organs constrict when exposed to serotonin, but their individual receptor type and sensitivity differs (1). In the isolated perfused hindlegs of rat the contractile response by serotonin is mediated predominantly by 5-HT$_2$ receptors. MCI-9042 potently inhibited the vasoconstrictory effects of serotonin in isolated perfused hindlegs of rat (Table 1). Concentrations of MCI-9042 in the range of 0.01–1 μmol/l caused a dose-dependent inhibition

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of the vasoconstrictory effect of serotonin (1–30 μg/0.1 ml). The maximal inhibitory effect of MCI-9042 was about 97% (1 μmol/l). As shown in Table 2 serotonin produced a concentration-dependent constriction of isolated tail arteries. MCI-9042 caused a concentration-dependent shift to the right of concentration-response curve to serotonin in the rat tail artery.

Table 1

Influence of MCI-9042 on the vasoconstrictory effect of serotonin in the isolated perfused hindlegs of the rat

	n	Concentration of serotonin [μg/0.1 ml]			
		1.0	3.0	10	30
Control	10	23.7±2.7	43.4±4.3	76.7±6.2	100.0±8.2
MCI-9042 0.1 nM/l	4	22.3±3.4	38.6±6.0	78.0±6.0	101.7±7.7
MCI-9042 1 nM/l	6	19.2±2.4	39.8±3.8	54.5±3.1*	76.7±8.6
MCI-9042 10 nM/l	5	9.4±1.2*	17.7±3.1**	39.5±4.6**	54.9±4.6*
MCI-9042 100 nM/l	5	2.3±1.2***	7.4±1.5***	10.0±1.7***	19.2±2.4***
MCI-9042 1 μM/l	5	0	0.9±0.9***	2.3±0.9***	3.4±1.0***

Value are given as percentage of the maximum response in the control. The data given as the mean ± SEM. Significance of the difference from the control: *p<0.05, **p<0.01, ***p<0.001

Table 2

Influence of MCI-9042 on the vasoconstrictory response of rat tail arteries to cumulative doses of serotonin

	Concentration of serotonin (μM/l)							
	0.1	0.3	1.0	3.0	10	30	100	300
Control	2.3±0.4	11.2±1.4	14.7±1.8	22.3±2.3	46.8±3.5	80.8±4.9	100.0±5.2	99.6±5.1
MCI-9042 0.01 μM/l	1.4±0.3	6.6±2.5	9.7±2.2	14.6±3.5	37.5±5.0	74.4±8.4	82.8±7.3	87.1±5.7
MCI-9042 0.1 μM/l	1.5±0.8 *	2.8±1.1 *	5.7±1.3 *	10.0±1.5	24.9±2.3 *	53.8±3.7 *	73.3±6.1	86.4±2.5 *
MCI-9042 1 μM/l	0	0.9±0.3 **	2.2±0.6 **	3.7±0.8 **	10.4±2.0 ***	31.7±3.2 ***	50.1±3.8 ***	69.0±5.2 ***
MCI-9042 10 μM/l	0	0	0.9±0.3 ***	1.5±0.4 ***	3.2±0.6 ***	4.8±0.8 ***	13.4±2.5 ***	17.2±3.2 ***

Value are given as percentage of the maximum response in the control. The data given as the mean ± SEM. Significance of the difference from the control: *p<0.05, **p<0.01, ***p<0.001

The compound did not affect the maximal response to serotonin (E_{max} control – 182.0± 9.5 mm Hg, E_{max} MCI-9042 0.1 μM – 156.5±4.6 mm Hg. These results suggest that MCI-9042 is a 5-HT2 receptor antagonist, exhibiting the inhibition of blood vessel constriction mediated by serotonergic receptor.

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EFFECT OF DV-7028, A NOVEL SEROTONIN 5-HT$_2$ RECEPTOR ANTAGONIST ON THE CARDIOVASCULAR SYSTEM IN RATS

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Abstract: The response of the cardiovascular system to DV-7028 demonstrated the complex action of this substance. In anaesthetized rats DV-7028 decreased the blood pressure and heart rate. These effects did not occur in pithed rats. The hypotensive action and bradycardia was partially reduced in vagotomized animals. DV-7028 potently inhibited the vasoconstrictory effects of serotonin in isolated perfused hindlegs of rat and caused a concentration-dependent shift to the right of response curve to serotonin in the rat tail artery. The present data demonstrate that DV-7028 is a potent 5-HT$_2$ receptor antagonist. Besides its peripheral action, DV-7028 exerts central effects which cause hypotension and bradycardia.

Keywords: DV-7028, cardiovascular system

Introduction

5-HT$_2$ receptors are suggested to be involved in cardiovascular diseases such as ischemic heart disease, peripheral vascular disease and cerebral vasospasm. These observations provide a rationale to explore a highly selective 5-HT$_2$ receptor antagonist for the therapy of cardiovascular diseases. DV-7028, (3-[2-[4-(4-fluorobenzoyl) piperidin-1-yl] ethyl]-6,7,8,9-tetrahydro-2H-pyridol [12,-a]-1,3,5-triazine-2,4 (3H)-dione maleate, is a newly synthesized potent and selective 5-HT$_2$ receptor antagonist, whereas it has no affinity for 5-HT$_{2A}$, 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. The affinity of the compound is 14–26 times greater for the 5-HT$_2$ receptors when compared to 5-HT$_{1C}$, adrenergic α1, dopamine D$_2$ and histamine H$_1$ receptors (2). The aim of the present study was to examine the influence of DV-7028 on the cardiovascular system.

Materials and methods

The experiments were performed on Wistar rats. Animals were anaesthetised with pentobarbital 75 mg/kg (i.p.). DV-7028 (Daiichi Pharmaceutical Co., Japan) was administrated i.v. in a dose of 1 mg/kg. The blood pressure and heart rate control, pithing of rats, and the preparation of the rat tail artery has been described in full elsewhere (1). Isolated perfused hindlegs of the rat was carried out according to Smid et al. (3). Results were expressed as mean ± SEM and were evaluated using Student's *t*-test.

Results and discussion

The response of cardiovascular system to DV-7028 demonstrated the complex action of this substance. In anaesthetized rats DV-7028 decreased the blood pressure and heart rate (Table 1). The hypotensive action and bradycardia was partially reduced in vagotomized rats since the reflex response is mediated by vagus nerves, we suggested that DV-7028 induced hypotension and bradycardia is partially induced by the reflex response of cardiovascular system. In our experiments we used the laboratory model of pithed rats. This allowed us to study only the peripheral influence of DV-7028 on vessels and heart. The hypotensive action and bradycardia did not occur in pithed rats. We suppose that DV-7028 possesses a complex action. Beside the known direct peripheral activity (selective 5-HT$_2$ receptor antagonist, blood platelets and the

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inhibitor of blood vessel constriction mediated by 5-HT_2 receptor stimulation) it possesses a central effect, causing hypotension and decrease heart rate after its intravenous injection. In the isolated perfused hindlegs of rat the contractile response to serotonin is mediated predominantly by 5-HT_2 receptors. DV-7028 potently inhibited the vasoconstrictory effects of serotonin in isolated perfused hindlegs of rat (data not shown). Stimulation of 5-HT_2 receptors also mediates the constriction of the rat tail artery (4). We showed that DV-7028 caused a concentration-dependent shift to the right of response curve to serotonin in the rat tail artery (Table 2). These findings indicate the competitive antagonism of DV-7028 against 5-HT_2 receptor. In conclusion, the present data demonstrate that DV-7028 is a potent 5-HT_2 receptor antagonist, and besides its peripheral action, DV-7028 exerts central effects which cause hypotension and bradycardia.

Table 1

Influence of DV-7028 (1 mg/kg i.v.) on blood pressure (RR) and heart rate (HR)

	Control n-10	DV-7028 1 mg/kg n-8	Vagotomy + DV-7028 n-6	Control pithed n-5	Pithed + DV-7028 n-4
Δ RR [mm Hg]	2.9±1.6	–33.0±4.6**	–19.0±4.3**$^{\#}$	1.2±0.6	1.3±0.2$^{\#\#\#}$
RR before exp. [mm Hg]	98.2±3.5	106.8±3.9	96.5±9.6	96.6±3.9	95.0±5.4
Δ HR [beats/min]	4.2±1.5	–57.6±5.4**	–17.3±6.1*$^{\#\#}$	2.0±0.6	1.5±0.8$^{\#\#\#}$
HR before exp. [beats/min]	391.7±6.7	405.0±6.8	391.3±12.7	295.8±7.9	291.8±9.7

The data given as the mean ± SEM. Significance of the difference from the control: *p<0.01, **p<0.001, and from the group receiving only DV-7028 (1 mg/kg iv): $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001

Table 2

Influence of DV-7028 on the vasoconstrictor response of rat tail arteries to cumulative doses of serotonin

	Concentration of serotonin (μM/l)							
	0.1	0.3	1.0	3.0	10	30	100	300
Control	2.3±0.4	11.2±1.4	14.7±1.8	22.3±2.3	46.8±3.5	80.8±4.9	100.0±5.2	99.6±5.1
DV-7028 0.01 μM/l	1.4±0.4	6.4±1.1 *	13.9±2.6	17.2±2.8	37.6±5.9	59.9±6.5 *	82.8±6.4	86.8±4.7
DV-7028 0.1 μM/l	0.5±0.3 *	1.5±0.6 **	4.0±1.0 **	8.4±1.5 *	23.0±1.8 **	52.2±2.0 **	71.8±5.7 *	79.2±5.7 *
DV-7028 1 μM/l	0	0	1.7±0.6 ***	5.8±1.2 ***	12.5±1.5 ***	19.2±2.2 ***	27.4±2.4 ***	38.9±3.3 ***
DV-7028 10 μM/l	0	0	0	0	0.4±0.3 ***	2.8±0.6 ***	6.6±1.0 ***	18.4±2.5 ***

The data given as the mean ± SEM. Significance of the difference from the control: *p<0.05, **p<0.01, ***p<0.001

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INFLUENCE OF CAPTOPRIL ON SOME HAEMOSTATIC PARAMETERS IN RATS WITH ONGOING PROCESS OF VENOUS THROMBOSIS

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Abstract: Recent laboratory findings strongly suggest that renin-angiotensin system plays an important role in regulation of haemostasis and fibrinolysis. In our previous study we showed that captopril exerts antithrombotic effect in venous thrombosis in rats. In this study we demonstrated that this effect is not a result of changes in platelet count, fibrinogen level alterations in blood coagulation pathways and fibrinolytic activity of the plasma. Further investigations are necessary to elucidate the mechanism of antithrombotic action of captopril.

Keywords: venous thrombosis, captopril, coagulation parameters, rat

Introduction

Pfeffer et al. showed that long-term administration of captopril to patients with left ventricular dysfunction after myocardial infarction reduces the rate of recurrent coronary thrombosis (3). The above finding may suggest potential antithrombotic action of this drug. Other studies demonstrated that captopril suppresses plasminogen activator inhibitor-1 activity and diminishes tissue plasminogen activator antigen level, what partially explain reduction in risk of reinfarction (1, 6). In our previous study we showed that captopril exerts antithrombotic effect in venous thrombosis in rats (2). Thus, we decided to investigate the influence of captopril on platelet count (PLT), fibrinogen (FIB) level, fibrinolytic system (euglobulin cloth lysis time; ECLT) and intrinsic and extrinsic pathway of blood coagulation measured by means of activated partial thromboplastin time (APTT) and prothrombin time (PT), respectively.

Materials and methods

Experiments were conducted on male Wistar rats 200–280 g of weight. The animals were housed as appropriate, were given tap water to drink and were fed a standard rat chow. 24 hours before venous thrombosis induction rats were deprived of food and had only free access to water. Animals received captopril chronically (25 mg/kg twice daily, per os, for 10 days) or acutely (100 mg/kg p.o. 1 h before venous thrombosis induction) or were given vehicle (the same volume and route). In chronically treated rats venous thrombosis was induced on the 11th day, 16 hours after the last captopril administration. Venous thrombosis was induced as previously described by others: rats were anesthetized with pentobarbital 45 mg/kg i.p., the abdomen was opened, vena cava was carefully cleaned from surrounding tissues and then tightly ligated with a cotton thread just below renal vein (4). Abdomen was closed with double layer of sutures (peritoneum with muscles and separately skin). After two hours blood for laboratory analysis was collected straight from the heart. FIB, APTT, PT and ECLT were measured with routine laboratory assays. Data were evaluated using ANOVA and post tests when appropriate. Significance was defined at a value of $p<0.05$. All data were expressed as the means ± S.E.M. and represented the results of at least 8 experiments.

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Results and discussion

In the present study we demonstrated that captopril did not alter PLT, FIB, APTT, PT and ECLT neither in acute nor in chronic administration in animals with ongoing process of venous thrombosis (Table 1). These findings show that antithrombotic action of captopril in venous thrombosis in rats reported in our previous study (2) is not a result of changes in platelet count, fibrinogen level; alterations in blood coagulation pathways and fibrinolytic activity of the plasma. This is in agreement with previous reports that other angiotensin-converting enzyme enalapril did not alter various coagulation parameters in humans (5) and that lisinopril administered chronically did not change platelet count in hypertensive subjects (7).

Table 1

Changes in PLT, FIB, APTT, PT, ECLT after acute and chronic captopril treatment

Group	PLT ($x\ 10^3/\mu l$)	Fibrinogen (mg/dl)	APTT (s)	PT (s)	ECLT (min)
Control	840±19	355±18	24±2	22±0.8	170±4
Captopril acute	832±29	365±23	24±1	21±0.5	176±24
Captopril chronic	846±12	332±15	30±1*	24±0.8	170±16

Results are means ± S.E.M. and represent the result of at least 8 separate experiments

This is also in concordance with our observation that antithrombotic action of captopril is mediated rather by nitric oxide (unpublished data) and that this property is, in main part, due to the presence of sulfhydryl group in the moiety of captopril (2). The exact mechanism of antithrombotic action of captopril therefore requires further investigations.

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EFFECT OF ENDOTHELIN-1 ON SOME HEMOSTATIC PARAMETERS IN NORMOTENSIVE RATS

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Abstract: Some parameters of hemostasis and fibrinolysis were investigated in rats administered with endothelin-1 (ET-1). ET-1 (0.5, 1.0, 5.0 nmol/kg) dose-dependently shortened the bleeding time (BT). Concomitantly significant shortening of the clotting time (CT) was observed. ET-1 produced prolongation of the activated partial thromboplastin time (APTT), whereas prothrombin time (PT) remained unchanged. ET-1 did not influence in vitro platelet aggregation induced by ADP and collagen. The euglobulin clot lysis time (ECLT) was significantly shortened after ET-1 administration. Our results suggest that ET-1 modulates the process of hemostasis and fibrinolysis in the rat.

Keywords: endothelin-1, hemostasis, fibrinolysis, rat

Introduction

ET-1 is claimed to be the most potent constrictor of both arterial and venous vessels yet identified (2, 3, 11). ET-1 binds to specific cell surface receptors (8, 9), stimulates the phosphoinositol pathway (8) and thereby causes the release of intracellular calcium stores (6, 8). It seems that changes in ionized calcium are important for the effect of endothelin. On the other hand, it is well known that changes in ionized calcium are very important for coagulation and blood platelets function. Therefore we can speculate that ET-1 may play a role in hemostasis. Recent data suggest that ET-1 inhibits rabbit and dog platelet aggregation in vivo (10), whereas no effect was observed in vitro (1). In addition ET-1 releases tissue plasminogen activator and prostanoids from the endothelium (7). This effect accounts for the increase in plasma fibrinolytic activity brought about by endothelin. The present study was designed to evaluate the role of ET-1 in hemostasis and fibrinolysis of the rat.

Methods

The studies were performed on 6–8 week old male Wistar rats (body weight 250–300 g). The animals received intravenously ET-1 in a dose 0.5, 1.0 and 5.0 nmol/kg. The tail bleeding time was determined using a method of Dejana et al. (4) 5 min after ET-1 administration. In other experiments blood was collected from ether-anesthetised rats by intracardiac puncture and decalcified with 3.13% sodium citrate, 1:9. The clotting time, the prothrombin time, the activated partial thromboplastin time and the euglobulin clot lysis time were measured by standard laboratory methods. All results were expressed as means ± standard error and statistical analysis was performed using the one way analysis of variance (ANOVA).

Results and discussion

The present study demonstrates that ET-1, a member of the endothelin family caused dose dependent changes in the hemostatic and fibrinolytic system of the rat (Table 1). Pretreatment of rats with ET-1 produced dose-dependent shortening of the bleeding time. It seems that ET-1 interacts with platelets and blood vessels. ET-1 is well known constrictor of both arterial and venous vessels from many species (3, 11). Moreover, ET-1 increases endothelial expression of

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von Willebrand factor, which facilitates the attachment of platelets and thrombus formation (5). The animals obtaining ET-1 showed significantly prolonged APTT and shortened the CT. The results of our study clearly show for the first time that ET-1 interacts with the coagulation phase of hemostasis. Since ET-1 caused changes in APTT having no effect on PT, it seems that this peptide participates in the regulation of intrinsic coagulation system.

The mechanism by which endothelin significantly shortens the clotting time and prolongs APTT remains to be elucidated. Recent data suggest the ET-1 modulates fibrinolytic system of a variety of species (7). In the present experiments we have shown that administration of ET-1 caused significant shortening of ECLT. This observation is in line with data described by Lidbury et al. (7). Taking all these data into account, a possible role of ET-1 in hemostasis and fibrinolysis of the rat is suggested.

Table 1

Effect of ET-1 on some hemostatic parameters

Dose of ET-1	BT (sec)	CT (min)	PT (sec)	APTT (sec)	ECLT (h)
0	248±42	6.3±1.1	16±0.6	24±1.0	5.4±1.2
0.5 nmol/kg	211±27	6.0±1.4	17±0.4	28±0.9*	5.2±0.9
1.0 nmol/kg	180±30*	5.3±1.8*	17±0.5	30±0.8**	4.9±0.7*
5.0 nmol/kg	181±23*	5.1±1.7*	16±0.3	32±0.8**	4.5±0.9*

*$p<0.05$, **$p<0.01$

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NON-ADRENERGIC REGULATION OF MICROCIRCULATION EVOKED BY ANTIDROMIC STIMULATION OF THE SAPHENOUS NERVE IN THE RAT SKIN

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Abstract: Electrical stimulation of the peripheral stump of the cut and perineurally capsaicin-pretreated saphenous nerve evokes antidromic vasodilatation preceded by a short vasoconstriction in the dorsal skin of the hindpaw in the rat. These microcirculatory changes were measured by laser-Doppler flowmetry. Blood flow increase induced by nerve stimulation was completely abolished by 1 μg/kg resiniferatoxin (RTX), while the inicial blood flow decrease was significantly reduced or totally inhibited by subsequent treatments with an α adrenergic receptor antagonist (GYKI-12743) and a neuropeptide Y functional antagonist (α-trinositol) in response to 10 Hz and 3 Hz stimulations, respectively.

Keywords: antidromic vasodilatation, laser-Doppler flowmetry, neuropeptide Y

Introduction

Changes in microcirculation evoked by electrical stimulation of peripheral nerves are due to vascular actions of neurotransmitters released from afferent and autonomic efferent nerve endings. The aim of the present study was to determine the role and origin of the released neuromediators which could participate in the regulation of cutaneous microcirculation.

Methods

Perineural pretreatment with capsaicin: Wistar rats (200–300 g) were anaesthetised with 40 mg/kg pentobarbital i.p. The left saphenous nerve was perineurally pretreated with capsaicin (2% solution was applied for 30 min). *Laser-Doppler flowmetry:* 5–7 days after the perineural capsaicin pretreatment the experiment was made in thiopental-sodium (Trapanal) anaesthesia (100 mg/kg s.c.). One carotid artery, an external jugular vein and the trachea were cannulated for injecting drugs, recording the arterial blood pressure and ventillation, respectively. The left saphenous nerve was reexposed, cut, and the peripheral stump was stimulated (20 V, 0.5 ms, 3 or 10 Hz, 100 pulses). Cutaneous blood flux of the dorsal skin of the hindpaw was detected by a laser-Doppler flowmeter (Moor Instruments Ltd, England, MBF3D). Physiological parameters (blood pressure, heart rate, respiration, respiration rate and microcirculation) were recorded by a specific computer program (Geopolita Ltd, Hungary). For quantitative evaluations the integrated blood flux responses were compared to the prestimulated control values and expressed as percent changes in cutaneous blood flux.

Results

After perineural capsaicin pretreatment electrical stimulation of the peripheral stump of the cut saphenous nerve evoked a moderate, late antidromic vasodilatation preceded by a short decrease of the blood flux. The long-lasting vasodilatatory response was completely abolished by subsequent l μg/kg i.v. resiniferatoxin (RTX). The remained vasoconstrictor response evoked by both 3 Hz and 10 Hz stimulation was significantly reduced by a new selective postsynaptic α-adrenergic blocking agent (GYKI-12743, 1 mg/kg i.v.). The rest of the vasoconstriction was

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markedly decreased by 50 mg/kg i.v. α-trinositol, a non-peptide NPY antagonist (Perstorp Pharma, Sweden). Guanethidine (8 mg/kg, i.v.) completely abolished the vasoconstrictor effect of nerve stimulation (Tables 1, 2).

Discussion

It is concluded that perineural capsaicin pretreatment is insufficient to completely abolish antidromic vasodilatation. The remaining response is eliminated by additional acute treatment with RTX. Vasoconstrictor response is mediated by sympathetic adrenergic fibres.

The incomplete inhibition of α-adrenoceptor antagonists and the potent effectiveness of α-trinositol suggest an important role for NPY in sympathetic regulation of cutaneous microcirculation.

Table 1

Effect of adrenergic blocking agents on the decrease in cutaneous microcirculation in response to saphenous nerve stimulation. Inhibition in % of control response. (Antidromic vasodilatation was abolished by capsaicin and RTX)

	3 Hz	10 Hz
Phentolamine 1 mg/kg	37.4	4.8
Prazosin 0.5 mg/kg	42.2	30.2
GYKI-12743 0.5–1 mg/kg	65.8	36.1
Propranolol 0.01 mg/kg	1.6	2.9
Guanethidine 8 mg/kg	100.0	100.0

Table 2

Effect of GYKI-12743 (a potent α-blocking agent) and α-trinositol (a functional NPY-antagonist) on the decrease of cutaneous microcirculation in response to saphenous nerve stimulation. Inhibition in % of control response (antidromic vasodilatation was abolished by capsaicin and RTX)

	3 Hz	10 Hz
GYKI-12743 0.5–1 mg/kg	62.68	37.47
GYKI-12743 0.5–1 mg/kg + α-Trinositol 50 mg/kg	89.92	82.57

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Acta Physiologica Hungarica, Volume 84 (3), pp. 333–335 (1996)

DOSE-DEPENDENT INTESTINAL AND HEPATIC GLUCURONIDATION AND SULFATATION OF P-NITROPHENOL IN THE RAT

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Abstract: The jejunum was able to metabolize p-nitrophenol (PNP) rapidly and to transport the metabolites efficiently back into the luminal solution. About 21, 16, 6 and 3.5% of recirculated amount of PNP could be detected in 90 minutes as glucuronide in the lumen of jejunal loop, when 20, 100, 500 or 1,000 μM PNP was perfused, which shows that the luminal appearance of p-nitrophenol-glucuronide (PNP-G) tended to saturability. Biliary excretion rate of PNP-G was lower than the luminal appearance of this metabolite, when PNP was recirculated at 20 or 100 μM concentrations, however, at higher concentrations (500, 1,000 μM) the biliary excretion exceeded the luminal appearance of this conjugate and no saturability was observed in the biliary glucuronidation of PNP. Biliary excretion of sulfate conjugate of p-nitrophenol (PNP-S) exceeded the luminal appearance of this metabolite.

Keywords: glucuronidation, sulfatation, p-nitrophenol, rat

Introduction

The role of hepatic biotransformation and biliary excretion of drugs has been already studied and it was presently recognized that intestinal metabolism and excretion may also influence the disposition of xenobiotics [1, 2, 3, 4, 5, 6]. The present investigations were designed to study the intestinal and hepatic glucuronidation and sulfatation of PNP and transport of these metabolites into the intestinal lumen and the bile simultaneously to compare the relative importance of intestinal and hepatic elimination of drugs. It is known that is metabolized almost exclusively by conjugation with glucuronic acid and sulfate, therefore it can be used as a model compound to study the role of these types of drug biotransformation.

Methods

Male Wistar rats weighing 220–250 g, were used. The animals were unaesthetized with urethane (1.2 g/kg i.p.), the abdomen was opened and a jejunal loop was cannulated. Perfusion through the lumen of intestine with isotonic medium containing different concentrations (20, 100, 500 and 1,000 μM) of PNP was carried our at a rate of 13 ml/min in a recirculation mode. For the investigation of biliary excretion the bile duct was cannulated with a polyethylene (PE-10) tubing and bile was collected. Concentrations PNP of and its metabolites were determined in samples obtained from the recirculated intestinal solution and bile by HPLC using external standards. Data are expressed as the mean ± SEM.

Results

Dose-dependent changes of the cumulative luminal appearance of PNP-G are demonstrated in Figure 1. Luminal appearance of PNP-G was elevated by increase of the dose of PNP, however, it tended to saturability: 5-fold higher PNP concentration (100 μM) produced 3–8 fold increase compared to the value measured at 20 μM PNP concentration in 90 minutes, it was found 76% difference between the values measured at 100 and 500 μM PNP and only 15% increase was observed when the PNP concentration was elevated from 500 up to 1,00 μM. Luminal

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appearance of PNP-S is not shown in Figure 1, because these values were very low, undetectable at lower (20, 100 μM) PNP concentrations.

Dose-dependent biliary excretion of PNP, PNP-S and PNP-G after luminal perfusion of PNP is shown in Figure 2. Biliary excretion rate of PNP was low in unchanged form the highest rate was measured at PNP-G. No saturability was found in are intestinal, glucuronidation of PNP. Biliary excretion of PNP-S was lower than that of PNP-G, however, it exceeded the luminal appearance of this metabolite.

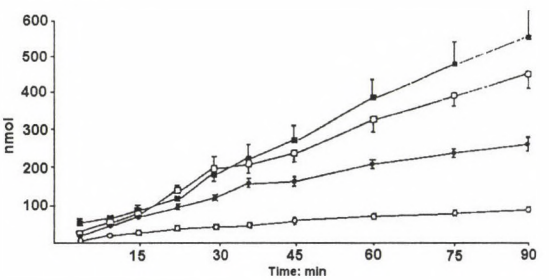

Fig. 1. Dose-dependent luminal appearance of PNP-G after luminal perfusion of PNP in a concentration of 20 (○—○), 100 (●—●) 500 (□—□) and 1,000 (■—■) μM

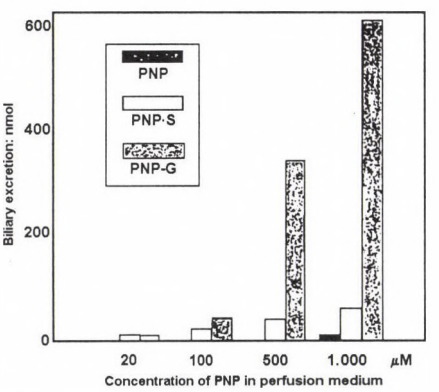

Fig. 2. Dose-dependent biliary excretion of PNP, PNP-S and PNP-G after luminal perfusion of PNP in a concentration of 20, 100, 500 and 1,000 μM

Discussion

These observations show significant qualitative and quantitative differences between the intestinal and hepatic elimination of PNP after luminal perfusion of a jejunal loop in the rat. These findings indicate that the small intestine may play an important role in the overall elimination of phenolic drugs after oral or luminal administration.

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ON THE CARDIOPROTECTIVE EFFECT OF ADENOSINE

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Abstract: In isovolumically perfused Langendorff heart preparations of guinea pigs adenosine – depending on the experimental protocol – more or less could prevent the hypoxia-induced decrease in myocardial adenosine triphosphate [ATP], creatine phosphate [CP], glycogen and increase in lactate, i.e. showed cardioprotection.

Keywords: hypoxia, adenosine, cardioprotection

Introduction

It is supposed that one of the possible mediators of the phenomenon called "preconditioning" is adenosine (1). The aim of our present work was to test this hypothesis and to study the effect of adenosine in isovolumically perfused Langendorff heart preparations of guinea pigs during hypoxia and reoxygenation. The prevention of the hypoxia-induced changes in biochemical parameters [myocardial ATP, CP, lactate and glycogen] was regarded as the criterion of cardioprotection.

Methods

For biochemical analysis, isovolumically perfused Langendorff heart preparations of guinea pigs were used under normoxic (95% O_2 + 5% CO_2) and hypoxic (95% N_2 + 5% CO_2) conditions. Hearts were excised from anesthetized, heparin-treated (1000 U/kg) guinea-pigs, moved quickly to a Langendorff apparatus and perfused at a constant flow rate of 12 ml/min with Krebs solution. In the 1st protocol, the 20 min normoxic preperfusion was followed by a 20 min duration of normoxic or hypoxic perfusion. In the 2nd protocol, a 120 min normoxic preperfusion was followed by a 20 min duration of normoxic or hypoxic perfusion, while in the 3rd protocol the 20 min normoxic preperfusion +20 min of normoxic or hypoxic perfusion were followed by a 30 min duration of reoxygenization. The experiments were terminated by freeze-clamping the ventricles used for biochemical determinations (Fig. 1).

Adenosine (Sigma) at a concentration of 15 μmol/L was present in the nutrient solution during the full experimental period. For the statistical analysis of the results, non-parametric methods (Kruskal-Wallis test and multiple comparisons according to Conover ref. 5) were used.

Results and discussion

In the myocardium of guinea pigs perfused according to the 1st protocol, hypoxia significantly reduced ATP, CP, glycogen and increased lactate. Of these four hypoxia-induced changes, only the decrease in CP and increase in lactate remained significant after a prolonged preperfusion (2nd protocol). In the 3rd protocol hypoxia caused significant decreases in ATP and glycogen levels.

In normoxia, in the 1st and 2nd protocol adenosine did not change significantly the biochemical parameters measured. In the 3rd protocol, however, it significantly elevated ATP, CP and decreased lactate.

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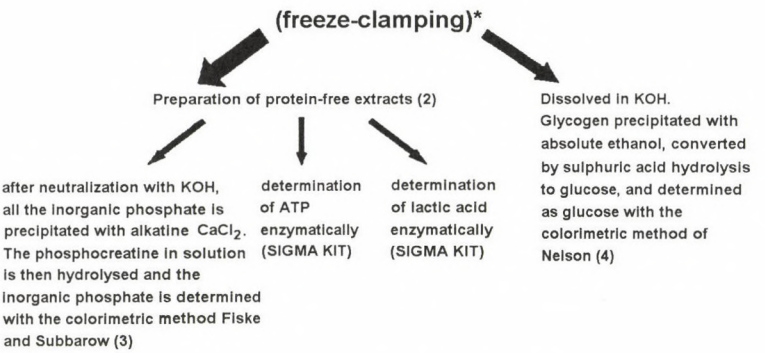

Fig. 1. Preparation of tissue for analysis

In hypoxia, in the 1st protocol adenosine could not prevent the hypoxia-induced decrease in ATP and CP and increase in lactate: the only manifestation of its cardioprotective effect in this protocol was the prevention of hypoxia-induced glycogen depletion. Prolongation of the normoxic preperfusion (120 minutes in the 2nd protocol) has resulted in no protection. In the 3rd protocol, however, adenosine could completely prevent the hypoxia-induced decrease in ATP, CP and glycogen and increase in myocardial lactate.

Conclusion

On the basis of our results, in accordance with the results of preliminary experiments with non-metabolizing adenosine-analogues, a metabolic rather than a receptorial effect of adenosine is supposed to be in the background of the protection.

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REGULATION OF PURINOCEPTORS IN GUINEA PIG PULMONARY ARTERY: FUNCTIONAL EVIDENCES

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Abstract: In isolated guinea pig pulmonary arteries (precontracted with 1 μM noradrenaline) N^6-cyclopentyladenosine (CPA), a selective A_1 adenosine receptor agonist, exerted a concentration-dependent contraction, whereas 5'-N-ethylcarboxamidoadenosine (NECA), a non-selective A_1/A_2 receptor agonist, in the presence of DPCPX (a highly selective A_1 receptor antagonist), produced a concentration-related rapid relaxation. Pulmonary arteries obtained from guinea pigs treated with aminophylline (APH) or 8-phenyltheophylline (8-PT) for 10 consecutive days, displayed more pronounced contraction in response to CPA compared to those of solvent-treated animals. Relaxant action of NECA was, however, attenuated in arteries prepared from methylxanthine-treated guinea pigs. Opposite changes were found in vascular tissues excised from chronically dipyridamole(DP)-treated guinea pigs.

Keywords: pulmonary artery, regulation of adenosine receptors, methylxanthines

Introduction

A number of studies have demonstrated the regulation of adenosine receptors under chronic treatment with adenosine receptor agonists or antagonists. The bulk of the papers, however, has been restricted to investigation of brain purine receptors and there have been only a few studies of adenosine receptor regulation in the cardiovascular system (for refs see 1, 2). The aim of the present study was to evaluate the action of chronic treatment with methylxanthines (adenosine receptor antagonists) and dipyridamole (a substance elevating the endogenous tissue adenosine concentration by inhibiting the membrane adenosine transporter) on A_1 and A_{2b} receptor-mediated functional responses of guinea pig pulmonary artery.

Methods

Female guinea pigs (320–480 g) were injected i.p. once daily with either 25 mg/kg APH (n=5), 2.5 mg/kg 8-PT (n=9), 1 mg/kg DP (n=6) or DMSO as solvent (n=5) for 10 consecutive days. Treatments were discontinued 48 h before the animals were killed and the pulmonary arteries were removed. Circular segments were prepared from the proximal part of the main pulmonary arteries according to the method described earlier (3). The preparations were mounted in an organ chamber containing Krebs solution bubbled with 95% O_2 and 5% CO_2 (37 °C). Mechanical responses were measured via an isometric mechano-electric transducer and recorded on chart recorder.

Results

In pulmonary arteries obtained from solvent-treated guinea pigs (precontracted with 1 μM noradrenaline) CPA induced a concentration-dependent (1 nM–100 μM) contractile response. As shown in Fig. 1, long-lasting *in vivo* APH pretreatment increased the sensitivity of the vessels at lower (1–10 nM) concentrations of CPA. In vascular tissues obtained from 8-PT-treated guinea pigs an enhancement of the contractile response was found at each concentration of CPA and the maximal effect of CPA was also increased.

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As described previously (3) in guinea pig pulmonary arteries, in the presence of 0.3 μM DPCPX, NECA induces a rapid, A_{2b} receptor-mediated relaxation. In the present study, –log EC_{20} values (EC_{20} refers to the concentration of NECA required to relax the noradrenaline-induced contraction by 20%) for NECA in pulmonary arteries obtained from solvent-, APH-, 8-PT- and DP-treated guinea pigs were 5.52±0.05, 5.20±0.09 ($p<0.05$), 4.76±0.28 (<0.05) and 5.91 ±0.10 ($p<0.01$), respectively.

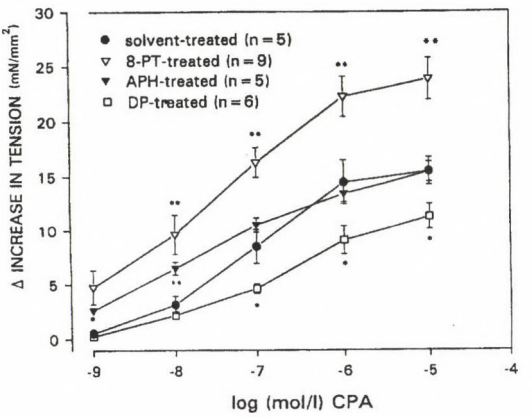

Fig. 1. Contractile action of N^6-cyclopentyl adenosine on pulmonary arteries obtained from guinea pigs in vivo pretreated with aminophylline, 8-phenyltheo-phylline, dipyridamole and solvent. Statistically significant differences with the solvent-treated controls: * $p<0.05$, ** $p<0.01$

Discussion

The results presented here clearly demonstrate that long-lasting treatment with adenosine receptor antagonists enhances the susceptibility of A_1 receptors to CPA, a specific A_1 receptor agonist, whereas significantly attenuates the A_{2b} receptor-mediated vascular responses. These findings are in concert with the previous data according of which A_1 adenosine receptors have been upregulated both in the brain and the heart after chronic exposure to methylxanthines (1, 2). To our knowledge there are no data available about regulation of A_{2b} receptors in vascular tissues.

Long-lasting treatment with DP, possibly by elevating the extracellular adenosine concentration, suppressed the CPA-induced A_1 receptor-mediated contractile response of pulmonary vascular tissues, while the rapid relaxation, elicited by activation of A_{2b} receptors, was enhanced.

It has been reported that A_1 and A_2 purinoceptors could be regulated differentially (4). Our present results provide functional evidence for this differential regulation, but the exact mechanism of this process remains to be elucidated.

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PROLONGED TREATMENT WITH ANTIDEPRESSANTS INCREASES THE 5-HT_{1A}-MEDIATED INHIBITION OF HIPPOCAMPAL NEURONS WITHOUT CHANGING THE 5-HT_{1A} RECEPTOR BINDING

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Abstract: The effect of repeated treatment with antidepressant drugs imipramine, (+)oxaprotiline and paroxetine on neuronal responsiveness to 5-HT and the 5-HT_{1A} receptor agonist 8-OH-DPAT was examined in the hippocampal slice preparation from the rat. 5-HT and 8-OH-DPAT decreased the amplitude of population spikes evoked in the CA1 cell layer by electrical stimulation of the stratum radiatum. The antidepressant drugs, administered for 2 weeks, produced a significant increase in the inhibitory effect of 5-HT and 8-OH-DPAT. Repeated treatment with imipramine did not change the density of 5-HT_{1A} receptors in the hippocampus suggesting that the increase in 5-HT_{1A} responsivity may not involve an increase in the receptor density.

Keywords: antidepressants, 5-HT, 8-OH-DPAT, 5-HT_{1A} receptors, hippocampal slice

Introduction

It has been established that central serotonergic (5-HT) systems are involved in the pathophysiology of major depression. As a result, it has been a matter of considerable interest to investigate adaptive changes in the serotonergic transmission following long-term treatment with antidepressants (4). 5-HT_{1A} receptors may be involved in the antidepressant action, since 5-HT_{1A} receptor agonists show antidepressant-like effects in some animal models (5), as well as in clinical studies (1). Localization of the 5-HT_{1A} receptors that mediate the putative antidepressant effect is still a matter of dispute, yet it has been suggested that postsynaptic 5-HT_{1A} receptors may be involved (5). The density of postsynaptic 5-HT_{1A} binding sites is particularly high in such limbic areas as the hippocampus, septum or entorhinal cortex. In these areas, activation of 5-HT_{1A} receptors has an inhibitory effect on principal neurons due to a membrane hyperpolarization mediated by an increase in potassium conductance (2).

We studied the effect of repeated treatment with antidepressant drugs on the 5-HT_{1A} receptor-mediated inhibition of a population spike in the rat CA1 hippocampal region ex vivo. Three drugs were chosen for the study: imipramine, a classic tricyclic antidepressant (a 5-HT and norepinephrine uptake inhibitor), (+)oxaprotiline (a selective norepinephrine uptake inhibitor) and paroxetine (a selective 5-HT uptake inhibitor).

Methods

The experiments were performed on male Wistar rats. Antidepressants were administered twice daily for 14 days (10 mg/kg, PO) or acutely. The rats were killed 48 h after the last dose. Non-treated animals served as a control. Hippocampal slices were prepared, transferred to a recording chamber and the response of CA1 neurons to electrical stimulation of Schaffer collaterals was recorded extracellularly. 5-HT and 8-OH-DPAT were applied by superfusion. Autoradiography was performed using [^{3}H]8-OH-DPAT and quantified by a computerized densitometry.

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Results

5-HT and the 5-HT_{1A} receptor agonist 8-OH-DPAT decreased dose-dependently the amplitude of population spikes; that effect was blocked by the selective 5-HT_{1A} receptor antagonist (S)-WAY 100135. Repeated (14 days, twice daily), but not single, administration of the antidepressant drugs (10 mg/kg) enhanced the effect of 5-HT_{1A} receptor activation. While studied in detail, imipramine shifted the dose-response curves for serotonin and 8-OH-DPAT to the left.

Having found an increase in the response to the 5-HT_{1A} receptor activation we wanted to ascertain whether the functional changes resulted from an increase in the binding of 5-HT_{1A} receptors. Repeated treatment with imipramine did not change the density of 5-HT_{1A} receptors in the hippocampus as measured by autoradiography.

Discussion

Our results suggest that repeated treatment with antidepressant drugs of different mechanism of action induces sensitization to the inhibitory effects of 5-HT_{1A} receptor agonists in the hippocampus. The antidepressant-induced increase in the responsiveness of hippocampal neurons to stimulation of 5-HT_{1A} receptors may not involve an increase in the density of this receptor subtype, as suggested by our autoradiography data. The observed effect of repeated treatment with antidepressants may result from an increased efficacy of 5-HT_{1A} receptor transducing mechanism i.e. the G-protein coupling to the K-channel. This would be in line with the postulated effects of antidepressants on cell signalling via direct action on G-proteins (6). If such an effect is induced by the studied antidepressants, it seems to be specific to 5-HT_{1A} receptors since we demonstrated (3) that the inhibitory effect of the $GABA_B$ receptor agonist baclofen, which also hyperpolarizes CA1 cells by activating the same type of the G-protein-linked K-channel as does 5-HT, is not affected by treatment with imipramine.

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THE EFFECT OF CHRONIC DIAZEPAM TREATMENT ON HYPOXIA-INDUCED ALTERATIONS IN FUNCTIONAL ACTIVITY OF GUINEA PIG MYOCARDIUM

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Abstract: The concentration-related sensitization of guinea pig left atrium to adenosine in the presence of diazepam is well established. It was found in our experiments that the cardiodepressive action of hypoxia is significantly enhanced by diazepam in the left atrial myocardium. In atrial preparations obtained from guinea pigs treated with diazepam for 10 days, the hypoxia-induced depression of myocardial contractility was not altered. These results indicate that diazepam-treatment does not impaire the hypoxic tolerance of myocardium.

Keywords: adenosine, myocardium, diazepam, hypoxia, reoxygenation

Introduction

It has been shown that under hypoxic conditions the increased tissue level of adenosine contribute to the functional impairment of the guinea pig myocardium (4). The removal of adenosine from the vicinity of its receptor by adenosine transporter is inhibited by benzodiazepines resulting an increased adenosine concentration at the site of the action (2). During the last decade a number of studies have supported that benzodiazepines potentiate the action of adenosine on cardiac and smooth muscle (1), as well as cortical neurons (3). The aim of our present experiments was to investigate, whether acute *in vitro* and chronic *in vivo* treatment with diazepam could influence the responsiveness of myocardium to hypoxia and subsequent reoxygenation.

Methods

Experiments were carried out in isolated, electrically driven (3 Hz, 1 ms, at twice threshold voltage) left atrial myocardium of female guinea pigs (350–470 g). The preparations were mounted in an organ chamber containing Krebs solution bubbled with 95% O_2 and 5% CO_2 during normoxia, with 95% N_2 and 5% CO_2 under hypoxia and 30% O_2–65% N_2 and 5% CO_2 under moderate hypoxia (for analyzing the acute action of diazepam) (37 °C). Mechanical responses were recorded via an isometric mechano-electrical transducer. The animals were injected with 10 mg/kg/day diazepam intraperitoneally for 10 consecutive days, while for control guinea pigs DMSO as solvent was administered. Treatments were discontinued 48 h before the animals were killed.

Results

Adenosine produced a concentration-dependent depression of the contractile force of guinea pig left atria. Adenosine dose-response curve was significantly shifted to the left in the presence of different concentrations (3–30 μM) of diazepam (not shown).

The hypoxia-induced depression of myocardial contractility was significantly enhanced by 30 μM diazepam in specimens excised from untreated animals. It is to be noted that recovery

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of the contractile force was significantly stronger in the early phase of reoxygenation compared to that of the control (Fig. 1a).

In contrast to the results found in the acute *in vitro* experiments with diazepam, there were no differences in the hypoxia-induced decline of contractile tension in myocardial tissues removed from guinea pigs chronically treated with diazepam and solvent. The recovery of contractile force during reoxygenation was, however, improved in diazepam-treated specimens (Fig. 1b).

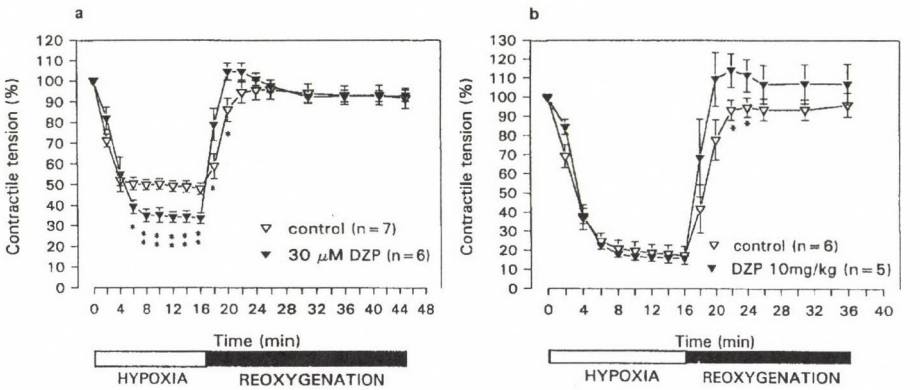

Fig. 1. Effect of hypoxia and reoxygenation on the mechanical activity of atrial myocardium obtained from (a) untreated and (b) diazepam (DZP)-treated guinea pigs

Discussion

Our results obtained from *in vitro* experiments are consistent with the previously demonstrated potentiating effect of benzodiazepines on adenosine actions in myocardium (4). Under hypoxic conditions the more pronounced decrease in myocardial contractility in the presence of diazepam (as adenosine-uptake inhibitor) supports the hypothesis that adenosine can play a role in the mechanical insufficiency of hypoxic myocardium. The findings of our recent study conducted on myocardial preparations, removed from diazepam-treated animals, clearly show that the hypoxic tolerance of myocardium has not been modified. The rate of recovery on the reoxygenation was, however, slightly improved. Therefore, our present data does not confirm the hypothesis (5) according which previous diazepam intake impairs the ischaemic damage of the heart in human.

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THE ROLE OF NMDA RECEPTORS IN CENTRAL ACTION OF ANGIOTENSIN II

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Abstract: The influence of noncompetitive (MK-801), competitive (AP-7) and the antagonist of polyamines site of NMDA receptor (arcaine) on the central activity of angiotensin II (A II) was studied. The open field test, conditioning of active avoidance responses (CARs) and passive avoidance situation was used to investigate learning and memory in rats. All used antagonists decreased beneficial action of A II on these processes.

Keywords: angiotensin II, MK-801, AP-7, arcaine, memory and learning

Introduction

NMDA receptor and exciting amino acids play an essential role in memory and learning. Angiotensin II (A II) – the peptide existing in central nervous system (CNS) has also beneficial effect on these processes (1, 2, 3, 4). The mechanism of action of A II in memory and learning is not precisely known. Central action of A II may be connected not only with direct activation of specific receptors. Effects of A II are partially mediated by dopaminergic receptors and adrenergic receptors (4). One indicates the colocalization of A II and glutaminic acid in neuronal terminations in hippocampus (4). So it is very interesting to study an interaction between A II and NMDA receptor in learning and memory. It may be that the beneficial effect of A II on these processes is exactly connected with glutaminergic system.

Methods

Male Wistar rats (160–180 g) were prepared according to the standard surgical procedure. All behavioural experiments were carried out in a standard conditions. Locomotor and exploratory activity of rats was measured in an "open field" which was a square 100 cm × 100 cm of white floor divided by 8 lines into 25 equal squares and surrounded by a 47 cm high walls. Crossings, rearings and bar approaches were counted manually for 5 min.

CARs were studied in a shuttle-box (60×28×24 cm) divided in two equal parts by a wall 6 cm wide and 8 cm high, opening in the middle of its length. CAR acquisition training consisted of 5 daily 20-trial sessions. The rats using in this experiment were preselected: each rat was tested for 3 days with 10 trials per day. The number of (+) CARs was recorded every day and expressed as per cent of the total number of trials.

Passive avoidance behaviour was studied in a one trial learning, step-through situation by the method of Ader et al., which utilizes the natural preference of rats for a darkness.

Statistical comparisons were made by the analysis of variance (ANOVA) followed by Newman–Keuls test, when multiple means were compared (active avoidance situation and "open field" test). The medians (passive avoidance situation) were compared by the Kruskal–Wallis nonparametric analysis of variance, and in the subsequent analysis of differences between groups nonparametric Mann–Whitney U test was used. In all comparisons between particular groups a probability of 0.05 was considered significant.

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Results

A II did not change locomotor activity of rats in open field test. MK-801 enhanced mobility of animals in this test and after using both compounds locomotor activity was also enhanced. AP-7 given before A II caused that the number of crossed fields and bar approaches was significantly bigger then in control group. Arcaine did not change locomotor activity of rats. MK-801 given alone significantly impaired the acquisition of conditioned avoidance responses (CARs) in rats in comparison to the control group. It also significantly decreased the beneficial action of A II on learning in CARs. AP-7 and arcaine given before A II significantly diminished the action of peptide in CARs. MK-801 and AP-7 did not influence on the remembering and retrieval of passive avoidance behaviour, when they were given alone, but decreased the possitive effect of A II in these tests. Arcaine diminished the improving effect of A II on the remembering of passive situation.

Discussion

The obtained results show that competitive (AP-7), noncompetitive (MK-801) and antagonist of polyamines site of NMDA receptor (arcaine) influence on central activity of angiotensin II. This influence is generally inhibitive. They diminish beneficial effects of A II on learning and memory in all used tests. MK-801 more strongly then AP-7 decreased the action of A II in active avoidance behavioural test. MK-801 is known to form its own activity (5). It may interact with dopaminergic system, causes hyperactivity of animals in lower doses (up to 0.1 mg/kg), produces muscle relaxation and ataxia (5). In our investigations MK-801 given icv enhanced locomotor activity of rats in "open field" test. MK-801 given alone did not influence on learning and memory in passive avoidance situation, but significantly diminished beneficial action of A II in this test. AP-7 also did not evoke significant effect in this test, when was given alone, but also diminished beneficial action of A II on learning. The conclusion of our investigations is that antagonists of NMDA receptor diminished beneficial action of angiotensin II on learning and memory processes. So it suggests that glutaminergic system may be engaged in central activity of A II. In memory and learning processes are included many other neurotransmitters (NO, GABA, vasopressin, ACh, katecholamines) (4). The balance between them is very complicated. Angiotensin II stimulates the vasopressin release, which also improves memory (2, 4). The colocalization of angiotensin-vasopressin receptor was shown (1, 4). The connection of all compounds engaged in memory and learning processes is impressing and it is difficult to say which system of transmitters is most important.

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Book reviews

Charis Roussos (Ed.)
The Thorax

Lung Biology in Health and Disease (Ex. ed. Claude Lenfant). Volume 85.
Marcel Dekker, Inc., New York – Basel – Hong Kong. 1995. Second edition (revised and expanded).
2880 pages, illustrated. In three parts including Part A: Physiology, Part B: Applied Physiology,
Part C: Disease. Price: U.S. $495 (sold only as a set).
ISBN#: 0-8247-9647-0

The 91 chapters of this three-part book edited by Charis Roussos provide a comprehensive and state-of-the-art overview of the physiology (Part A) and pathophysiology (Part B) of the chest wall as well as an overview of the diagnostic and therapeutic modalities (Part C). The first edition of The Thorax was published about a decade ago, and since then the literature relating to the chest wall expanded rapidly. The second edition contains almost double number of pages to reflect this trend. It was completed with the help of over 125 authors, who are highly respected experts of their respective fields. The individual chapters are integrated into a book, which can be regarded as an encyclopaedia of the thorax.

The Thorax, Second Edition successfully integrates recent spectacular successes of molecular biology and genetics with the physiology and clinical aspects of the thoracic function. The first volume, Part A, updates information on the striated muscles and relates it to the respiratory muscles (Chapters 1–12), leading to the complex subsequent chapters on mechanics and energetics (Chapters 13–25) and control (Chapters 26–34) of respiratory muscles. The second volume, Part B, reviews extensively the methods of measurement of chest wall function, including illustrates to clinicians the pitfalls and limitations of each method (Chapters 35–44). The relation between the chest wall and common activities or conditions such as speech, dyspnea, exercise, diving, and anaesthesia are discussed in Chapters 45–60. The third volume, Part C, focuses on disease states. The described diagnostic methods of the chest wall function offer practical information to the clinicians (Chapters 61–65). Chapters 66–76 describe the pathophysiology of the respiratory muscles and the chest wall in various clinical conditions. The final chapters describe therapeutic approaches necessary to improve ventilatory function in various disease states.

This book will be invaluable to clinical investigators studying thoracic function. Purchase of this book is also recommended for libraries and for clinicians, scientists and professors with an interest in respiration and thoracic function.

L. HUNYADY

Akadémiai Kiadó, Budapest

Adhesion Molecules and the Lung

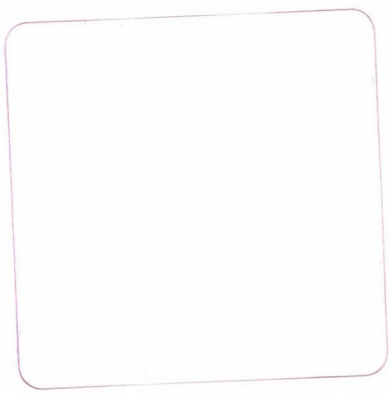
(Lung Biology in Health and Disease Series/89). Edited by: P. S. Ward and Joseph C. Fantone.
416 pages; illustrated. ISBN: 0–8247–9517–2. 165.00 USD

This book undertakes to present the role of adhesion molecules in the morphogenesis, pathological processes, and reparation of the pulmonary tissue. The text is into 13 chapters divided. Considering the volume of the chapters some disproportion could be observable. This presents itself in the number of literary citations, too. It alternates between 29 and 255. The individual chapters have usually more authors. Their number alternates between 1 and 7. In the first chapter the structural basis and regulation of β2integrin interactions are summarized. After discussing the structure, tissue distribution, transcriptional regulation of β2integrins, their functions, the regulation of β2integrin-ligand interactions and the β2integrin-mediated signalling are overviewed. The heading promises the surveying of the β2integrins, whereas come repeatedly up other (β1, β3, β7) integrins. Thus the title is not entirely adequate, without mentioning their existence are with other integrins dealt with. The outlined, deficient second chapter is intented for the selections. The chapter of β2integrins contains 43, while that of selectins 18 sides. Among the L-selectin ligands the E- and P-selectins are not referred to. Nor the essential role of sialic acid and fucose turns out. In connection with the selectin ligands the notion of sialoadhesin (sialomucin) is not mentioned at all. The third chapter deals with the signal transduction of leukocyte adhesion molecules. The reader meets once again with the short recapitulation of integrins, selectins, just as their ligands. Perhaps it might be more proper to discuss the themes in a single chapter in detail to be the repetitions avoidable. This chapter summarizes very well the knowledges connected with the regulation of expression of the leukocyte adhesion molecules, the adhesion molecule-dependent leukocyte activation, their costimulatory role. The connections of the adhesion molecules and the lung morphogenesis are briefly surveyed in the fourth chapter. Repetition takes once more place. The reader regrets to succeed slightly in brief the next chapter, which discuss the connections of the pulmonary epithelial cells and the extracellular matrix. The research of the correlations of the decorin-TGβ-metalloproteinase – metalloproteinase inhibitor system would promise substantial knowledges. The brief recapitulation of the interrelations between the adhesion molecules and the respiratory pathogens is very interesting. In the chapter discussing the relationship between the adhesion molecules and the experimental pulmonary inflammations emerges an amazing statement. According to this the adhesion molecule constellations of the lung injury mediated by IgG-, respectively, IgA-immunecomplexes are different. In the first the selectins having, while in the second case having nothing role, and the contributions of β2integrins are also various. Only the αLβ2 participates in the formerly, while both the αLβ2 and αMβ2 in the latter case. Exceptionally valuable chapter is the following in which the cell adhesion-wound healing-pulmonary fibrosis is analyzed. One can meet with the crowd of knowledges exceeding the scope of the concrete topic. Such are the organization of the extracellular tissues, the adhesion molecules and their extracellular ligands, the sequences recognized by the integrins, correlation of cytokines and growth factors with adhesion molecules. Having everyday significance in clinical practice the chapter analyzing the role of adhesion molecules in the establishing of tomour metastasis is very valuable. The statement, that albeit pulmonary capillaries are narrower, than the diameter of an average tomour cell, still not the mechanical trap, rather the role of constitutive and the cytokine-induced adhesion molecules is distinctive. The cytokines are from the tomour cells (and endothelial cells?) originating. The constitutive Lu-ECAM1 occurring mostly in postcapillary venules and the DPPIV occurring different cell types are in detail characterized. Remarkable that both of them are sialoglycoproteins and the terminal structure of oligosaccharide on the Lu-ECAM1 is very similar to sialyl-LewisX. Among the cytokine-inducible endothelial cell adhesion molecules are the selectins mentioned. The P-selectin does indeed not require new protein synthesis, but the E-selectin does. The hypothesis, that ICA1, VCAM1 and E-selectin being present in significant concentrations in the serum of cancer patients may act as competitive inhibitors in tomour cell interactions with cells of the immune system and could contribute to the immunological escape of tomour cells is very impressive. Well-written is the chapter summarising the role of ICAM1, but its role is perhaps

overestimated. Truly being an important adhesion molecule, though occurring in great number on the alveolar cells, notwithstanding is a single factor only in mediating the pathological processes. The α4integrins obtain independent chapter. Being able to attach to both VCAM1 and CS1 containing fibronectin it undoubtedly has significant role in the pulmonary processes, too, nevertheless the former comments are also in this case valid. The twelfth chapter deals with the role of adhesion molecules and cytokines in lung transplantation. Some error has crept into the Table 1. P-selectin occurs in platelets and megakaryocytes, too, TCR occurs naturally only on T lymphocytes, while MHCI correctly on all cells except erythrocytes, MHCII on cells taking in the immune response part. The last chapter includes the role of adhesion molecules in the bacterial pneumonia. The most detailed is the explanation of the migration of neutrophils and their two sword-edged nature. Noticeable that the emigration of leukocytes in the lung takes not in the postcapillary venules place, but in the capillaries. The ICAM1- and β2integrin-dependent character of the leukocyte migration is peculiar.

The reader keeps an excellent book in his hands and can recommend it with the best convictions to all, who are interested in adhesion molecules and in their clinical relations.

LAJOS JAKAB



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SHORT COMMUNICATIONS

Vth Joint Meeting of the Hungarian, Polish and Italian Pharmacological Societies (Pécs, 1996), Hungary, (Part II.)

Acta Physiologica Hungarica, Volume 84 (4), pp. 353–360 (1996)

Evidence for genetic variability in venous responsiveness

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Alterations in adrenergic mediated vascular responsiveness and glucose metabolism have been implicated in the pathogenesis of essential hypertension. We sought for a human model to study possible interactions between the two pathogenetical factors. In a group of Latin-Americans there is a predominance of diabetes mellitus, impaired glucose tolerance and obesity, while the rate of hypertension is unexpectedly low (lower than in the normal population). Here we demonstrate attenuated alpha1 adrenergic mediated vasoconstriction in the above group, suggesting a possible protective mechanism against the development hypertension.

Keywords: essential hypertension, vascular responsiveness, glucose metabolism

Alterations in adrenergic mediated vascular responsiveness, resulting in increased total peripherial resistance plays a role in the development of essential hypertension in a considerable number of patients. Similarly, reduced compliance of the venous system has been suggested among the possible mechanisms responsible for initiating and/or maintaining essential hypertension [12, 20]. Moreover, a genetically determined decreased venous compliance has been previously demonstrated in young, normotensive patients with family history of hypertension [14].

Several recent reports have addressed the possible association between hyperinsulinemia, insulin resistance and hypertension. Most [15, 25], but not all [22] studies have found such a relationship. This relationship was not present in a study of Latin American subjects [10]. The association between abnormal oral glucose tolerance

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tests and hypertension has been extended further to include normotensives who have a family history of hypertension [9].

We sought for possible interactions between the above factors in the development of hypertension. Therefore, we intended to find a human model, in which all these pathogenetical changes might be present. Latin Americans of Mexican descent, whose racial ancestry are of Native American (also called North American "Indians") and White descent (3) comprise 5.4% of the population of the United States [30]. Compared to Whites, Latin Americans are generally more obese, have a higher incidence and prevalence of non-insulin dependent diabetes mellitus, yet paradoxically have a lower prevalence [15] and a similar incidence of hypertension [16]. In face of the above, it is puzzling why Latin Americans do not have greater rates of hypertension than the population at large.

It is possible that a protective effect from the development of hypertension may arise from different responsiveness to vasoactive substances. Alpha-adrenergic receptors have been implicated in the development of hypertension as hypertensive subjects are more sensitive to the vasoconstrictive responses produced by alpha adrenergic stimulation than normotensive subjects [13, 19]. Ethnic variability in the response to alpha and beta-adrenergic mediated stimulation has been previously described in black subjects [6, 11, 31].

Our hypothesis was, that alterations in adrenergic mediated venous responsiveness in Latin Americans might play a role in the different prevalence of hypertension observed in this group, when compared to Whites. To test this hypothesis, responses to the alpha1-adrenergic agonist, phenylephrine, were studied in Latin Americans and Whites.

The dorsal hand compliance vein technique is a simple, mildly invasive method to study the response to vasoactive substances, *in vivo* [1, 21]. This technique has been used to compare the responses between several different physiologic and pathologic states. Complete dose-response curves can be constructed and the venodilatory and venoconstrictive effects of pharmacologically active agents can be studied and compared between individual subjects and between groups of subjects. Among the previous studies in which the technique has been used, has been to investigate age-related changes in responsiveness to isoproterenol [17, 24], alpha1-adrenergic responsiveness in mild hypertensives [10], and lack of effect of aging on responsiveness to nitroglycerin (9), PGE1 [17] and bradykinin (5). Since very small, sub-therapeutic quantities (1/50–1/1000 of normal intravenous dosages) of the test agents are used, systemic effects, such as adverse reactions and compensatory responses are virtually absent. The technique has been validated by several investigators, including Alradi and Carruthers [2] who found little diurnal, day-to-day or intrasubject variability in the response to norepinephrine. Vincent et al [32] found a

significant correlation between the blood pressure response to systemically infused phenylephrine and the dose of phenylephrine required to produce half maximal venoconstriction in the dorsal hand vein, indicating that the response in the hand vein mirrors that of the body as a whole.

Methods

Drugs

All study medications were diluted in normal saline. Phenylephrine hydrochloride (1% injection) was obtained from Winthrop Laboratories, New York City, USA. Phenylephrine was infused in the dosing range of 1–6,800 ng/min.

Subjects

Studies were performed on healthy, non-obese volunteers. 10 White (7 male, 3 female–mean age 26.7 ± 2.1 years) and 10 Latin American subjects (5 male, 5 female, mean age 25.4 ± 3.0 years) were studied. To preserve homogeneity, Latin American subjects included only those of Mexican descent dating back three generations. The subjects were admitted on the morning of the study to the Drug Evaluation Unit at Maricopa Medical Center. The subjects signed a written informed consent and underwent a complete physical examination, ECG and routine laboratory tests (SMA-20, CBC, urinalysis). Pre-menopausal female subjects were screened for a negative serum pregnancy test within 24 hours before the study period. Exclusion criteria included a past history of any significant disease state, drug addiction, alcoholism or chronic use of any medication that could affect the vasculature. All subjects were non-smokers and were asked to refrain from caffeine for at least 12 hours prior to the study.

Dorsal hand vein compliance technique

The dorsal hand vein technique was modified slightly from that originally described by Aellig in 1981 (1). The technique has the advantage of being able to quantitate responsiveness of the dorsal hand vein to small amounts of vasoactive drugs, often by constructing full dose-response curves, without confounding systemic effects. Studies are conducted with each subject in the supine position with one arm placed on a padded support sloping upwards at an angle of 30 from the horizontal to allow complete emptying of the veins. The temperature of the room is maintained at 22 ± 2 °C during the study period. A suitable vein is chosen on the dorsum of the hand and a

23 gauge needle is inserted. A normal saline infusion is started at 0.30 ml/min using a syringe infusion pump. The tripod holding a linear variable differential transformer (LVDT, Shaevitz Engineering, Pennsuaken, NJ) is mounted on the back of the hand with the central aperture of the LVDT over the vein under investigation at a distance of 10 mm downstream from the needle. The central aperture of the LVDT contains a freely movable core; vertical movement of the core is directly proportional to the signal output of the LVDT, which is recorded on a strip chart recorder. Recordings of the position of the core located on top of the vein are made both before and after inflation of a sphygmomanometer cuff on the arm to 40 mm Hg. This baseline vasodilation during saline infusion with the cuff inflated is defined as 100% relaxation; the recording obtained with the cuff not inflated (and the vein emptied) is defined as 100% constriction (or 0% relaxation). The difference between the two positions of the core gives a measure of the diameter change of the vein under the given congestion pressure. Baseline recordings are obtained during normal saline infusion after approximately 30 minutes to allow for equilibrium of the vein after the initial vasoconstriction produced by insertion of the needle. Phenylephrine, an alpha1 selective agonist, is used to produce vasoconstriction of the hand vein. A dose-response curve to phenylephrine is performed in each subject (dose range 2–6,800 ng/min); and the dose of phenylephrine that produced 50% constriction determined. Response to each concentration of the drug is recorded after infusing for at least 5 minutes, which allowed sufficient time to reach the maximum effect at each infusion rate. Blood pressure and heart rate are regularly monitored in the opposite arm throughout the study period.

Data analysis

Individual dose-response curves are analyzed with a sigmoid EMAX model utilizing the computer program, MKMODEL (18). This iterative nonlinear curve-fitting program provides an estimate of the maximal response (EMAX) and the infusion rate producing half maximal response (ED50). A log transformation is performed on individual ED50 values to obtain geometric means. An unpaired two-tailed t test is used to compare the individual ED50 values (after log transformation) and the EMAX values for the subject groups. A value of $p < 0.05$ is considered significant.

Results

As shown on figure 1., the maximal venoconstriction for phenylephrine in the Latin American subject group was less than that of the White group (71.2 ± 20.1% vs.

89.4 ± 10.9%) ($p < 0.05$). In addition, we compared the log of the dose of phenylephrine that produced half maximal response (log ED50). It was not statistically different between the two subject groups: 2.16 (145 ng/min) ± 0.56 for the Latin American group vs 1.91 (80 ng/min) ± 0.49 for the White group (p=ns.). There was no difference in baseline hand vein diamater in the two subject groups (data not shown).

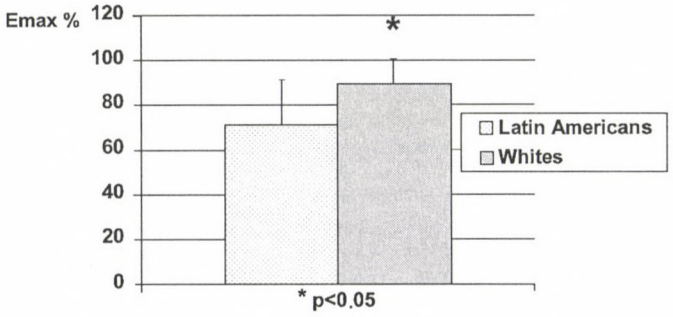

Fig. 1. Phenylephrine-induced maximal venodilation (Emax) in Latin Americans and Whites.

Discussion

In our study, there was a difference in venous responsiveness between Latin Americans and Whites to both the alpha1-adrenergic agonist, phenylephrine and the beta2-adrenergic agonist, isoproterenol. The Latin American group had less of a maximal venoconstrictive response to phenylephrine, and required a greater infusion rate of isoproterenol to produce a half maximal response. It is possible that one or both of these differences in responsiveness may underlie the unexpectedly low rate of development of hypertension in the Latin American population.

Various studies have proposed reasons for interethnic differences in the incidence of hypertension. These factors include environment, plasma renin activity, plasma volume, the kallikrein-kinin system, red cell membrane function and the adrenergic nervous system [6]. These studies have largely been conducted using normotensive and hypertensive white and black patients. We are unaware of comparative studies of the adrenergic nervous system involving Latin Americans. Factors which have been used to attempt to explain the lower than expected rate of hypertension in Latin American individuals include genetic influences, life-style and cultural factors [28].

In our study, that the maximal venoconstrictor response to phenylephrine for the Mexican-Americans was less than that obtained in Whites. An increase in peripheral vascular resistance is the underlying hemodynamic abnormality in most individuals with essential hypertension. Hyper-responsiveness of vascular smooth muscle to alpha1 adrenoceptor mediated vasoconstriction has been proposed as a factor in maintaining this increased resistance [23]. It is possible that hypo-responsiveness may play a protective role. Other investigators have found that vascular responsiveness to alpha1-adrenergic stimulation with norepinephrine and phenylephrine is enhanced during the development of hypertension [29]. However, a previous study utilizing phenylephrine in hypertensives failed to show an increase in responsiveness to stimulation of postsynaptic vascular alpha1 adrenergic receptors in essential hypertension [10].

Although one cannot necessarily extrapolate the results of studies in the venous system to diseases of the arteries, the dorsal hand vein technique may be a better model than arterial infusions to study the effects of adrenergic responsiveness *in vivo*. Interpretation of results in hypertensive arteries are complicated by the confounding pathophysiologic changes that occur in hypertension. In hypertension, there is arterial wall thickening and vascular smooth muscle medial cell hypertrophy and hyperplasia [26], whereas changes of the venous system in hypertension appear to be due to increased neurogenic tone or enhanced contractile responses only [32]. Previous studies investigating adrenergic responsiveness, found no generalized decrease in alpha1 receptor responsiveness in the dorsal hand vein of hypertensive subjects, a result consistent with previous *in vitro* studies and with *in vivo* arterial studies [10].

Our study suggested that normotensive Latin American subjects may have different peripheral vascular responsiveness to adrenergic agonists. This change in responsiveness may help explain why factors that normally predispose to the development of hypertension in the White population are not as predominate in the Latin American population. The exact mechanism regarding a protective role against the delopment of hypertension warrants further investigation.

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Effect of ACE inhibition by benazepril, enalapril and captopril on chronic and post exercise proteinuria

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Although post exercise proteinuria has long been known, its exact pathophysiology is unclear. Our objective was to determine whether long-term angiotensin converting enzyme (ACE) inhibition by different ACE inhibitors had an influence on post exercise proteinuria. We studied 14 patients who also had mild, chronic proteinuria caused by diabetes mellitus or chronic glomerulonephritis. We compared changes both in chronic (baseline) and post exercise proteinuria, during and after treatment with three different ACE inhibitors, with appropriate washout periods for the three drugs to all 14 patients. Proteinuria (mg/24 hours +/- SD), prior to the treatment was 682 +/- 92. Proteinuria after treatment for 30 days with benazepril was 464.4 ± 82.6 ($p<0.001$), with enalapril: 477.1 ± 105.5 ($p<0.001$), and captopril: 504.7 ± 100.1 ($p<0.001$). Proteinuria three days after discontinuing the treatment with benazepril was 532.4 ± 113.5, ($p<0.01$), with enalapril: 561.3 ±128.5, ($p<0.01$), and with captopril: 620.8 ± 101.8, p = n.s. Post exercise proteinuria prior to treatment (mg/min. +/- SD) was: 1.38±0.32, vs. after a 30-day treatment period with benazepril: 0.81±0.19 ($p < 0.001$), enalapril: 0.95±0.24, ($p < 0.001$), captopril: 1.09±0.27 ($p < 0.02$). Post exercise proteinuria three days after discontinuing the treatment was (blood pressure already back to baseline): in case of benazepril: 1.26 +/- 0.36 (p=n.s.), of enalapril: 1.17 +/- 0.46 (p=n.s.), and of captopril: 1.34 +/- 0.41 (p=n.s.). We conclude that the renin-angiotensin system plays a significant role in the pathogenesis of post exercise proteinuria; the antiproteinuric effect of ACE inhibition in exercise-induced proteinuria seems to be associated chiefly with the hemodynamic changes due to these drugs, whereas in chronic proteinuria the antiproteinuric and antihypertensive effects are, at least partially, dissociated.

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Although post-exercise proteinuria has long been known, its exact pathophysiology is unclear. A few theories and studies have been published related to this phenomenon, investigating possible mechanisms leading to post-exercise proteinuria [2, 3, 6, 9, 11]. The possible role of the renin-angiotensin system has been suggested [2], however a recent study failed to show changes in post-exercise proteinuria after administration of a single dose of captopril [6].

Our objective was to determine whether long-term angiotensin converting enzyme (ACE) inhibition by different ACE inhibitors had an influence on post-exercise proteinuria. We also attempted to evaluate, whether possible changes seen with ACE inhibition persisted after the discontinuation of the treatment, when the systemic hemodynamic conditions had returned to baseline.

We selected patients who had mild, chronic proteinuria caused by diabetes mellitus or chronic glomerulonephritis. We compared changes both in chronic (baseline) and post-exercise proteinuria, during, and after discontinuing treatment with ACE inhibitors. We studied three different ACE inhibitors, to determine, whether the possible effects are drug-specific, or class-specific.

Materials and Methods

Subjects

Studies were performed on 14 patients (8 males, 6 females, age 36 +/- 8.4 years). The patients had mild to moderate proteinuria (682 +/- 97 mg/24 hours) secondary to type II. diabetes mellitus or chronic glomerulonephritis proven by renal biopsy. The creatinine clearance was greater than 60 ml/min. in each patient. The baseline diastolic blood pressure of the patients was 103.64 +/- 4.8 mmHg. The subjects signed a written informed consent. Premenopausal female subjects had a negative serum pregnancy test. The patients were on no diuretics or vasoactive medications prior to the study and had no previous history of side effects, or allergic reaction, related to ACE inhibitors. The institutional committee on human research approved the study protocol and the procedures followed were in accordance with institutional guidelines.

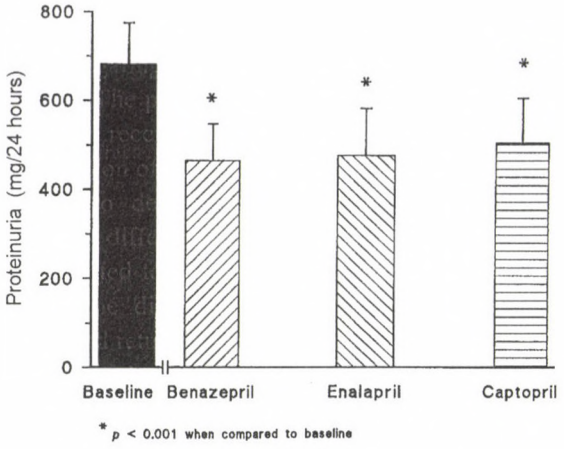

Fig. 1. Proteinuria (mg/24 hours), prior to treatment (baseline), vs. after a 30-day treatment period with: benazepril, enalapril and captopril.

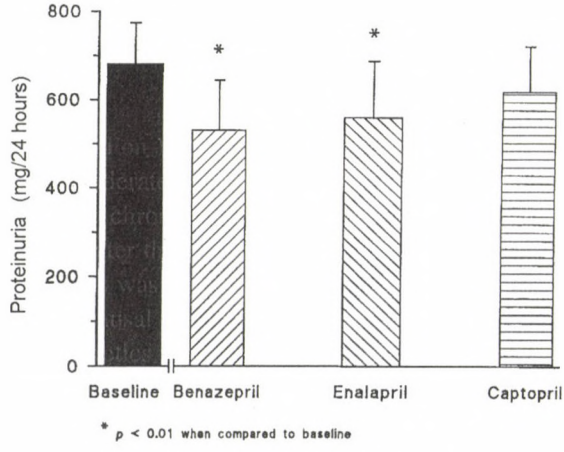

Fig. 2. Proteinuria (mg/24 hours) prior to the treatment (baseline), and 3 days after discontinuing the treatment with: benazepril, enalapril and captopril.

Study drugs

Benazepril (CIBA), enalapril (Merck Sharp and Dohm) and captopril (Bristol Myers Squibb) were administered orally, in equivalent doses, which was determined by

lowering of the diastolic blood pressure by 15 % compared to baseline. This required doses of 13.75-20 mg/day of benazepril, 27.5-40 mg/day of enalapril and 137.5-200 mg/day of captopril. Each patient received each medication in randomized order for 30 days for each drug, with washout periods of 10 days between the three 30-day treatment intervals.

Measurements

Proteinuria was measured by the Biuret method prior to and after the 30-day treatment period with each drug for 24-hour periods. Proteinuria was also determined after moderate, standardized physical activity (1.44 kJ/kg for one hour, by stepmaster) prior to, and after the treatment with each drug. On the third day after discontinuing the treatment, when the blood pressure had already returned to baseline, proteinuria was also measured with and without exercise.

Data analysis

An unpaired two-tailed t test was used to compare proteinuria prior to and after treatment with each drug under study and patient setting. A value of $p < 0.05$ was considered significant. All mean values are given as mean±SD if not indicated otherwise.

Results

Post-exercise proteinuria prior to treatment (mg/min. +/- SD) was: 1.38±0.32, vs. after a 30-day treatment period with benazepril: 0.81±0.19 ($p < 0.001$), enalapril: 0.95±0.24, ($p < 0.001$), captopril: 1.09±0.27 ($p < 0.02$) (Fig 3.). The post-exercise values of proteinuria three days after discontinuing the treatment were as follows (blood pressure already at baseline): benazepril: 1.26 +/- 0.36 (p=n.s.), enalapril: 1.17 +/- 0.46 (p=n.s.), captopril: 1.34 +/- 0.41 (p=n.s.) (Fig 4).

Chronic proteinuria (mg/24 hours +/- SD), prior to the treatment was 682 +/- 92. Chronic proteinuria after treatment for 30 days with benazepril proved to be 464.4 ± 82.6 ($p<0.001$), with enalapril 477.1 ± 105.5 ($p<0.001$), and with captopril 504.7 ± 100.1 ($p<0.001$) (Fig 1.). Chronic proteinuria three days after termination of the treatment with benazepril was 532.4 ± 113.5, ($p<0.01$), with enalapril 561.3 ±128.5, and with ($p<0.01$), captopril 620.8 ± 101.8, (p = n.s.) (Fig 2.).

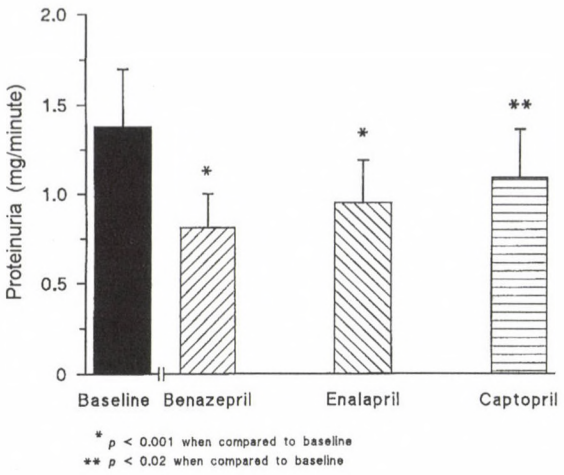

Fig. 3. Post exercise proteinuria (mg/min) prior to the treatment (baseline), vs. after a 30-day treatment period with: benazepril, enalapril, captopril.

Discussion

Exercise induces profound changes in renal hemodynamics. Although, the renal blood flow and the glomerular filtration rate decrease, the filtration fraction may increase [1, 4, 8]. A variety of theories have been suggested to explain post-exercise proteinuria. Robertshaw et al suggested that post-exercise proteinuria can be attributed at least partially to reduced tubular reabsorption [11]. Poortmans et al concluded that both increased glomerular permeability and decreased tubular reabsorption may play a role in the pathomechanism of post-exercise proteinuria [8, 9]. The role of the renin-angiotensin system in the development of the phenomenon is an alternative hypothesis, but a consensus in the literature has not been reached.

After a 30-minute exercise with cycle ergometer, Mittelman and Zambarski found no alteration of post-exercise proteinuria following administration of captopril (6). In their study, however, there was a significant attenuation of post-exercise proteinuria associated with inhibition of the prostaglandin system by indomethacine, suggesting the possible role of that system [6].

Ninety minutes after administration of captopril, Cosenzi et al have demonstrated a complete resolution of post-exercise proteinuria, supporting the theory that the renin-angiotensin system is involved in the development of the phenomenon [2].

Unlike Mittelman and Zambarski [6], but similar by to the results of Cosenzi et al (2), we found that ACE inhibition reduced post exercise proteinuria. In our study, all

three ACE inhibitors resulted in a significant reduction of proteinuria. These findings pertained for both chronic- and post-exercise situations. Changes associated with captopril were less significant than with benazepril or enalapril in exercise-induced proteinuria (Fig. 3). In chronic proteinuria, there was no significant difference between the antiproteinuric action of the three different ACE inhibitors. Three days after discontinuing the treatment, when systemic hemodynamic conditions had returned to baseline, there was still a significant reduction detectable in chronic proteinuria. This was, however, not the case with post exercise proteinuria. This suggests that the pathomechanism of post-exercise proteinuria differs from chronic proteinuria, associated with diabetes mellitus or chronic glomerulonephritis.

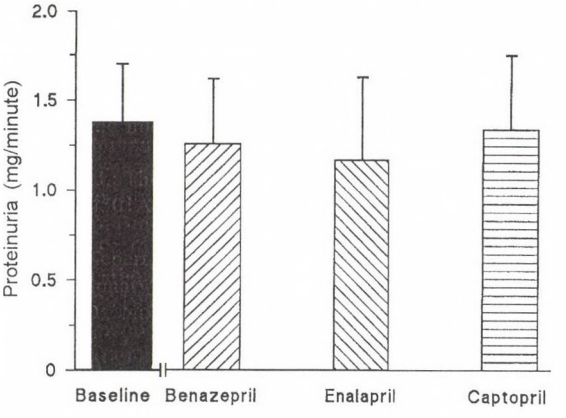

Fig. 4. Post exercise proteinuria (mg/min) prior to the treatment and 3 days after the discontinuing the treatment with: benazepril, enalapril, and captopril.

We conclude that:

a.) the renin-angiotensin system plays a significant role in the pathogenesis of post exercise proteinuria;

b.) the antiproteinuric effect of ACE inhibitors in exercise-induced proteinuria seems to be associated chiefly with the hemodynamic changes due to these drugs,

c.) whereas in chronic proteinuria the antiproteinuric and antihypertensive effects are, at least partially, dissociated. Our observations in humans confirm similar previous theories (5, 12) and the results of animal model studies by Remuzzi et al, demonstrating dissociation of the antiproteinuric and antihypertensive effects of lisinopril in rats (10). Our findings are also consistent with the results of Palla et al (7). In their study, a dissociation of the antiproteinuric and antihypertensive effects of ACE inhibition was shown by using increasing doses of lisinopril, resulting in an increasing antiproteinuric effect, without significant changes of the blood pressure [7].

In accordance with the above, we hypothesize, that patients with chronic proteinuria, who are physically active, due to their occupation or recreation, may benefit from being treated with ACE inhibitors. Furthermore, if the above patients are hypertensive, an ACE inhibitor might be considered as antihypertensive medication. This may prevent exercise-induced exacerbations of chronic proteinuria.

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Xenobiotic metabolizing enzymes in fish: diversity, regulation and biomarkers for pollutant exposure

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Cytochromes P450 play key roles in biotransformation of pollutant chemicals and in the activation or inactivation of many toxic or carcinogenic compounds. Multiple P450 isozymes have been purified from different fish species. Fish monooxygenase activity shows temperature compensation and sex-related variation. Several xenobiotics can induce cytochrome P450 monooxygenases altering toxicity of chemical contaminants. Polycyclic aromatic hydrocarbons can increase transcription of CYP1A gene in fish as it has been observed in mammals, but phenobarbital-type agents do not induce in fish at all. The presence of conjugation enzymes in fish has also been proved, although their induction by xenobiotics is poorly investigated. Since exposure of fish to environmental contaminants can result in the induction of specific cytochrome P450 enzymes, monitoring of their catalytic activities can identify polluted areas.

Keywords: cytochrome P450, fish, biotransformation, induction, biomonitoring

The release of chemicals to aquatic environment by industry and agriculture as well as urban communities is causing serious problems; aquatic ecosystems are heavily contaminated with lipophilic organic compounds (xenobiotics). Many of the pollutants, such as polyaromatic hydrocarbons (PAHs) and their halogenated counterparts, are chemically stable, and owing to their lipophilic nature they can easily penetrate biological membranes and accumulate in organisms. At the top of the food chain they may reach concentrations high enough to bring about toxic effects. If the chemicals are acutely toxic, the consequences are clear. However, acute toxicity expressed as LC_{50} is insufficient for assessing the impact of various pollutants and does not say anything about the early or the long-term effects. The chemicals coexist in mixtures and the

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chemical analysis of the pollutants might not reveal the actual health risks. On the other hand the biological response is a complex outcome of the genetic background and exposure history [68, 43]. Continuous monitoring of the water quality by measuring physico-chemical parameters is essential, but it must be completed by sensitive biological methods. Bioindicator systems are a promising tool that enables early detection of biochemical responses to aquatic pollution before structural changes occur or diseases appear [56].

The biotransformation of environmental chemicals is an adaptive strategy in most species. This ability can lower toxicity and eliminate xenobiotics which can have harmful effects. Most of our present knowledge of xenobiotic metabolism has been derived from studies on liver, although metabolism of pollutants and other environmental chemicals is not restricted to the liver. In lower vertebrates such as fish, high levels of xenobiotic metabolizing enzymes have been found in extrahepatic tissues: in kidney, gastrointestinal tract and gill [69].

Biotransformation reactions can be classified into two types: phase I and phase II reactions. Phase I enzymes, mainly cytochrome P450 enzymes introduce a polar group into the lipophilic substrate via oxidative processes [33]. Phase II reactions involve the conjugation of xenobiotics or their phase I metabolites with polar, endogenous constituents such as glucuronic acid, sulfate or glutathione. These reactions are catalyzed by conjugating enzymes: UDP glucuronosyl transferase, sulfate transferase and glutathione S-transferase. Phase I metabolites or conjugated products are water-soluble, usually less toxic and rapidly excreted [11, 17, 31, 50].

Activity and concentration of enzymes responsible for xenobiotic metabolism are under extensive exogenous and endogenous control and can be used successfully as markers for environmental abnormalities. Exposure to xenobiotics often involves alteration in enzyme function and expression. The consequences of changes (increase or decrease in activity or concentration) make these parameters a suitable tool for biochemical monitoring.

1. Cytochrome P450 enzymes

Fish have been in focus of the campaign to protect waters from harmful effects of environmental chemicals. The response of the biotransformation enzyme systems of fish exposed to water pollutants has been studied extensively [22]. The cytochromes P450 (P450) are the major enzymes involved in the oxidative metabolism of both endogenous and exogenous compounds in fish as well as in other animals. The cytochrome P450 haemoprotein is the terminal oxygenase catalyzing the insertion of one atom from molecular oxygen into substrate (while other is used to form water).

Reactions of cytochrome P450 system require two electrons transferred from NADPH by NADPH-cytochrome P450 reductase. Cytochrome P450 is the substrate binding component of the system and determines the specificity of the monooxygenase reaction. In the cell, xenobiotic metabolizing P450s are localized in the endoplasmic reticulum [13]. Multiple forms of cytochrome P450 exist in combination with the broad substrate specificity of many P450 forms [48]. It results in the ability of organisms to metabolize a wide variety of lipophilic molecules. Cytochromes P450 (CYP) purified from fish (Table 1) are identified by catalytic assays, antibodies to mammalian P450s, sequence comparison of cDNAs or functional similarities [60, 64, 65].

Table 1

Cytochrome P450 subfamilies purified from fish

Subfamily	Name in fish species		Specific activity	Inducers
CYP1A	trout:	P450LM$_{4b}$	ethoxyresorufin	polycyclic aromatic
	scup:	P450E	O-deethylase (EROD)	hydrocarbons,
	cod:	P450c	benzo[a]pyrene	polychlorinated biphenyls
	perch:	P450V	hydroxylase (AHH)	(TCDD)
CYP2B	trout:	P450LMC1	?	non inducible
	scup:	P450B		
CYP2E		?		
CYP2K	trout:	P450LM2	lauric acid hydroxylase	β-naphtoflavone
	trout:	P450KM2	aflatoxin B_1 activation	
CYP3A	trout:	P450LMC5	steroid	
	scup:	P450A	6β-hydroxylase	
	cod:	P450b		

Trout: Oncorhynchus mykiss
Scup: Stenotomus chrysops
Cod: Gadus morhua
Perch: Perca fluviatilis

The regulation of P450 expression in cells is complex and can occur at many levels ranging from specific cytosolic receptor mediated gene transcription to posttranscriptional and posttranslational control. Induction of P450 by xenobiotics is one of the most characteristic features of the enzyme system. During the inductive process a chemical enhances the rate of gene transcription resulting in elevated levels

of mRNA and synthesis of P450 enzyme [51, 47]. The induction process in fish is not completely understood, however, the mechanism of mammalian P450 expression can be used for regulation by polyaromatic hydrocarbons of CYP1A orthologue in fish.

CYP1 gene family in fish

CYP1A gene subfamily is prominent in the activation of many environmental carcinogens. The P450 forms purified from several fish species: rainbow trout $P450LM_{4b}$, scup P450E, cod P450c and perch P450V [71, 36, 20, 74] all have physico-chemical, catalytic and regulatory properties in common with each other and with mammalian CYP1A forms. Trout CYP1A has 57–59% of the amino acid residues identical to mammalian CYP1A1 and 51–53% of the residues identical to mammalian CYP1A2. The two members of mammalian CYP1A subfamily have been diverged from a common ancestor. There are some speculations that fish CYP1A could represent a type ancestral to both CYP1A1 and CYP1A2 in mammals [28, 61]. On the other hand there are continuing arguments that a CYP1A2-like form exists in fish [73] based on different induction patterns to the typical inducers of CYP1A1 and CYP1A2: β-naphtoflavone and isosafrole [10]. The results concerning induction of multiple CYP1A enzymes in fish are ambiguous.

All fish CYP1A enzymes are inducible by polyaromatic hydrocarbons and by planar halogenated aromatic hydrocarbons (including polychlorinated biphenyls [PCBs] or 2,3,7,8-tetrachlorodibenzodioxin [TCDD]). The induction of these forms of cytochrome P450 can indicate the exposure of fish to carcinogens and other toxic compounds in the environment. Fish CYP1As catalyze ethoxyresorufin O-deethylation (EROD), 7-ethoxycoumarin O-deethylation (ECOD) and aryl hydrocarbon hydroxylation (AHH) [33, 53, 12]. Measurement of these activities is the most sensitive method to determine the inductive response in fish. CYP1A enzyme activities occur at low, often undetectable, levels in control or untreated fish. Following β-naphtoflavone or TCDD treatment, the rapid increase of CYP1A mRNA and CYP1A protein content is due to the transcriptional activation of CYP1A gene. Inducers are recognized by a cytosolic receptor (the Ah-receptor) that is identical in fish and mammals. Activated receptor (receptor-inducer complex) binds to the xenobiotic response element (XRE) in the 5' region of fish CYP1A gene and increases transcription of the gene. In fish hepatocytes β-naphtoflavone or TCDD cause a rapid rise of CYP1A mRNA that is followed by the increase in CYP1A protein concentration and EROD activity [42, 27]. *Devaux and Pesonen* [14] showed that dexamethasone potentiated CYP1A induction caused by β-naphtoflavone in trout hepatocytes, suggesting that glucocorticoid response elements (GRE) exist in fish CYP1A gene as it was found in mammals.

CYP1A induction is not restricted to the liver, it is expressed in most extrahepatic organs: in gill, gut or in kidney [58]. The regulation of extrahepatic CYP1A is linked to protective mechanisms in some cases. Low concentrations of PAHs in the diet of fish induce intestinal CYP1A without any changes in liver. Enhanced metabolism of the inducer in the gut prevents active concentration from reaching the liver [67].

CYP2 and CYP3 gene families

A major difference between fish and mammalian P450s is the lack of induction by phenobarbital in teleosts. Although pentoxyresorufin O-dealkylase activity was observed in untreated fish liver; pentoxyresorufin O-dealkylation is catalyzed by CYP2B in mammals. In addition it has been proved by immunochemical identification that fish have P450 forms (P450LMC1 of rainbow trout; P450B of scup) homologous to mammalian CYP2B [45, 62]. Nucleic acid probes for mammalian CYP2B provided further evidence of these orthologues in fish liver. Phenobarbital or phenobarbital-type inducers, however, do not cause an increase of pentoxyresorufin O-dealkylation activity, the amount of CYP2B orthologues or CYP2B mRNA. Thus the lack of phenobarbital-type induction is not due to the lack of structural CYP2B gene, but rather the lack of a regulatory mechanism like that acts to control expression of CYP2B in mammals [15, 34]. (The mechanism in mammals is not completely understood.) However, there are several other animal species lacking phenobarbital-type induction and the common feature in these groups is a general absence of terrestrial plants in the diet [47].

Fish liver microsomes are able to dealkylate diethylnitrosamine (a fish carcinogen) that indicates a cytochrome P450 form related to CYP2E. Furthermore, antibody to rat CYP2E1 recognizes a single protein in the liver [32]. Although the enzyme has not been purified and further studies are required for identification.

Several lines of evidence is of the presence of CYP3A forms in fish. P450LMC5 in trout and P450A in scup catalyze steroid 6β-hydroxylation that is attributed to CYP3A in mammals. Immunological cross-reactivity was detected between P450LMC5 and rat CYP3A1 or human CYP3A4 [46].

Cytochrome P450s in kidney

Among extrahepatic tissues, kidney is studied extensively. High content of P450LM2 and P450LMC5 was observed in the kidney of mature male trouts, but not of females or juveniles [72, 44]. P450LM2 has been classified as a CYP2K. Two more novel P450 forms: P450KM1 and P450KM2 have been purified that are highly expressed in male trout kidney, but are absent from females and immatures. P450KM2, however, can be induced in juveniles by 11-ketotestosteron (potent androgen for fish) [6].

2. Regulation of cytochrome P450 enzymes in fish

A number of factors (endogenous processes or external factors) can modulate xenobiotic metabolizing enzyme levels and their activities. These factors (developmental stages, sex and seasonal cycles, temperature, nutritional status) may influence biotransformation enzymes directly and/or by altering inducibility [7].

Regulation in early developmental stages

The early life stages are very sensitive to toxic effects, particularly to carcinogens [59]. P450 enzyme system in fish embryos can metabolize several compounds (such as aflatoxin B1 or dimethylnitrosamine) to reactive intermediers which bind to DNA and cause carcinomas [29]. Fish-eggs and sac-fry take up lipophilic xenobiotics directly from water or these compounds are transported from mother to gonads before ovulation [24, 70]. In early sac-fry period the yolk sac confines lipophilic xenobiotics, but as the yolk sac is consumed these compounds are transported to the fry which is a critical stage for toxic effects. During the development xenobiotic metabolizing enzyme system becomes more efficient and can eliminate toxic and carcinogenic chemicals [25]. In addition, inducibility of P450 system by PAHs was higher around the development of sac-fry and fry than in embryos [21].

Regulation during sexual development and seasonal cycle

Strong sexual differences have been observed in P450 enzyme levels in fish liver especially during the spawning time. In general, male fish have higher P450 content than females and several P450 enzyme activities (benzo[a]pyrene hydroxylase, ECOD, aminopyrine demethylase, steroid 6β-hydroxylase) are 2–10-fold higher in mature males than in mature females [18, 40]. In rainbow trout sexual differences in P450 levels are greater in kidney than in liver [72, 5].

The major constitutive trout P450 form called P450LM2 also contributes to the sex difference. Higher level of hepatic P450LM2 has been detected in males than in females by immunochemical methods. However, it is absent in female kidney microsomes indicating that this P450 form is strictly regulated in kidney [72].

Altering plasma hormone level is likely to be involved in the sex-related differences in P450 enzyme levels during seasonal cycle. In male rainbow trout increasing hepatic P450 level and activities are parallel to the increase in plasma androgen level. In females estradiol down-regulates P450 levels and several P450 activities [18]. Estradiol exerts suppression of P450 system in both maturing fish and estradiol treated juvenile fish [52].

Hormonal factors during sexual development also influence the induction of P450 enzymes by PAHs or PCBs. Estradiol pretreatment suppresses ethoxyresorufin O-dealkylase induction by β-naphtoflavone and the suppression was observed in CYP1A content as well as in CYP1A mRNA indicating a pretranslational block [16].

The effect of ambient temperature

Many fish species are exposed to a wide range of temperatures and they can survive and remain active in large fluctuations of water temperature. It is due to some adaptive mechanisms that compensate widely differing body temperatures by regulating enzyme levels and the microenvironment of the enzymes. Although the field situation is complex, temperature compensatory mechanisms for P450 enzyme activities are proved in laboratory experiments [2, 8, 57]. The rate of ethoxyresorufin or benzo[a]pyrene metabolism is higher in liver cells of cold-acclimated fish than of warm-acclimated animals [4]. The effect of change in water temperature is not prompt, but the changes occur over an acclimation period. The compensation to lower temperatures does not mean the increase in total P450 level, but it is accompanied with the change in the isoenzyme pattern in fish liver. In warm-acclimated rainbow trout (18 °C) the $P450LM_{4b}$ (CYP1A) content is lower than in cold-acclimated fish (10 °C), while P450LM2 is not affected. The temperature compensation of CYP1A explains the higher rate of ethoxyresorufin O-dealkylation or benzo[a]pyrene hydroxylation in cold-acclimated trout liver [9]. As changes in ambient temperature cause alterations in the composition of membranes, it has been proposed that the phospholipids surrounding P450 enzymes in endoplasmic reticulum may influence the optimal conditions for P450 activities [26]. The ambient temperature can therefore highly modulate the enzyme activities in vivo and influences the fate of toxic or carcinogenic compounds in fish. In addition, NADPH-cytochrome P450 reductase that reduces P450 enzymes, also showed temperature compensation. It supports electrons for P450 enzymes with increased activity in cold-acclimated fish [2].

The induction of P450 enzymes by xenobiotics is also affected by water temperature. Low temperature increases the time necessary to reach maximal induction by PAHs. CYP1A content and activity rose slowly over time after β-naphtoflavone treatment in cold-acclimated rainbow trout (5 °C), while it was quickly induced in warm-acclimated fish (17 °C). However, CYP1A mRNA content was elevated to the same level in both warm- and cold-acclimated fish, the half-life of mRNA was longer in the cold fish. Thus the induction (maximal CYP1A activity) in cold-acclimated fish was about twice as high as in warm-acclimated animals [35].

The role of temperature in biotransformation and induction is evident, but further work is required for clear understanding the temperature compensation mechanisms.

3. Conjugation reactions in fish

In phase II reactions the xenobiotic or its metabolite is linked to an endogenous molecule producing a conjugate. Thus phase II products become more polar and generally less toxic than the unconjugated, original compound. Fish are capable of forming glucuronide, glutathione and sulfate conjugates.

In most fish species glucuronidation catalyzed by UDP-glucuronosyl transferase, has been found one of the most important pathways for the conjugation of several xenobiotics. As in mammals, UDP-glucuronosyl transferase activity in fish is localized in endoplasmic reticulum. 4-nitrophenol is generally used as a substrate for determination of UDP-glucuronosyl transferase level in vitro [31, 11]. There are other substrates such as 1-naphtol, 4-methylumbelliferone or bilirubin used successfully in studies on fish microsomes. Multiple forms of UDP-glucuronosyl transferases have been isolated and partially purified from 3-methylcholanthrene induced fish [38]. In fish high UDP-glucuronosyl transferase activities have also been found in extrahepatic tissues: gut or gills [37, 39].

Glutathione S-transferases are a family of enzymes that conjugate reduced glutathione (GSH) with wide range of electrophilic compounds generated by phase I enzymes. Glutathione conjugates are further metabolized to mercapturates and excreted in bile or urine. Glutathione S-transferase (GST) activities towards several substrates have been determined in freshwater and marine fish livers and extrahepatic tissues as well [38, 31]. The glutathione S-transferase activity in rainbow trout liver towards 1-chloro-2,4-dinitrobenzene was about the same as measured in rat liver [49]. In fish liver GST activity is located primarily in the cytosol, but activity has also been detected in the microsomal fraction. Multiple hepatic glutathione S-transferases have been isolated from different fish species [66, 54, 55].

Sulfate conjugation is detected in some freshwater species: goldfish (Grassius auratus), guppy (Poecilia reticulata) and roach (Rutilus rutilus), but this enzymatic step appears to be of minor importance.

Many cytochrome P450 inducers also induce phase II enzymes [51]. Details derive from studies mainly on mammals, the induction of conjugating enzymes in fish is poorly investigated. In rainbow trout liver, glutathione S-transferase activity towards 1-chloro-2,4-dinitrobenzene and UDP-glucuronosyl transferase activity towards 4-nitrophenol have been shown to be induced by β-naphtoflavone. However, the magnitude of induction is lower and the response comes later than induction of hepatic cytochrome P450 activities [3].

4. Environmental induction and monitoring

Evidence of biotransformation and induction in fish has resulted in the interest in the use of these mechanisms as biomarker to monitor xenobiotics in the environment. Monitors or early warnings to identify contamination is important in analysis of waters. Cytochrome P450 induction in fish has been suggested to indicate the exposure of organisms to chemical contaminants in the environment. The induction can be detected by the analysis of catalytic activity or by the increase of PAH-inducible CYP1A form. Close correlation has been shown between CYP1A induction and the levels of PAHs or PCBs either in the organisms or in their environment. The results are convincing enough and CYP1A induction can safely determine aquatic pollution [19, 30, 23].

It is worth emphasising, that cytochrome P450 induction in fish by xenobiotics is a general indicator of environmental contaminants rather than for the identification of specific chemicals. The transition of detection to identification the cause of environmental induction is more difficult [41]. However, the significance of environmental induction is central to environmental toxicology and induction measurements can be a complement of conventional analytical chemistry measurements using as an indicator of aquatic pollutants [1].

Finally it should be mentioned that P450 enzymes are involved in the activation of some procarcinogens. As CYP1A is a main catalyst through which some polyaromatic hydrocarbons are activated in fish, the induction of CYP1A enhances the transformation into a carcinogenic derivative. Although detoxification and repair processes can decrease the risk of carcinogenic activation by CYP1A [63]. In addition to monitoring of water quality, CYP1A induction can indicate the levels of contaminants and possible risk of consumption of fish from highly contaminated places. While fish may contain small or sometimes undetectable amount of parent compound, carcinogenic metabolites may be present. Unfortunately, there is little information about whether such molecules would occur in carcinogenic state in fish products available for human consumption [63].

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Effects of administration of temazepam on blood glucose and serum lipid levels in hyperlipidemic rats

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In rats rendered hyperlipidemic by the administration of Triton WR-1339 temazepam induced significant reductions of serum lipids. The effects was more reduced than of diazepam. The blood glucose level failed to be affected by most of the doses.

Keywords: Rats, hyperlipidemic, benzodiazepines, temazepam

In a previous paper [4], have shown that oxazepam as many other benzodiazepines (BZD) had the property to lower the serum lipids, when they were elevated by Triton WR-1339 administration. However, oxazepam was less effective than other BZDs, for example diazepam, lorazepam and chlordiazepoxide.

The purpose of this study was to present the results obtained on influence of the temazepam, a benzodiazepine closely related to oxazepam on serum lipids. In the present study, the effects of this drugs on the blood glucose level were also investigated.

Materials and Methods

The experiments were carried out in male Wistar rats weighing 160–200 g. They were fed a normal rat chow and housed at a constant temperature (20–22 °C). The

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Table I

Effects of the acute intraperitoneal administration of various doses of temazepam on blood glucose level and serum lipids

	Blood glucose level (mmol/l)	TL (mg%)	TCHOL (mmol/l)	TG (mmol/l)
Control Hyperdyslipidemic (Triton WR-1339, 200 mg/kg)	5.71±0.26	2838.9±174.74	7.12±0.53	20.7±1.09
Reference (Fenofibrate 200 mg/kg)	5.16±0.35 NS	651.2±26.5 (p<0.001)↓	3.16±0.137 (p<0.001)↓	1.015±0.15 (p<0.001)↓
Temazepam (30.0 mg/kg)	5.36±0.28 NS NS	2653±164.54 NS (p1<0.001)↑	6.02±0.32 NS (p1<0.001)↑	18.02±1.89 NS (p1<0.001)↑
(20.0 mg/kg)	3.28±0.204 (p<0.001)↓ (p1<0.001)↓	1067.81±76.06 (p<0.001)↓ (p1<0.01)↑	3.26±0.109 (p<0.001)↓ NS	4.35±0.55 (p<0.001)↓ (p1<0.001)↑
(15.0 mg/kg)	5.04±0.186 NS NS	3210±208.56 NS (p1<0.001)↑	5.95±0.298 NS (p1<0.001)↑	17.08±1.67 NS (p1<0.001)↑
(12.5 mg/kg)	6.42±0.31 NS (p1<0.01)↑	2540±174.89 NS (p1<0.001)↑	3.26±0.109 NS (p1<0.001)↑	4.35±0.55 NS (p1<0.001)↑
(10.0 mg/kg)	3.64±0.22 (p<0.001)↓ (p1<0.001)↓	1938.08±211.02 (p<0.001)↓ (p1<0.001)↑	5.82±0.47 NS (p1<0.01)↑	10.15±1.45 (p<0.001)↓ (p1<0.001)↑
(7.5 mg/kg)	5.52±0.21 NS NS	682.0±83.56 (p<0.001)↓ NS	2.70±0.17 (p<0.001)↓ (p1<0.01)↓	2.15±0.36 (p<0.001)↓ (p1<0.001)↑
(5.0 mg/kg)	5.58±0.25 NS NS	2896.66±109.06 NS (p1<0.001)↑	8.25±0.248 NS (p1<0.001)↑	18.52±0.24 NS (p1<0.001)↑
(2.5 mg/kg)	5.17±0.23 NS NS	2112.04±278.17 (p<0.01)↓ (p1<0.001)↑	4.58±0.41 (p<0.001)↓ (p1<0.001)↑	9.8±1.28 (p<0.001)↓ (p1<0.001)↑
(1.25 mg/kg)	5.45±0.3 NS NS	2013±178.2 (p<0.01)↓ (p1<0.001)↑	5.02±0.31 (p<0.001)↓ (p1<0.001)↑	8.8±1.38 (p<0.001)↓ (p1<0.001)↑

All the groups consisted from 10 rats.
NS: Nonsignificant.
p: Compared with the hyperlipidemic control.
p1: Compared with the reference group (fenofibrate).
↓: Decrease when compared to the control.
↑: Increase when compared to the control.

TL: Total lipids.
TCHOL: Total cholesterol.
TG: Triglicerides.

hyperlipidemia was induced by an intraperitoneal (ip.) injection of an aqueous 10% solution of Triton WR-1339 (200 mg/kg). Then, the rats were fasted till blood sampling, water was allowed ad libitum.

18 hours later, blood was collected by the punction of the retroorbital sinus. From the whole blood the glucose content was measured by the enzymatic method described by Merck [7]. From the separated serum, total lipids (TL) were determined by the procedure of Zöllner and Kirsch, total cholesterol (TCHOL) and triglycerides (TG) by the enzymatic method (Reanal's and Merck's Test). The following groups were formed:

1) Control hyperlipidemic rats received two hours prior to the injection of Triton WR-1339 the same volume of the mixture used for suspension of temazepam (methylcellulose 0.2% + glycerin 1%).

2) Reference group: the animal were injected i. p. two hours prior to Triton WR-1339 administration with fenofibrate (200 mg/kg) suspended in the above mentioned mixture.

3) Temazepam administered groups: the following doses of temazepam were administered 1.25–2.5–5.0–7.5–10.0–12.5–15.0–20.0 and 30.0 mg. The remainder of the procedure was identical to that described in the case of fenofibrate. In all cases the blood sampling was performed 18 hours after Triton WR-1339 administration. The results were expressed as mg% for (TL) and as mmol/l for other compounds.

The significance of differences between Triton WR-1339 treated rats and those which received fenofibrate or temazepam (p) as well as the significance of the differences between fenofibrate and temazepam (p1) was evaluated by the "t" test [9].

Results

I) In temazepam administered rats compared with the hyperlipidemic control group, blood glucose levels were reduced by the doses of 20.0 mg/kg and 10 mg/kg while no alteration was recorded at other doses. TL were decreased by 20.0 mg/kg, 10.0 mg/kg, 7.5 mg/kg, 2.5 mg/kg, 1.25 mg/kg, at other doses no decrease was found. TCHOL was reduced by the doses of 20.0 mg/kg, 7.5 mg/kg, 2.5 mg/kg and 1.25 mg/kg, at other doses it remained unchanged. TG were decreased by the doses of 20.0 mg/kg, 12.5 mg/kg, 10.0 mg/kg, 7.5 mg/kg, 2.5 mg/kg and 1.25 mg/kg. Other doses were ineffective.

II) In comparison with the findings obtained in the reference group, i.e. fenofibrate treated rats, temazepam induced the following changes. The blood glucose level was diminished by the doses of 20.0 mg/kg and 10 mg/kg, other doses being ineffective. TL were increased by all the doses, while 7.5 mg/kg which did not exert

significant change. TCHOL was decreased by the same dose, it remained unchanged by the dose of 20 mg/kg and was increased by the other doses. TG were elevated by all doses.

Discussion

Temazepam differs from oxazepam only by the presence of a methyl group in the position 1 of the BZD ring. Moreover, its biotransformation yields oxazepam [8] as it results from Figure 1. So, temazepam can be regarded, at least partly as a prodrug, the active compound being oxazepam. However, the transformation of temazepam into oxazepam is only one of the metabolic pathways (Fig. 1). Therefore, it is not sure that the effects of temazepam on blood glucose level and serum lipids are entirely due to its conversion in oxazepam.

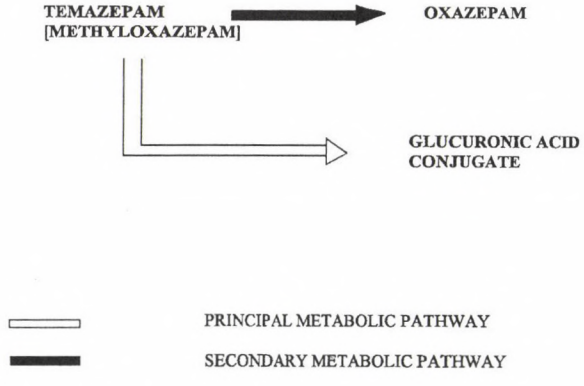

Fig. 1. Metabolic pathways of temazepam, according to Marmo [8]

Concerning the relationship between doses and effects, it must be emphasized that most BZD present a bell-shaped relationship, i.e. the doses lower or higher than the optimal dose (usually a 5 mg/kg) have a lower activity. Temazepam doesn't share this property. Indeed, if we examine the dose-response relationship for serum lipids of this drug, it results that there are two peaks of hypolipidemic activity. The most active dose was 7.5 mg/kg. The lower and higher doses were less effective. Another peak is represented by the dose of 20.0 mg/kg. It is noteworthy that a similar aspect, i.e. the existence of two peaks of action was observed with lormetazepam, a lorazepam derivative [5]. It seems to be very probable that both drugs act in two ways: directly

(for the lowest peak) and indirectly (for the highest peak). Temazepam, as well as oxazepam is a less hypolipidemic agent than diazepam, lorazepam and chlordiazepoxide. It seems that the introduction of an OH group at the position 3 of the BZD ring reduced the intensity of the hypolipidemic activity.

Regarding the effects on blood glucose level, it is obvious that they are not dose related. From this investigation as well as from other studies [1] it can be concluded that no clear dose-response was visible.

In conclusion, temazepam has a modest lipid-lowering activity following acute administration in hyperlipidemic rats. Concerning the mechanism of action of temazepam on serum lipids in hyperlipidemic rats, it is very probably identical to that responsible for other BZD clonazepam [6], diazepam [3], 4'chlorodiazepam [2] i.e. the stimulation of the peripheral type BZD receptors. The mechanism of BZD on the blood glucose level is still completely unknown.

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Effects of midazolam on glycemia and serum lipids in rats

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Midazolam administered ip. in albino rats (each group consisted from 10 animals rendered hyperdyslipidemic by the administration of Triton WR-1339) induced at most doses a significant reduction of glycemia ($p<0.001$). However, the reduction of blood glucose level was outside of the dangerous level. Midazolam elicited also very significant decrease of the elevated serum lipids ($p<0.001$).

The pharmacological analysis of these phenomena by using the peripheral type benzodiazepine (BZD) receptors antagonist PK 11105, the central BZD receptor antagonist flumazenil and the purinergic P1 receptors antagonist aminophylline has shown that the effects on serum lipids were due, very probably to the stimulation of the peripheral type BZD receptors. Aminophylline seems to have the property to block the peripheral type BZD receptors. The effects on blood glucose level were very variable.

Keywords: Rats, hyperlipidemy, midazolam, PK-11195, flumazenil, aminophylline.

Midazolam is one of the most recent development in the class of benzodiazepines (BZD). It has similar properties to diazepam and other BZD. However, its duration of action is shorter. Midazolam is viewed by many authors as a possible successful follower of diazepam, in the medical practice (for a review see Dormicum, 29). Our previous investigations [6–8] have shown that almost all BZD have the property to lower the serum lipids levels when they were artificially augmented. These findings were confirmed by other authors [1, 2, 3, 5, 20, 24, 25, 28]. Preliminary clinical results confirmed this statement [14]. At the same time, glycemia was variably influenced [3, 4, 7]. Other studies [16] have shown that the antihyperlipidemic activity of BZD was

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due, at least partly to the stimulation of the peripheral type BZD receptors, since PK 11195 (RP 52028) a specific antagonist of these receptors [8, 17] greatly diminished or even reversed the effects of most doses of BZD on serum lipids.

On the contrary, flumazenil, a selective antagonist of the central BZD receptors [12] failed to antagonize the actions of BZD. Moreover, this compound mimicked the lipid lowering activity of BZD (16]. Since these effects could be blocked by PK 11195, it appears that, at least in some doses, flumazenil is capable to stimulate the peripheral type BZD receptors.

The aims of this paper were:

1) To investigate the effects of various doses of midazolam on glycemia and serum lipids in hyperdyslipidemic rats.

2) To see whether PK 11195 could block the metabolic effects of midazolam.

3) To study the actions of flumazenil administered jointly with midazolam.

4) To investigate the possible interactions between midazolam and aminophylline, a P1 purinergic receptor antagonist (for the explanation of the reason of this association see discussion).

General Procedures, Materials and Method

The experiments we carried out in male Wistar rats, weighing 150–200 g. Prior to the experiments the rats were fed a common rat chow, with water *ad libitum*. The ambient temperature was kept constant (20–22 °C).

The hyperdyslipidemia was induced by an intraperitoneal (i. p.) injection of an aqueous solution of Triton WR-1339 (200 mg/kg) [10, 22]. The food was then withdrawn till blood sampling, but the water was allowed *ad libitum*. 18 hours after Triton WR-1339 administration the blood was sampled by the punction of the retro-orbital sinus. Blood glucose was determined using the enzymatic method of Ashwell (Merck's test). From the serum, the following measurements were carried out : total lipids by the procedure of Zöllner and Kirsch, total cholesterol (TCHOL) with the enzymatic method of Roschlau et al. (Reanal test). Triglycerides by the enzymatic technique of Epstein (The methods were described by Manta et al., 19). The results were expressed as mmol/1 with the exception of TL which were expressed as mg%.

Experimental groups

The experiments were carried out in the following groups of rats:

1) Control rats which were treated as described above. In addition two hours prior to i.p. injection they received (in sites different from those used for Triton WR-1339 administration) an i.p. injection of the mixture used for suspension of drugs (with the exception of aminophylline which was dissolved in saline). The mixture consisted in methylcellulose 0.2% + glycerin 1%. It was administered in volumes equal to those used for i.p. drug administration. The control group for aminophylline consisted of rats which received i.p. saline. Since this was practically without any effects, the results were pooled with those obtained in control rats, treated with the mixture.

2) Reference group: the rats were administered two hours prior to Triton WR-1339 injection fenofibrate i.p. (200 mg/kg). The remainder of the procedures was identical to that already mentioned.

3) PK 11195 (RP 52028) (25 mg/kg, i.p.) treated rats which were processed as above.

4) Flumazenil (R015-1788) (10 mg/kg P) was given under the same conditions

5) Aminophylline (10 mg/kg i.p.) was administered in another group.

6) Midazolam was injected i.p. in the following doses: 2.5 mg/kg – 4.0 mg/kg – 5.0 mg/kg – 7.5 mg/kg and 10.0 mg/kg. The remainder of the procedures was identical to that described previously.

7) Two hours prior to the administration of various doses of midazolam, PK-11195 (25 mg/kg) or flumazenil (10 mg/kg) were given i.p.

8) One hour prior to midazolam, aminophylline (10 mg/kg) was injected i.p. The procedures were identical to those mentioned.

Each group consisted of 10 rats with the exception of the control group which was formed from 20 rats.

Statistics

The results were compared in the following manner, by using the unpaired "t" test (Snedecor and Cochran, 26): Each experimental group was compared with the control group (P).

Besides, midazolam administered rats groups were compared with the groups of rats which received in addition PK 11195, flumazenil or aminophylline. Each dose of midazolam alone was compared with the same dose of midazolam associated with the antagonists (P1). The differences between groups were considered to be significant when $P<0.05$.

Drugs

Midazolam and flumazenil were gifts of Hoffman – La Roche Co., Basle, Switzerland, PK 11195 (RP 52028) was donated by Rhone – Poulenc – Rorer Co. Neuilly-sur-Seine, France. Fenofibrate was purchased from Ratio Pharm Co. Blaubeuren, Germany.

Aminophylline was a Romanian product.

Results

I. Fenofibrate induced the following changes in comparison with the control (hyperdyslipidemic) group: very significant decreases of glycemia, TL, TCHOL, TG. PK 11195, elicited a very significant decrease of glycemia, a significant elevation of TCHOL, whereas TL and TG were unaffected. Flumazenil brought about only a significant reduction of glycemia, other parameters remained unchanged. Aminophylline failed to induce any significant alteration (Table I).

Table I

Effects of fenofibrate, PK 11195, flumazenil and aminophylline on blood glucose and serum lipids in hyperdyslipidemic rats

Group/dose (mg/kg)	Glycemia (mmol/l)	TL (mg %)	TCHOL (mmo/l)	TG (mmol/l)
Control Hyperdyslipidemic (Triton WR-1339, 200 mg/kg)	5.71±0.26	2838.9±174.8	7.12±0.53	20.7±1.09
Reference (Fenofibrate 200 mg/kg)	3.90±0.056 P<0.001↓	614.8±23.7 P<0.001↓	2.15±0.12 P<0.001↓	1.77±0.18 P<0.001↓
PK 11195 (25 mg/kg)	4.88±0.17 P<0.01↓	2990.8±127 NS	9.39±0.28 P<0.01↑	22.6±1.18 NS
Flumazenil (10 mg/kg)	4.74±0.13 P<0.001↓	2915.3±163 NS	6.76±0.47 NS	23.28±1.86 NS
Aminophylline (10 mg/kg)	5.18±0.22 NS	2858±76.7 NS	7.044±0.28 NS	18.36±0.88 NS

All the groups consisted of 10 rats, with the exception of the control group which consisted of 20 animals.

NS: Nonsignificant

↓: Decrease when compared to the control.

↑: Increase when compared to the control.

II. Midazolam induced the following modifications of the investigated parameters when they were compared with those of the control group: glycemia was diminished by doses of 2.5–5.0 and 10.0 mg/kg and it was not changed by other doses. TL were not modified by the lowest and highest doses and reduced by other doses, TCHOL was changed in a similar manner. TG were decreased by all doses. The most prominent changes of the serum lipids were elicited by the dose of 4.0 mg/kg (Table II).

III. When the effects of various doses of midazolam were compared with the same doses to which PK 11195 was added, the following results were obtained: glycemia was very significantly raised by the lowest dose (2,5 mg/kg) and by the highest one (10 mg/kg). It was very significantly diminished by the doses of 4 mg/kg and 7.5 mg/kg and remained unchanged by 5 mg/kg. TL were increased by 4 and 5 mg/kg, reduced by 7.5 and 10 mg/kg and not modified by 2.5 mg/kg. TCHOL and TG behaved like TL (Table III).

Table II

Effects of various doses of midazolam on blood glucose and serum lipids in hyperdyslipidemic rats

Group/dose (mg/kg)	Glycemia (mmol/l)	TL (mg %)	TCHOL (mmo/l)	TG (mmol/l)
Control Hyperdyslipidemic (Triton WR-1339, 200 mg/kg)	5.71±0.26	2838.9±174.8	7.12±0.53	20.7±1.09
Midazolam (2.5 mg/kg)	4.57±0.19 P<0.001↓	2110±354 NS	6.64±0.44 NS	13.4±1.47 P<0.001↓
Midazolam (4.0 mg/kg)	5.68±0.12 NS	440.2±57 P<0.001↓	2.13±0.26 P<0.001↓	1.57±0.45 P<0.001↓
Midazolam (5.0 mg/kg)	4.14±0.14 P<0.001↓	744.11±75.5 P<0.001↓	2.96±0.161 P<0.001↓	2.99±0.49 P<0.001↓
Midazolam (7.5 mg/kg)	5.42±0.22 NS	971±37.2 P<0.001↓	4.99±0.177 P<0.001↓	4.51±0.20 P<0.001↓
Midazolam (10.0 mg/kg)	4.82±0.17 P<0.001↓	3026±180 NS	7.65±0.39 NS	16.65±1.20 P<0.01↓

Foot notes: See Table I

Table III

Effects of the association of PK 11195 on the activity of various doses of midazolam in hyperdyslipidemic rats

Group/dose (mg/kg)	Glycemia (mmol/l)	TL (mg %)	TCHOL (mmo/l)	TG (mmol/l)
Midazolam (2.5 mg/kg)+ PK 11195 (25 mg/kg)	5.52±0.127 P1<0.001↑	1948±272 NS	5.88±0.27 NS	12.00±0.88 NS
Midazolam (4.0 mg/kg)+ PK 11195 (25 mg/kg)	4.30±0.140 P1<0.001↓	1805±97.4 P1<0.001↑	5.90±0.30 P1<0.001↑	12.06±0.85 P1<0.001↑
Midazolam (5.0 mg/kg)+ PK 11195 (25 mg/kg)	4.52±0.17 NS	1620±206 P1<0.001↑	6.66±0.55 P1<0.001↑	13.63±2.07 P1<0.001
Midazolam (7.5 mg/kg)+ PK 11195 (25 mg/kg)	4.36±0.11 P1<0.001↓	551±45.45 P1<0.001↓	2.137±0.07 P1<0.001↓	1.69±0.33 P1<0.001↓
Midazolam (10.0 mg/kg)+ PK 11195 (25 mg/kg)	6.85±0.41 P1<0.001↑	608±54.8 P1<0.001↓	3.95±0.39 P1<0.001↓	2.80±0.61 P1<0.001↓

Foot notes: See Table I

IV. The association of flumazenil with various doses of midazolam, yielded the following results: glycemia was reduced by all doses, TL were diminished by the doses of 5 and 10 mg/kg and not changed by other doses. TCHOL was decreased by 10–7.5 and 5 mg/kg and not modified by other doses. TG were reduced by 10 and 5 mg/kg, other doses being ineffective (Table IV).

Table IV

Effects of the combination of flumazenil on the metabolic activity of various doses of midazolam in hyperdyslipidemic rats

Group/dose (mg/kg)	Glycemia (mmol/l)	TL (mg %)	TCHOL (mmo/l)	TG (mmol/l)
Midazolam (2.5 mg/kg)+ Flumazenil (10.0 mg/kg)	4.03±0.10 P1<0.01↓	2171±124 NS	6.32±0.37 NS	15.37±1.11 NS
Midazolam (4.0 mg/kg)+ Flumazenil (10.0 mg/kg)	4.33±0.11 P1<0.001↓	415±13.5 NS	2.67±0.08 NS	0.78±0.09 NS
Midazolam (5.0 mg/kg)+ Flumazenil (10.0 mg/kg)	4.28±0.16 NS	453±240 P1<0.001↓	1.93±0.165 P1<0.001↓	1.35±0.18 P1<0.001↓
Midazolam (7.5 mg/kg)+ Flumazenil (10.0 mg/kg)	4.83±0.13 P1<0.01↓	933±66.7 NS	3.23±0.19 P1<0.001↓	5.47±0.42 NS
Midazolam (10.0 mg/kg)+ Flumazenil (10.0 mg/kg)	4.24±0.12 P1<0.01↓	595±67 P1<0.001↓	3.12±0.46 P1<0.001↓	2.85±0.42 P1<0.001↓

Foot notes: See Table I

Table V

Effects of aminophylline on the metablic activity of various doses of midazolam in hyperlipidemic rats

Group/dose (mg/kg)	Glycemia (mmol/l)	TL (mg %)	TCHOL (mmo/l)	TG (mmol/l)
Midazolam (2.5 mg/kg)+ Aminophylline (10.0 mg/kg)	5.18±0.173 P1<0.01↑	1678±134 P1<0.001↓	5.85±0.25 NS	9.69±1.51 P1<0.001↓
Midazolam (4.0 mg/kg)+ Aminophylline (10.0 mg/kg)	5.20±0.188 P1<0.01↓	2580±130 P1<0.001↑	5.91±0.18 P1<0.001↑	20.39±1.28 P1<0.001↑
Midazolam (5.0 mg/kg)+ Aminophylline (10.0 mg/kg)	4.84±0.17 P1<0.001↑	1085±140 P1<0.01↑	3.49±0.16 P1<0.01↑	4.33±0.98 P1<0.001↑
Midazolam (7.5 mg/kg)+ Aminophylline (10.0 mg/kg)	5.14±0.26 NS	517±66.1 P1<0.001↓	2.66±0.35 P1<0.001↓	1.86±0.38 P1<0.001↓
Midazolam (10.0 mg/kg)+ Aminophylline (10.0 mg/kg)	4.37±0.14 NS	1421±105 P1<0.001↓	5.91±0.44 P1<0.01↓	12.58±1.29 P1<0.01↓

Foot notes: See Table I

V. The combination of aminophylline with various doses of midazolam yielded the following modifications: glycemia was increased by the doses of 2.5 and 5.0 mg/kg, significantly reduced by 4 mg/kg and unaffected by other doses. TL were decreased by 2.5–7.5 and 10.0 mg/kg and very significantly augmented by 4 and 5 mg/kg. TCHOL and TG behaved like TL, with the exception that the dose of 2.5 failed to affect TCHOL (Table V).

Discussion

Our results have shown that in Triton WR 1339 administered rats, midazolam had at least from a qualitative point of view approximately the same activity on serum lipids as other BZD [3, 7]. Indeed, the dose–effect curve had a bell shaped aspect. Moreover, the parameter which was most affected was the TG level. However, there is an important difference: The peak of TG and TCHOL changes was 4 mg/kg for midazolam and 5 or 7.5 mg/kg for other BZD.

The effects of midazolam on blood glucose level were not dose-dependent. For most doses (2.5–5.0 and 10.0 mg/kg) a hypoglycemic response was observed, other doses failed to affect glycemia. This statement is in consensus with other results which have shown that blood glucose level is very variably influenced by BZD [8]. Fenofibrate (200 mg/kg) which was used as a reference hypolipidemic compound

induced very significant reductions of TL, TCHOL and TG. The blood glucose level was also very significantly diminished. The effects of PK 11195 and of flumazenil on blood glucose level and serum were already presented (see results).

Aminophylline was used in these experiments for the following reasons: according to some authors [4, 11] adenosine would play a role in the mechanism of action of BZD on the central nervous system. For this assumption, the obvious antagonism of the central depressive effect of BZD by methylxanthines, mainly aminophylline seems to be confirmatory. This hypothesis is not supported by most investigators. However, it can explain some anomalies of the action of BZD on the central nervous system (Bidart et al, [4]). It seems that the effects of aminophylline on the metabolic effects of BZD were still not investigated. Admitting the hypothesis that the action of BZD on glycemia and/or serum lipid are mediated by purinergic P1 receptors, the use of a low dose (10 mg/kg) of aminophylline in order to antagonize the effects of midazolam was logical since in the used dose, aminophylline was without any effects on the investigated parameters.

Thus, at used doses, the antagonists of the receptors possibly involved had only minimal agonistic activity or even are completely inactive on the levels of the serum lipids. PK 11195 and flumazenil diminished glycemia, whereas aminophylline was ineffective.

If the effects of midazolam (and other BZD) would be due to the stimulation of the peripheral type BZD receptors, the administration of PK 11195 a competitive antagonist should abolish or at least diminished the effects on serum lipids and blood glucose. This assumption was only partly confirmed. The effects of the optimal dose, the dose which induced the most marked diminution of the serum lipids were diminished. The same was valid also for the next dose (5.0 mg/kg). On the contrary the effects of higher doses (7.5 mg/kg and 10.0 mg/kg) on the serum lipids were enhanced. The causes of this particular behavior are still unclear. It is noteworthy that these doses had a less tendency to the reduction of serum lipids than the lower ones. So, it is possible that the antagonism of this trend to increase serum lipids would explain this behavior. An alternative explanation would be as follows: according to newer data [15] PK 11195 is not a pure antagonist of the peripheral type BZD receptors, it is rather a partial agonist. So, it is conceivable that in association with certain doses of BZD, PK 11195 enhances the lipid lowering activity of these compounds instead of antagonizing it.

Flumazenil, in sharp contrast to PK 11195 induced for most doses of midazolam a potentiation of the lipid lowering activity. These results confirmed previous findings [16]. The explanation of this statement is as follows: flumazenil despite to be considered as acting only on central BZD receptors as a competitive antagonist, [12] has very probably a stimulatory effect on the peripheral type BZD receptors. Indeed, it

potentiated the effects of BZD on serum lipids and this property could be antagonized by PK 11195 [16]. It is noteworthy that according to some authors [21, 23, 27] flumazenil is not a pure antagonist, but a partial agonist.

Aminophylline, as other methylxanthines is viewed as a nonspecific BZD antagonist, [6]. It is noteworthy that the aforementioned conclusion concerns only the central BZD receptors, as far as we know, no investigations were undertaken in order to see whether it is acting on the peripheral type BZD receptors.

Our experiments have shown that aminophylline administered jointly with midazolam had partly effects similar to PK 11195. So, it potentiated the effects of the lowest dose (2.5 mg/kg) which in itself decreased only TG, at the dose of 7.5 mg/kg and at the highest dose (10.0 mg/kg). At the same time, it antagonized the effects of the medium doses (4.0 and 5.0 mg/kg). Thus, it can not be excluded that aminophylline would have some similar properties to PK 11195, i.e. it could antagonize the peripheral type BZD receptors when midazolam was administered in some doses and it could stimulate them in other doses. This assumption should be confirmed by other experiments, including the binding of the radioactive ligands.

With regard to the interactions of PK 11195 and aminophylline with the effects of midazolam on blood glucose, the results were very variable, so no firm conclusion could be drawn. On the other hand, flumazenil potentiated the hypoglycemic activity of midazolam at almost all doses.

The causes of these discrepancies are not known. It is worthwhile to remind that with the exception of diabetic rats in which diazepam had an obvious hypoglycemic activity (Cuparencu et al. [7]), in other experimental settings diazepam and other BZD had very variable effects on glycemia. Thus, the involvement of the BZD receptors in the activity of BZD on glycemia is a matter of debate.

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STUDIES ON THE SITES AND MECHANISMS OF 5-HT1A RECEPTOR-MEDIATED IN VIVO ACTIONS

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In the first study the possible role of the hypothalamic paraventricular nucleus (PVN) in 5-HT_{1A} receptor agonist-induced neuroendocrine responses was tested. Surgical lesions of the PVN completely blocked ACTH and corticosterone and markedly attenuated oxytocin but not prolactin responses to ipsapirone, providing evidence for a crucial role of the PVN in these responses. In the second study we have compared the effectiveness of intracerebroventricular and intravenous administration of 8-OH-DPAT in frequently used behavioural models of 5-HT_{1A} receptor activation, namely lower lip retraction, body temperature and tail flick responses. We have found marked differences in the rates of effectiveness. We conclude that these models measure the activation of different subsets with clearly separate location of 5-HT_{1A} receptors.

Keywords: Serotonin (5-HT), Hypotalamic paraventricular nucleus (PVN)

Serotonin (5-HT), involved in numerous physiological and pathophysiological processes, interacts with various distinct membrane receptors. 5-HT receptors are now divided into seven major groups (or classes, families) and at least 5-HT_1 and 5-HT_2 families are further subdivided. Activation of 5-HT_{1A} receptors in the central nervous system modulates the state of emotion and anxiety, causes several behavioural effects, changes physiological and neuroendocrine parameters (1,2,3). Studies about the sites of actions are often missing or conclusions are confusing (1,3). Possible methods to differentiate between the mechanisms of action include brain lesion studies and specific administration of selective compounds. In the first set of experiments we have used hypothalamic paraventricular nucleus lesions to find out more about the mechanism of hormonal responses. In the second, the effectiveness of intracerebroventricularly (i.c.v.) and intravenously (i.v.) administered 8-OH-DPAT were compared in frequently used behavioural models of 5-HT_{1A} receptor activation.

Methods

Male Sprague-Dawley rats were kept under standard condition. (±)8-Hydroxy-2-(di-n-propylamino)tetralin hydrobromide (8-OH-DPAT, R.B.I., Natick, MA, USA) and ipsapirone (Bayer AG, Wuppertal, Germany) were used in the studies.

Study I.: Paraventricular lesions were performed with a rotating knife destroying an inverted cone of tissue containing the PVN (3). Ipsapirone was administered into the femoral vein, blood was obtained from chronic cannula in the femoral artery in conscious, freely moving animals. Hormone measurements and statistical analysis were performed as previously reported (2,3). Net area under the curve (NET AUC) was calculated as the increase of concentration over baseline times length of testing.

Study II.: I.c.v. cannula was implanted under halothan anesthesia. The following coordinates were used (bregma 0,0): AP 1.3 mm, L 1.8 mm, DV 4.0 mm. Challenges with wide dose-ranges of i.c.v. or i.v. (tail vein) administered 8-OH-DPAT were done in gently restrained

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rats after at least one week recovery. Body temperature was continuously measured with rectal proves and recorded for at least 30 min. Lower lip retraction was scored based on the visibility of the lower incisors. Tail flicks were defined as the number of elevation of the tail over 30 min. Statistical analysis of the dose-response curves were done with ALLFIT.

Results and discussion

Surgical lesions of the PVN completely blocked ACTH and corticosterone, and markedly attenuated oxytocin but not prolactin responses to ipsapirone (2.0 mg/kg i.v., Fig. 1; *P<0.05). That means that stimulation of 5-HT_{1A} receptors causes CRH/ACTH/corticosterone and oxytocin releases mediated by the hypothalamic paraventricular nucleus. Prolactin responses are mediated, however, by other hypothalamic sites.

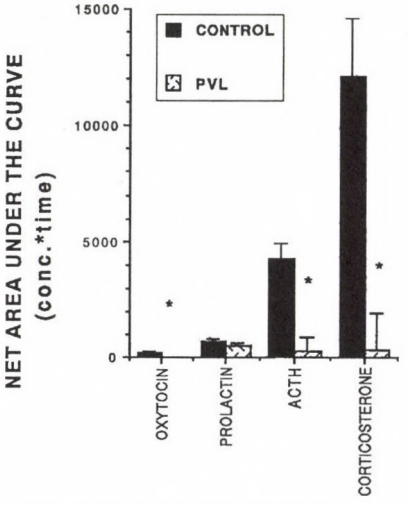

Fig. 1. Neuroendocrine responses to IPSAPIRONE (2 mg/kg, iv.) *: p<0.05 compared to control

Table I

ED_{50}s and ratios of effectiveness (ED_{50} i.v. per. ED_{50} i.c.v.) of intracerebroventricularly (i.c.v.) or intravenously (i.v.) administered 8-OH-DPAT inducing hypothermia, lower lip retraction and tail flick responses and their statistical comparisons. Values are given as means ± S.E.M.

	i.c.v. ED_{50} (μg/kg)	i.v. ED_{50} (μg/kg)	Ratio of effectiveness (i.v. ED_{50}/i.c.v. ED_{50})
Hypothermia	5.10±0.91*	49.9±4.24	9.78±1.68
Lower lip retraction	29.4±2.56*	86.0±7.63	2.92±0.363#
Tail flicks	408±133	193±12.5	0.47±0.14#

* Significantly different from i.v. ED_{50}, P<0.05

Significantly different from Ratio of effectiveness in hypothermia, P<0.05

Analysis of the dose response curves provided evidence for a 10 fold effectiveness of i.c.v. over i.v. administration in hypothermia and a 3 fold difference in effectiveness for lower lip retraction. In induction of tail flicks 8-OH-DPAT was less effective after i.c.v. than after i.v. administration (Table 1). We conclude that 5-HT_{1A} receptors mediating hypothermia or lower lip retraction are localized at different brain areas. Hypothermia but not lower lip retraction is mediated mainly by receptors in the close vicinity of the lateral ventricle. Receptor sites outside the brain are responsible for tail flick responses.

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PLATELET RICH PLASMA SEROTONIN CONTENT IN PATIENTS SUFFERING FROM DIFFERENT PSYCHIATRIC DISORDERS

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Platelet rich plasma serotonin contents were examined using HPLC-EC method in patients suffering from different anxiety states and compaired to age matched healthy controls.

We have found significant increase of PRP serotonin level in the group of schizophrenic patients and in patients suffering from dementia compaired to controls. PRP serotonin content was significantly lower in heavy drinkers but in patients suffering from panic disorder did not differ significantly from the controls.

Keywords: serotonin, anxiety, platelet model, HPLC-EC

Serotonin is one of the best-known and the most examined neurotransmitter, which may be involved in the pathogenesis of different anxiety states. In clinical studies platelets are often used as convenient peripheral model for serotonergic system as they have a similar mechanism of active uptake for, and storage of serotonin as have serotonergic neurons in the central nervous system.

In our present study platelet rich plasma (PRP) serotonin contents were measured in four group of patients suffering from panic disorder (PD n=7), schizophrenia (Sch n=32), dementia (Dem n=24) and alcoholism (Alc n=16) all showing anxiety symptoms. Psychiatric patients were diagnosed using DSM-III-R criteria and alcoholism was also confirmed by laboratory findings.

Age matched healthy controls were selected from the medical staff. Patients and controls were free of drugs known to after platelet functions or serotonin metabolism. All patients and controls were informed on the object of trial and written consent was obtained.

Methods

9 ml venous blood was drawn before meals between 8 and 10 a.m. into plastic tubes containing 1 ml ACD (Na-citrate, citric acid, D-glucose) as anticoagulant. The samples were centrifuged at 500 g for 10 minutes. PRP was transferred into Eppendorf plastic tubes and sonicated for 20 sec. (Labsonic 2000). Platelets were counted (CELL-DYN 400) before and after sonication. Samples were stored at –80 °C for assay no longer than 14 days and serotonin content was measured by the modified method of Ortiz et al. 1988.

1 ml aliquots were treated with equal volume of 0.8 N perchloric acid containing 5 ng/20 µl N-methyl-serotonin (SIGMA) as internal standard, vortexed for 20–30 seconds and particles were let to sediment at 0–4 °C for 20 minutes.

Samples were centrifuged again (1000 g, 15 min.) and the supernatant was directly injected onto HPLC column (SUPELCO LC-18-DB). Serotonin content was given in ng/10^9 platelets.

Data were analyzed by two tailed Student's test. The level of significance was 1%.

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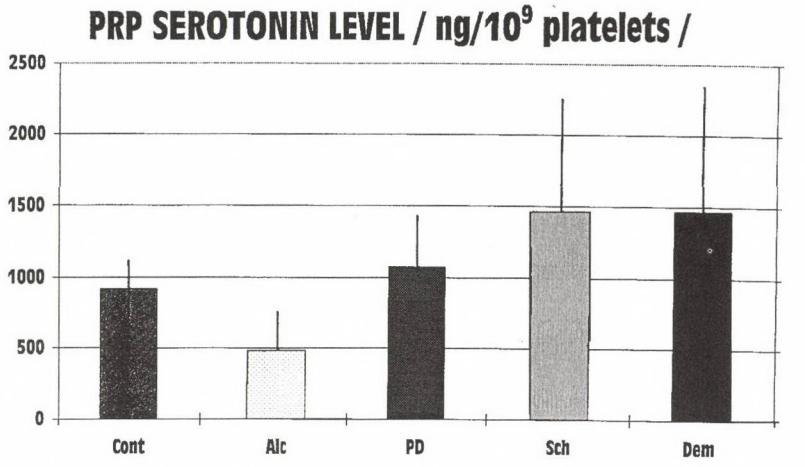

Fig. 1. Cont:control, Alc:alcoholism, PD:panic disorder, Sch:schizophrenia, Dem:Dementia

Results and discussion

We have found significant increase of PRP serotonin level in group of Sch patients (1463.2±793.9 ng/10^9 plat.) and in patients suffering from Dem (1467.0±880.2) compaired to healthy controls (917.1±189.9). However PRP serotonin content was significantly lower in Alc patients (482.1±271.4) than in controls. PRP serotonin level of (drug-free) PD patients (1074.0± 334.5) did not differ significantly from healthy controls (F=1.25, p>20% N.S.).

Our results show that anxiety states of different origin may be characterized by normal as well as increased or decreased PRP serotonic levels.

Accepting that platelets are good peripheral models for serotonergic neurons of the central nervous system our data suggest that anxiety of the patients studied is influenced with priority by other factors. Data may also indicate that platelets have limited value as peripheral models for central serotonergic neurons and PRP serotonin level is also regulated by peripheral metabolism of serotonin.

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ANTIESTROGENS, ANTIANDROGENS

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The aim of the study was to find new antiestrogenic and antiandrogenic structures. Out of the triphenyl-alkene derivatives Panomifene (EGIS-5660) proved to be the most active antiestrogenic compound which binds to specific estrogen receptors and exhibits inhibitory effects on experimental mammary tumors both in vitro and in vivo. The investigated antiandrogenic compounds were indol and imidazole derivatives. One of these compounds a di-imidazolil derivative, GYKI-24479 inhibited the in vitro androgen (testosterone and androstenedione) biosynthesis both in vitro and in vivo in concentration/dose dependent manner, and in these respects proved to be more active than the referent ketoconazole.

Keywords: antiestrogen, Panomifene, tumor inhibition, antiandrogen, steroid biosynthesis inhibition

In the 70-ies a project has been started in the Institute of Drug Research searching for new antihormones, mostly with antiestrogenic or antiandrogenic properties. Out of about 200 triphenyl-alkane and -alkene derivatives Panomifene (Pan, EGIS-5650, /E/-1,2-diphenyl-1-[4-(2-hydroxy-ethylamino-ethoxy)-phenyl]-3,3,3-trifluoro-1-propene) was selected for detailed investigation for its antiestrogenic and tumor inhibiting activities both in vitro and in vivo. It was expected, that the trifluoro-substitution might stabilise the receptor binding of the molecule ensuring a long-lasting antihormone and antitumor effect.

Methods and Results

The antiestrogenic/intrinsic estrogenic effects of Pan were tested in infantile rat uterine test, its antitumor activity in human mammary cell-line in vitro and NMU (Nitrosomethylurea) induced mammary cc. in rats. The compound inhibited the uterine growth induced by E_2 given in a dose-range of 0.03–1 mg/kg/day p.o. and reduced the uterine RNA, DNA and protein content in a dose-dependent manner. The compound bound to specific estrogen receptors prepared from uterine cell cytosolic fraction and displaced the tritiated E_2 from receptor sites competitively. The IC_{50} were 3.06×10^{-7}mol/l for Pan and 7.84×10^{-7}mol/l for tamoxifen. The attachment of Pan to specific nuclear receptors was long-lasting, the level of ligand-receptor complex did not decrease significantly until 48 hours. This long-lasting binding was reflected in the in vivo antiuterotrophic effect. Given at a single dose of 1 mg/kg p.o. the inhibition lasted at least 120 hours. In MCF-7 human mammary cell-line (E_2+, Prg+) Pan inhibited the proliferation in a concentration-dependent manner in 0.1–1 μmol/l concentration range. In 3 μmol/l concentration it exerted a cytotoxic effect, and also inhibited the proliferation in receptor negative breast cancer cell line, but only in a high concentration. Pan given at doses of 1–5 mg/kg p.o. three times a week elicited almost the same response rate of the animals. The results are shown in Table 1.

Summing up the results, Panomifene proved to be a potent estrogen antagonist with relatively low intrinsic estrogenic and high antitumor activities and can be regarded as a potential drug in the treatment of estrogen dependent tumors.

The second part of the program was to search for antiandrogen structures, which inhibit some key enzymes of androgen biosynthesis. Numerous imidazolil derivatives were synthesised in the institute of Drug Research in the last decade. A relatively simply screening system was elaborated for checking the enzyme inhibitory effect of the newly synthesised compounds. The

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screen started with an in vitro study in mouse Leyding cell short culture in the presence of 1 ng/tube LH for stimulating the androgen hormone biosynthesis, and continued with an in vivo one in infantile male rats stimulated by LHRH (10 ng/animal s.c.). The concentration of the end product (testosterone) and also some intermediers were detected by RIA in the medium and also in the plasma.

Table 1

Inhibition of the growth of NMU-induced mammary tumor cc in rats

response rate	Panomifene 1 mg/kg n=8	Panomifene 5 mg/kg n=10	tamoxifen 5 mg/kg n=9
complete	37.5%	40.0%	11.1%
partial	25.0%	20.0%	22.2%

the treatment was started when the tumor size had attained approximately 500 mm^3

Among of the compounds a di-imidazole derivative (GYKI-24479) proved to be the most active molecule. The compound inhibited the biosynthesis of testosterone (and that of androstenedione) in vitro in a concentration dependent manner, and in this respect proved to be more active than the referent ketoconazole in a concentration-range of 10^{-5}–10^{-8} mol/l. This effect was found to be selective, namely the compound did not block the production of progesterone (which is a key intermedier also of the corticosteroids). On the contrary ketoconazole inhibited the synthesis of this hormone as it could be expected. In vivo the lowest dose of GYKI-24479 which significantly decreased the serum testosterone level in infantile male rats stimulated by LHRH was found to be 1 mg/kg/day p.o. Given in the same dose the serum progesterone level increased significantly. The plasma corticosterone level of the animals was found to be very high in the GYKI-24479 treated animals (possible due to the stress effect), on the contrary, ketoconazole inhibited the function of the ACTH-adrenal axis. The results of the in vivo studies are shown in Table 2.

Table 2

Changes of the plasma levels of androgen hormones and corticosterone of infantile male rats treated with GYKI-24479 and ketoconazole

mg/kg/day p.o.	testosterone nmol/l±SE		corticosterone nmol/l±SE	
LHRH	55.9±2.8		136.6±7.7	
	24479	ketoconazole	24479	ketoconazole
0.2	53.3±1.9	49.1±2.8	303.3±27.1[ab]	105.9±9.6[a]
1.0	48.0±1.9[a]	49.7±2.9	316.5±17.2[ab]	104.8±8.6[a]
5.0	37.3±1.2[a]	41.1±0.8[a]	308.5±24.0[ab]	94.8±11[a]

[a] $p<0.05$ compared to LHRH [b] $p<0.05$ compared to ketoconazole

Discussion

The final conclusion of the study is that a new very active antiandrogen was found which is at least as active as the ketoconazole, what is more, its effect is more specific in respect of the biosynthesis of the testosterone than that of ketoconazole. Unfortunately the compound proved to be hepatoxic in rats, accordingly the development of this group of compounds was cancelled.

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ELECTROPHYSIOLOGICAL EFFECTS OF WAY 100635, A NEW 5-HT_{1A} RECEPTOR ANTAGONIST, ON DORSAL RAPHE NUCLEUS SEROTONINERGIC NEURONES AND CA1 PYRAMIDAL CELLS *IN VITRO*

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The novel 5-HT_{1A} receptor antagonist WAY 100635 [(N-(2-(-4(2-metoxyphenyl)-1-piperazinyl)ethyl)-N-(2-pyridinyl) cyclohexane carboxamide)] has been tested on 5-HT_{1A} receptor-mediated inhibition of firing and intracellularly recorded hyperpolarisation of serotoninergic cells of the dorsal raphe nucleus (DRN) and on hyperpolarisation of hippocampal CA1 pyramidal cells. WAY 100635 selectively blocked 5-HT_{1A} receptor-mediated responses of 5-HT, 8-OH-DPAT, lesopitron and 5-CT. The antagonism of the hyperpolarisation elicited by 5-CT was competitive in the DRN and non competitive in CA1, probably because of the existence of a 5-HT_{1A} receptor reserve in serotoninergic cells of DRN.

Keywords: 5-HT, 5-HT_{1A} receptor antagonist, WAY 100635, hippocampus, dorsal raphe

Most of serotonin (5-HT)-containing projections in the brain originate from the raphe nuclei, where 5-HT exerts a feed-back control of discharge through 5-HT_{1A} somatodendritic receptors. On the other hand, 5-HT_{1A} receptors exist on almost all known neurones which are targeted by serotoninergic neuromodulation, including hippocampal pyramidal cells. WAY 100635 is a recently synthesised compound which has been shown to efficiently and selectively block 5-HT_{1A} receptor-mediated responses, both in biochemical and behavioural studies [2]. This makes this drug a potential tool to assess the physiological implication of 5-HT_{1A} receptors in central serotoninergic neurotransmission. The aim of the present study was to investigate the effects of WAY 100635 on electrophysiological responses to 5-HT_{1A} agonists both on serotoninergic cells of DRN and on hippocampal CA1 pyramidal cells, *in vitro*.

Methods

Intracellular recordings were performed in rat hippocampal [4] or DRN [3] slices, using conventional recording techniques [4]. Synaptic potentials were evoked by stimulating the stratum radiatum of the CA1 hippocampal region. Agonists and/or antagonists were superfused in oxygenated artificial cerebrospinal fluid.

Results and Discussion

WAY 100635 (0.5–10 nM) neither affected the resting membrane potential (r.m.p.) nor the membrane input resistance (R_{in}) of intracellularly recorded CA1 pyramidal cells in the hippocampus. However it antagonised the hyperpolarisation and the decrease in R_{in} elicited by 5-HT (30–300 μM, Fig. 1) or 5-carboxamidotryptamine (5-CT, 50–300 nM). WAY 100635 did not antagonise (Fig. 1) the hyperpolarising effect of the $GABA_B$ receptor agonist baclofen, which opens potassium channels through G-protein-dependent mechanisms apparently held in common with 5-HT_{1A} receptors [1]. It is therefore unlikely that WAY 100635 blocked 5-HT_{1A} receptor-operated potassium channels or G-proteins. WAY 100635 blocked the 5-HT_{1A} receptor-

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mediated inhibitory effect of 5-HT on the evoked excitatory synaptic potentials. Cumulative concentration-response curves for the hyperpolarization or the decrease in R_{in} produced by 5-CT (3 nM–10 μM) were shifted to the right by WAY 100635, with a reduction of the maximal response to the agonist. In DRN slices, WAY 100635 (10 nM) prevented the decrease in the firing rate produced by 5-HT (3–15 μM), 8-OH-DPAT (10 nM), 5-CT (20 nM), or lesopitron (100 nM). The antagonism of 5-HT response could be surmounted by increasing the concentration of the agonist. Similarly, in intracellular recordings from DRN neurones, WAY 100635 shifted the concentration-response curve of the agonist 5-CT to the right without decreasing the maximal response.

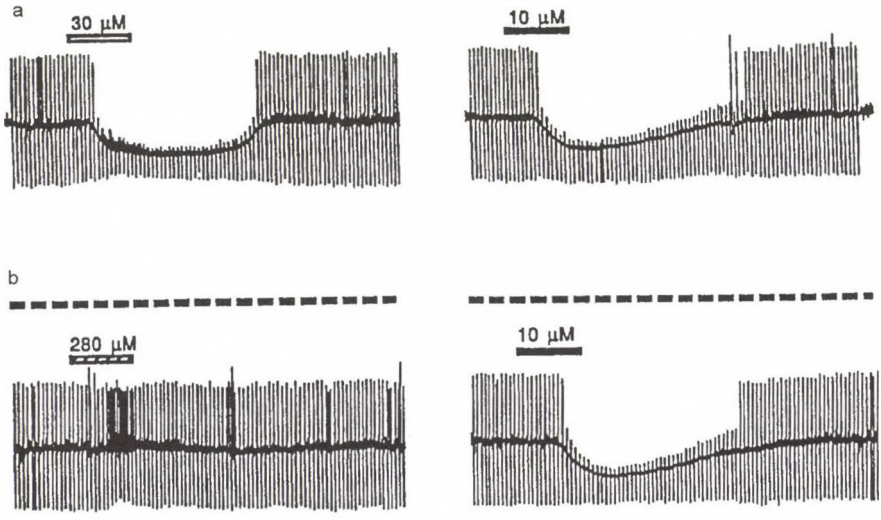

Figure 1. Way 100635 selectively antagonises the hyperpolarisation produced by 5-HT but not that evoked by baclofen in a CA1 pyramidal cell. (a) 5-HT (left, open bar) or baclofen (right, filled bar) both induced membrane hyperpolarisation and decrease in R_{in} revealed by the reduction of the electrotonic voltage (downward) deflections produced by injection of pulses of constant current (–300 pA, 400 ms) through the recording electrode. Upward deflections are truncated action potentials discharged on repolarization from pulse. Note the 5-HT also increases spontaneous GABA-mediated synaptic events (arrow, inverted in polarity by recording conditions [4]). (b) In the presence of WAY 100635 (10 nM, broken lines) 5-HT (left, hatched bar) failed to hyperpolarise cell membrane, whereas the effect of baclofen (right, filled bar) resulted unaffected. Note the increased concentration of 5-HT in the presence of WAY 100635, and that the increase in spontaneous depolarising synaptic events was unaffected. Calibrations: 20 mV, 3 min. KCl-filled electrode, r.m.p. –59 mV, R_{in} 48 MΩ.

Our findings support that WAY 100635 is a potent antagonist at 5-HT_{1A} receptors, both in the DRN and in the hippocampus. Binding studies have demonstrated that WAY 100635 is a selective ligand of 5-HT_{1A} receptors, showing a high-affinity, competitive and reversible binding [2]. In our hands, the antagonism exerted by WAY 100635 is competitive in the DRN and apparently non-competitive in the CA1 region of the hippocampus. Owing to its slow dissociation constant, WAY 100635 may act as a pseudoirreversible, albeit competitive, antagonist and affect maximal response to agonists in those brain areas devoid of a receptor reserve. These characteristics make WAY 100635 a suitable tool for investigating the existence

of a 5-HT_{1A} receptor reserve in different brain areas, and for identifying 5-HT_{1A} receptor-mediated responses, both *in vitro* and *in vivo*.

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THE XANTHINE DERIVATIVES AND CEREBRAL BLOOD FLOW (CBF) AFTER I.V. OR I.C. ADMINISTRATION IN SHR RATS

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It was shown, that TPH after i.v. administration causes significant decrease of CBF, but IBMX and P-23 had no effects on CBF. After i.c. administration of TPH and P-23 CBF decreased, whereas IBMX i.c. injection had no effect on CBF in SHR rats.

These results indicate that direct administration of TPH or P-23 into the cerebral vascular beds causes intensification of TPH activity and reveals the action of P-23.

These data suggest, that intracarotid administration of xanthine derivatives can change their cerebrovascular activity.

Keywords: methylxanthines, cerebral blood flow, adenosine receptor

Methylxanthines such as caffeine and theophylline act as antagonists at P_1 receptors, but are inactive at P_2 receptors. The P_1 receptors can be subdivided as either of the A_1 or A_2 adenosine receptor subtypes [Olah, 1992]. The most activity in relation to a xanthine structure activity relationship (SAR) depended on various alkyl substitutions in positions 1, 3 and 7 of the pharmacophore [Williams, 1989]. Deletion of the 7-methyl group in caffeine to give theophylline increases A_1 activity by up sixfold with an approximately fourfold increase in A_2 binding. IBMX, where an isobutyl group is substituted for the methyl in theophylline, is some fourfold more active at A_1 receptors with no real change in A_2 receptor binding.

Because stimulation of A_1-adenosine receptors leads to a decrease in c-AMP content and to a subsequent reduction of cerebral blood flow (CBF), the xanthines, via their ability to block A_1-adenosine receptors – mediated responses, should increase the cerebral blood flow.

However, caffeine and theophylline can decrease CBF in animals [Morri, 1987] and patients [Jensen, 1987].

The aim of this work, was to compare the cerebrovascular activity of three xanthine derivatives, such as TPH (1,3-dimethyl-xanthine theophylline), IBMX (3-isobutyl-1-methyl-xanthine) and P-23 [7-(beta-hydroxy-gamma-morpholino)-propylo-8-amino-1,3-dimethyl-xanthine] after intravenous (i.v.) or intracarotid (i.c.) administration.

Methods

The experiments were performed on male SHR Okamoto-Aoki rats. Animals were divided into three groups (n=24) and treated with TPH 10 mg/kg i.v. or i.c. in the first group, IBMX 10 mg/kg i.v. or i.c. in the second group, and P-23 10 mg/kg i.v. or i.c. in the third group. The animals were anaesthetized with 0.4% halothane in N_2O/O_2, paralyzed with suxamethonium (40 mg/kg) and ventilated on a respirator. Body temperature was kept at 37 °C. Mean arterial pressure (MAP) was recorded throughout the study and arterial tension of carbon dioxide ($PaCO_2$) and oxide (PaO_2), together with pH were measured immediately after each CBF determination. CBF was measured using the intracarotid ^{133}Xenon injection method

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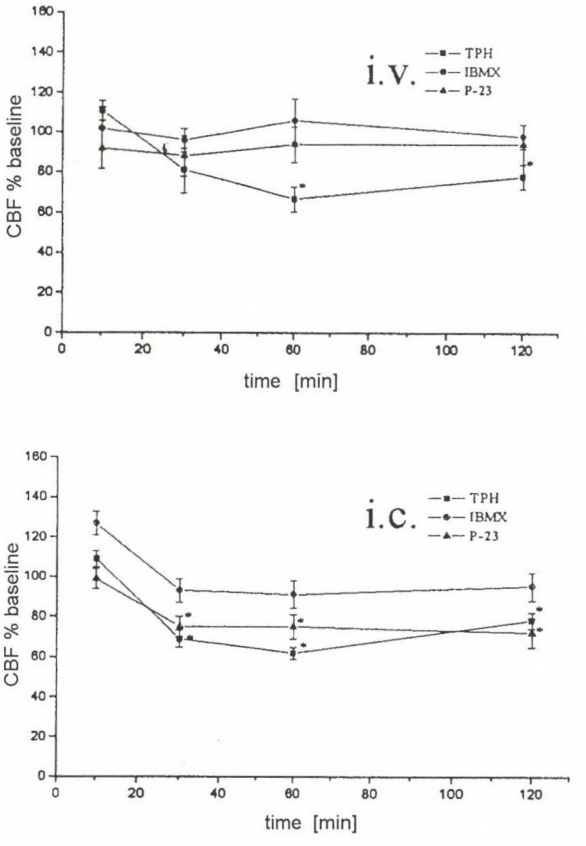

Fig. 1. The influence of TPH, IBMX and P-23 in doses 10 mg/kg on CBF after i.v. or i.c. administration in SHR rats, *CBF vs CBF baseline ($p<0.05$)

[Barry, 1982]. For each CBF determination 10–15 μCi ^{133}Xenon dissolved in 10–15 μl saline was injected into the carotid artery and the ^{133}Xenon activity in the brain measured with the heavily collimated scintillation detector. The clearance of the ^{133}Xenon from the brain was recorded semilogarithmically and CBF calculated from the 10–15 s initial slope of the clearance curve. Following stable base line CBF measurements, TPH, IBMX or P-23 were administered in doses 10 mg/kg (i.v. or i.c.), and CBF measured after 10, 30, 60, 90 and 120 min.

Results and Discussion

The xanthine derivatives TPH, IBMX besides their antagonistic effects to A_1-adenosine receptors can block the phosphodiesterase enzyme (PDE). Compound P-23 also increases c-AMP level in endothelium cells in SHR rats [Korzeniowska, 1994].

However, in this study it was shown, that TPH after i.v. administration causes significant decrease of CBF, but IBMX and P-23 had no effects on CBF. After i.c. administration of TPH and P-23 CBF decreased, whereas IBMX i.c. injection had no effect on CBF in SHR rats.

These results indicate that direct administration of TPH or P-23 into the cerebral vascular beds causes intensification of TPH activity and reveals the action of P-23.

These data suggest, that intracarotid administration of xanthine derivatives can change their cerebrovascular activity.

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MISOPROSTOL (CYTOTEC) IN THE TREATMENT OF PERIPHERAL ISCHAEMIC DISEASE

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The stable prostacyclin analogue – iloprost and prostaglandin E_1 (Alprostadil) showed a beneficial effect on activated platelets and leukocytes, and thrombocyte and leukocyte vessel interaction and damaged endothelium, improving microvascular perfusion and were useful in treatment of patients with peripheral arterial disease. The 4 weeks therapy with misoprostol caused a clinical improvement in all 14 patients and resulted in vasorelaxation and showed antiplatelet and fibrinolyitic effects.

Keywords: misoprostol, arteriosclerosis obliterans, blood platelets

In clinical trials prostacyclin (PGI_2) has shown a beneficial effect on activated platelets and leukocytes and thrombocyte and leukocyte vessel interaction and damaged endothelium, improving microvascular perfusion. The stable PGI_2 analogue – iloprost and prostaglandin E_1 (Alprostadil) showed similar properties and were useful in treatment of patients with peripheral arterial disease (1). However, these two drugs could be used only intraarterially or intravenously. The methyl estrification of the carboxyl group of alprostadil extended the duration of action following i.v. application (2). However, the methyl ester of alprostadil has still a relatively short duration of action following oral dosing. Transfer of the hydroxy group from C15 to C16 and addition of the methyl group at C16 – forming misoprostol – improved the oral potency and tolerabiality profile and extended duration of action. Misoprostol has been approved for many years for oral treatment of gastric and duodenal ulcers, asthma and for induction of abortion (3). The aim of our pilot study was to check if our suggestion that misoprostol could be useful in the treatment of peripheral ischaemic disease is right.

Patients and methods

Twenty patients with peripheral vascular disease according to Fountain's classification stages IIa and IIb were included into the study. In all patients the stenosis or occlusion was localized below the common femoral artery. Symptoms of the disease lasted from some months to 10 years. All patients received 200 mg of misoprostol 3 times a day, half an hour before the meal, for a month. Before and after termination of the therapy, patients were subjected to routine clinical and laboratory examinations. Following clinical estimations were performed: 1) pain-free and maximum walking distance on a horizontal treadmill at the speed of 4 km/h. 2) Time of pain relief in the ischaemic limb after maximum walking distance was expressed. 3) Arterial blood flow in both calves was measured using mercury gauge pletysmograph 4) Ratio of blood pressure on tibial posterior artery to blood pressure on brachial artery pressure (AAPR) using the ultrasound Doppler technique. Clinical estimation were performed before commencement of the therapy, after two weeks of the therapy and after finishing of the therapy. The following functional tests for fibrinolysis and platelet function were performed: 1) Euglobulin clot lysis time (ECLT) by the method of von Kaulla 2) Spontaneous platelet aggregation as measured by platelet aggregates ratio (PAR) using the method of Wu and Hoak 3) ADP-induced platelet

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aggregation by the method of Born. The blood for laboratory estimations was collected from antecubital vein before the treatment, 1 hour and 2 hours after administration of the first dose of the drug. These estimations were repeated on the 14th and 28th day of the therapy and on the next day after termination of the therapy. The study was approved by the Ethical Committee of the University and informed consent was obtained in all cases.

Results

The 4 weeks therapy with misoprostol caused a clinical improvement in all 14 patients. We observed an elongation of the pain-free walking distance (before treatment 132.69 m±96.4 m, after treatment 185.38 m±127 m, mean ±S.D., $p<0.01$). Also the maximal walking distance increased (before treatment 277.69 m±132.8 m, after treatment 451.95 m±313.8 m, mean ±S.D., $p<0.01$). At the same time of shortening of pain duration was observed (before treatment 105.23 sec±54.52 sec, after treatment. 75.07 sec±43.18 sec, mean ±S.D., $p<0.01$). The blood flow in the calves measured by venous occlusion pletysmography increased in both calves (more affected limb – before treatment 2.67±0.48 ml/dl/min, after treatment 3.0±0.4 ml/dl/min, mean ±S.D., $p<0.01$ less affected limb – before treatment 2.92±0.54 ml/dl/min, after treatment 3.15± 0.49 ml/dl/min, mean ±S.D., $p<0.01$). Misoprostol influenced also the laboratory tests. An activation of the fibrinolytic system was observed. The ECLT shortened on the 14th and 28th day of therapy (before treatment 93.33±31.43 min., after treatment 49.20±23.99 min, mean ±S.D., $p<0.01$). On the next day after finishing the therapy it returned to control values. The activation of the fibrinolysis may be connected with the release of t-PA from endothelium. A rise in the platelet aggregate ratio was observed. It indicates that a smaller number of platelets shows a tendency to spontaneous aggregation (before treatment 0.62±0.06, after treatment 0.74±0.05, mean ±S.D., $p<0.01$). The platelets were less sensitive to ADP after misoprostol intake (before treatment 0.62±0.06, after treatment 0.74±0.05, mean ±S.D., $p<0.01$).

Conclusions

Our Misoprostol therapy in patients with peripheral vascular disease resulted in: 1) improvement of subjective symptoms during exercise 2) elongation of pain-free and maximal walking distance on the treadmill 3) shortening of pain duration 4) increase in AAPR 5) increase in blood flow in the calves. Moreover, we observed inhibition of platelet function and activation of the fibrinolytic system.

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DIFFERENCES BETWEEN BDZ_1 SELECTIVE AND NON-SELECTIVE $GABA_A$/BDZ RECEPTOR LIGANDS IN DISCRIMINATIVE STIMULUS AND EtOH INTAKE/PREFERENCE PARADIGMS

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The present results showing a lack of considerable substitution of abecarnil and zolpidem for EtOH stimulus suggest that the EtOH cue is related to the non-selective full $GABA_A$-BDZ receptor agonism rather than selective action on $GABA_A$ isoreceptors.

Keywords: Ethyl alcohol (EtOH), GABA/benzodiazepine (BDZ) receptor complex

Several studies have shown that central GABA neurotransmission may be influenced by ethyl alcohol (EtOH) [2, 5]. EtOH was shown to increase Cl^- influx through the $GABA_A$ receptor complex [6, 1]. It has been proposed that the ability of EtOH to enhance GABA receptor complex function is corrected with [$^+$H] zolgiolem binding [2, 3]. The aim of the study was to examine the effect of diazepam, (the full agonist at the BDZ receptor), zolpidem, (the BDZ_1 receptor selective agonist) and abecarnil, (the partial agonist of the BDZ receptor) on EtOH preference and EtOH discriminative stimulus properties in rats.

Methods

Drug discrimination test. Male Wistar rats (300–330 g at the beginning of the study) were used. The animals were maintained at ~80% of their free-feeding weight and trained to discriminate between i.p. administered EtOH (1.0 g/kg, 10% v/v) and saline under a sweetened milk-reinforced fixed ratio 10 (FR10) schedule of reinforcement. Ethanol or saline were injected 15 min before the training session of another 15 min. Dose-response (0.25–1.0 g/kg of 10% EtOH) and substitution tests were carried out when the rats had achieved discrimination criteria: a) ≤2 incorrect responses before completing first FR10; b) ≥90% of the total session responses on the correct injection level; c) the response rate ≥0.45 responses/sec – in 9 out of 10 sessions. During the dose-response and substitution test sessions the lever on which first FR10 was completed continued to be reinforced for the remainder of the 15 min session. Diazepam (0.5–2.5 mg/kg), abecarnil (0.01–0.5 mg/kg) and zolpidem (0.005–0.5 mg/kg) were injected i.p. 30 min before start of the substitution test session.

Ethanol drinking test. Male Wistar rats (200–250 g at the beginning of selection) were used. Only EtOH high preferring (HP) rats were used in further study with the drugs. To obtain these rats, forced EtOH drinking procedure was introduced. Briefly, during 1st week rats were given 20% (v/v) EtOH intragastrically, b.i.d., in a total amount of 5.0 g/kg/24 h. During the 2nd and 3rd week the animals had access to water for 1 h a day only, while for the remaining 23 hours the only fluid available was 5% and 8% ethanol, respectively. During the 4th week the two-bottle preference test (8% ethanol vs. water) was performed. The animals consuming over 5.0 g/kg of pure alcohol (HP rats) were used in the drug test (5th week). During the drug test the HP rats

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received diazepam (0.001–0.5 mg/kg i.p.), abecarnil (0.01–0.5 mg/kg i.p.) or zolpidem (0.01–0.5 mg/kg i.p.) b.i.d. and EtOH intake/preference as well as total fluid intake (8% ethanol + water) were measured.

Results

Zolpidem (0.1 mg/kg) and diazepam (0.01 mg/kg) significantly decreased EtOH preference and intake, while abecarnil (0.01–0.5 mg/kg) did not influence these parameters. Higher doses of drugs (zolpidem 0.5 mg/kg, abecarnil 0.1 and 0.5 mg/kg) reduced the total fluid intake in rats; this effect, however was probably due to their sedative properties.

The ethanol discrimination required an average of 44 training sessions. The ED_{50} calculated on the basis of the dose-response tests was: 0.51 g/kg (C.L.: 0.39–0.71 g/kg). Ethanol (0.25–1.0 g/kg) did not affect the mean response rate. In rats trained to discriminate between EtOH (1 g/kg i.p.) and saline, zolpidem (0.005–0.5 mg/kg i.p.) and abecarnil (0.01–0.5 mg/kg i.p.) failed to substitute for EtOH. The highest dose of zolpidem (0.5 mg/kg) caused a significant reduction in the mean response rate. On the other hand, diazepam in the dose of 1.5 mg/kg partially (73%) substituted for EtOH with no apparent effect on the rates of responding.

Discussion

The most important finding of our study is that zolpidem, the selective BDZ-1 receptor agonist, does not substitute for EtOH stimulus in rats even at the dose significantly suppressing the mean response rate. Our results suggest that the subpopulation of $GABA_A$ receptors labelled by [^{3}H]zolpidem is not of critical importance for EtOH stimulus properties, in rats. The results of EtOH drinking test support this hypothesis since only one dose of zolpidem (0.1 mg/kg) moderately attenuated EtOH intake and preference in the HP rats.

The present results showing a lack of considerable substitution of abecarnil and zolpidem for EtOH stimulus suggest that the EtOH cue is related to the non-selective full $GABA_A$-BDZ receptor agonism rather than selective action on $GABA_A$ isoreceptors.

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THE HIPPOCAMPUS – THE KEY STRUCTURE IN PROCESSING OF EMOTIONAL INPUT IN THE CENTRAL NERVOUS SYSTEM

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Gray's conception hypothesizes that anxiolytics act indirectly to impair the behavioural inhibition system through GABA-ergic modulation of the ascending NA and 5-HT pathways to the hippocampus. The obtained results support this theory.

Keywords: hippocampus, emotional control, GABA, 5-HT and NMDA neurotransmitter systems

The hippocampus is considered a main limbic structure transmitting and transforming emotional input in the central nervous system. According to Gray (1), a septo-hippocampal behavioural inhibition system monitors environmental events and feedback from the animal's responses, and is particularly sensitive to signals associated with novelty and punishment. When these stimuli are detected, ongoing behaviour is inhibited, while attention and arousal are increased. Thus anxiety corresponds to the activity in the behavioural inhibition system. Since enhancement of inhibitory GABA-ergic neurotransmission leads to anxiolysis, it has been suggested that anxiolytic effects may be obtained also after inhibition of central excitatory inputs via blockade of the NMDA receptor complex (6).

Methods

Open field test (OFT). The behaviour of unhabituated rats was assessed for 10 minutes and the following parameters were scored:

General activity defined as the number of photobeams interruption.

Central entries defined as the number of entries to the central part of the open field.

The time in central sector defined as the total time (sec) spent in central part of the open field.

Vogel's test (VT). During 15 min long session, after 4 days of training, thirsty rats were exposed to conflict situation, in which a water deprivation was used as the incentive, and the punishment of drinking with mild electric shocks as an aversive stimulus. The amount of water intake (ml) during the test session was recorded and considered a measure of the conflict behaviour.

Drugs. Intra-hippocampal injections of drugs solutions were given bilaterally in a volume of 0.5 μg/site 5 min before the experiment. The following drugs were used in the treatment: midazolam, 8-OH-DPAT, buspirone, tropisetron, ondansetron, (+)–MK-801, AP7. p-chlorophenylalanine was administered intraperitoneally on the fourth and fifth day prior to behavioural testing.

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Results

The intra-hippocampal microinjections of benzodiazepine midazolam induced selective anxiolytic-like effect in the OFT (0.01, 10 μg), and in the Vogel test (10 μg). In serotonin depleted rats (pretreated with p-CPA), the effect of midazolam was even more potent. Injection of p-CPA alone, resulted in a smaller, although significant increase in conflict behaviour. In the OFT, 5-HT1A agonists: 8-OH-DPAT (0.1, 0.5, 1 μg) and buspirone (2.5, 5 μg) increased the number of entries and time spent in the central part of the testing arena. 8-OH-DPAT (0.5, 1 μg) decreased also shock-induced suppression of drinking. Injections of 5-HT3 antagonists tropisetron (0.005, 0.01 μg) and ondansetron (1, 2.5 μg) into the hippocampus increased punished consumption of water in the Vogel test. In the OFT both drugs did not produce anxiolytic effects. Both NMDA receptor antagonists (+/–) MK-801 and AP7 stimulated exploratory and locomotor activity (5 μg MK-801, 1 μg AP7), and attenuated the suppressive influence of a shock on drinking (1 and 0.5 μg, respectively).

Discussion

The results of the present study confirm and extend earlier observations suggesting that the hippocampus is related to emotional control, and that interaction between GABA, 5-HT and NMDA neurotransmitter systems in this structure contributes to the regulation of anxiety states. As in the case of peripheral administration (3), the OFT appeared to be more sensitive to the benzodiazepine derivative midazolam, in comparison to the Vogel test. Activation of 5-HT1A receptors within the hippocampus by 5-HT1A agonists 8-OH-DPAT and buspirone, produced marked behavioural disinhibition, presumably via mechanisms related to the hyperpolarization of postsynaptic neurons (4). 5-HT3 receptor antagonists were ineffective in the OFT, while the Vogel test revealed their anticonflict properties (5). The inhibition of glutamatergic neurotransmission in the hippocampus appeared to mediate locomotor stimulation, which is often observed after peripherally injected NMDA receptor antagonists (2). Gray's conception hypothesizes that anxiolytics act indirectly to impair the behavioural inhibition system through GABA-ergic modulation of the ascending NA and 5-HT pathways to the hippocampus. The obtained results support this theory.

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PRETREATMENT WITH INDOMETHACIN MODULATES THE EFFECTS OF SELECTIVE ADENOSINE RECEPTOR AGONISTS ON THE CONTRACTILITY AND EFFECTIVE REFRACTORY PERIOD IN THE GUINEA PIG HEART

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The effects of selective adenosine receptor agonists N^6-Cyclohexyladenosine (CHA, A_1-agonist) and 5'-(N-ethylcarboxamido)-adenosine (NECA, A_2-agonist) on the force of contraction (Fc) and effective refractory period (ERP) of isolated guinea pig papillary muscle were measured. Additionally, the influence of potent cycloxygenase inhibitor indomethacin on above mentioned parameters as well as on the effects of CHA and NECA were studied. It was found that CHA induced negative inotropic action and a shortening of ERP, opposite to NECA and indomethacin. The pretreatment with 10 μM of indomethacin shifted to the right the CHA effects and abolished the NECA effects.

Keywords: adenosine, indomethacine, papillary muscle, leukotriens

The involvement of adenosine receptor stimulation, activation of ATP-sensitive K^+ channels and translocation of protein kinase C in "preconditioning" phenomenon of heart is well known (3). Recently, it has been reported that stimulation of lipoxygenase metabolic pathway of arachidonic acid cascade could be one of the key event in preconditioning of the rat heart (2). The purpose of this study was to find out does a pretreatment with indomethacin influence the action of selective adenosine receptor agonists on the contractility and ERP duration.

Methods

Experiments were performed on mixed breed guinea pigs of both sexes, weighing 300–500 g. The animals were killed by cervical dislocation and papillary muscle was dissected out from right ventricle, attached to an isometric force transducer (F-30, HSE), superfused with modified Krebs-Henseleit solution (7 ml/min) at 35 °C, pH 7.4, preload 0.4 g, paced by square waves 1 Hz, threshold voltage +20% (DISA-Multistim). Preparation was allowed to equilibrate for 60 min. Then, the concentration-response curves to CHA (0.5–10 nM), NECA (1–30 nM in the presence of 2 nM of DPSX, antagonist of A_1 receptor) alone or after pretreatment with indomethacin, were constructed. The ERP duration was determined as the shortest interval between two stimuli (S_1–S_2), which evoked an increase in force amplitude of at least 25%, using digital storage oscilloscope VC-6525 with HIMES software (Hitachi).

Results

Figures 1a and 1b present the influence of 10 μM of indomethacin on the negative inotropic effect of CHA and positive inotropic effect of NECA. It can be seen that pretreatment with indomethacin shifted to the right the effects of CHA and almost completely abolished the effects of NECA. Indomethacin itself, at concentration of 10 μM induced the increase in the force of contraction for about 50% above control value (from 1.2±0.36 to 1.72±0.35, $p<0.05$).

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Only CHA produced the shortening of ERP duration (from 90±7.35 ms to 75±5.4 ms, $p<0.05$). NECA and indomethacin prolonged the ERP from 122±18.54 ms to 152±15.04 ms ($p<0.05$) and from 108±15.85 ms to 134±16.07 ms ($p<0.01$), respectively.

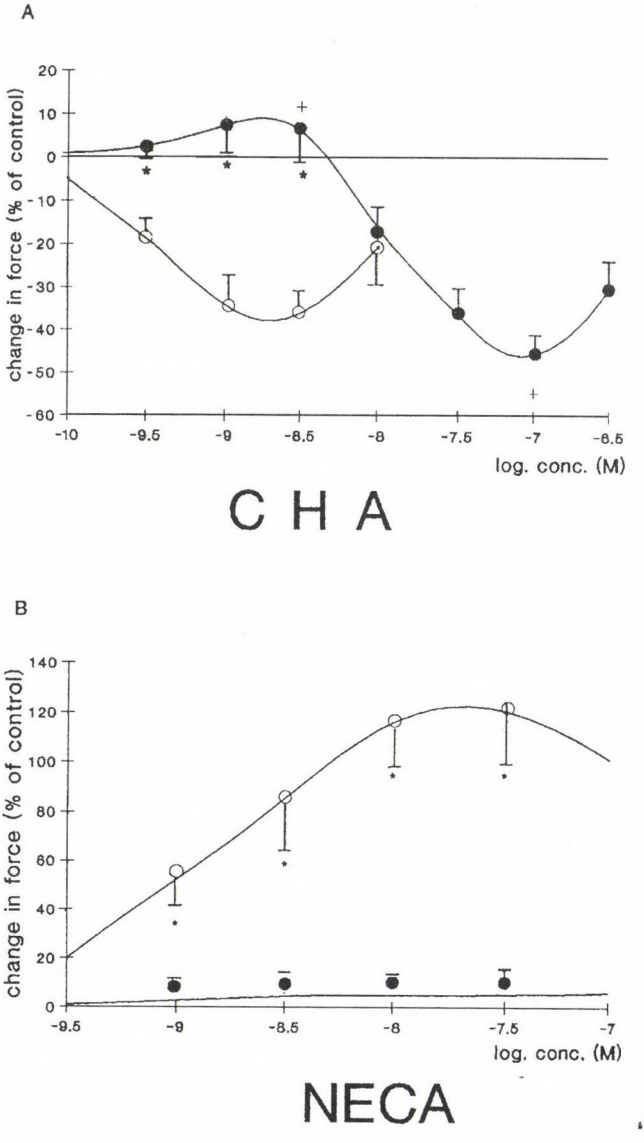

Fig. 1. Effects of CHA and NECA alone (O) or after pretreatment with indomethacin (•) on the force of contraction of the guinea pig papillary muscle. Each point represent mean ± SEM from 4-6 experiments. * $P<0.05$, t-test, unpaired data;

Discussion

In this study it has been demonstrated that pretreatment with 10 μM of indomethacin almost completely abolished the effects of A_2 receptor stimulation and strongly shifted to the right the effects of A_1 receptor stimulation on the contractility and ERP duration of guinea pig papillary muscle. This effect of indomethacin as a strong inhibitor of cycloxygenase, is probably connected with a driving the arachidonic acid cascade into the lipoxygenase pathway. Data obtained with leukotriene D_4 support this hypothesis (1). The explanation for the lack of ability of NECA to increase the maximum positive inotropic action and prolongation of ERP induced by indomethacin is probably due to the same metabolic pathway involved in the action of both agents. On the other hand, an antagonism of indomethacin to CHA effects is rather uncompetitive and requires further experiments to be cleared.

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ON THE REGULATION OF KYNURENIC ACID PRODUCTION IN THE RAT BRAIN SLICES: EVIDENCES FOR THE INVOLVEMENT OF METABOTROPIC GLUTAMATE RECEPTOR

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These data suggest that endogenous KYNA production may be controlled by neurotransmitter(s) acting at metabotropic glutamate receptor(s).

Keywords: Kynurenic acid, metabotropic glutamate receptor

Kynurenic acid (KYNA) is the only known endogenous antagonist of all 3 ionotropic excitatory amino acid (EAA) receptors in the brain (5). As a regular constituent of human brain tissue, KYNA may modulate EAA neurotransmission and thus participate in the pathogenesis of seizure phenomena and/or neurodegenerative processes (2). In the brain KYNA production occurs mainly in glial cells due to irreversible enzymatic transamination of L-kynurenine, the metabolite of amino acid tryptophan (1). KYNA levels may be affected not only by the alterations in KYNA biosynthetic enzyme activity (1), but also by factors influencing neuronal activity such as changes in potassium or sodium extracellular concentrations (5). Therefore, we decided to investigate the effects of substances depolarizing presynaptic endings and EAA agonists on the KYNA production in vitro.

Methods

The substances employed in the study included: L-glutamate, quisqualate, kainate, N-methyl-D-aspartate (NMDA), trans-(1S,3R)-amino-1,3-cyclopentanedicarboxylic acid (t-ACPD), (±)α-amino-3-hydroxy-5-methylisoxide-4-propionic acid (AMPA), 4-aminopyridine (4-AP), veratridine (all RBI) and L-kynurenine (Sigma).

The experiments were performed in cortical slices obtained from adult Wistar rats. KYNA production in vitro was estimated according to the method of Turski et al. (5). Briefly, slices prepared with a McIlwain chopper were placed in culture wells containing 1 ml of oxygenated Krebs-Ringer buffer. Tissue was preincubated for 10 min at 37 °C and incubated further for 2 hours at 37 °C in the presence of 5 μM L-kynurenine and tested drugs. After incubation, media were separated from the tissue and applied to Dowex 50W (H^+ form) cation-exchange columns. Subsequently columns were washed with 1 ml of 0.1 M HCl and 1 ml of water. The fraction containing KYNA was eluted with 2 ml of water. The eluate was applied to HPLC column operating with the mobile phase composed of 250 mM zinc acetate, 50 mM sodium and 4% acetonitrile, pH 6.2. KYNA was detected fluorimetrically according to the method of Shibata (2).

Results and discussion

The effects of EAA receptor agonists and depolarizing agents on KYNA synthesis are summarized in Table 1.

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Selective agonists of the EAA ionotropic receptors: AMPA, kainate and NMDA did not affect the KYNA production in vitro. In contrast, the synthesis of KYNA was dose-dependently inhibited by drugs interacting with metabotropic receptor agonist: t-ACPD which is a selective metabotropic receptor agonist, quisqualate which acts at metabotropic receptor and ionotropic AMPA receptor and L-glutamate – an endogenous nonselective agonist of metabotropic and all 3 ionotropic glutamate receptors. Similarly, the depolarizing agents such as 4-AP and veratridine which stimulate the presynaptic release of neurotransmitters, were able to diminish KYNA production in vitro. These data suggest that endogenous KYNA production may be controlled by neurotransmitter(s) acting at metabotropic glutamate receptor(s).

Table 1

Effect on KYNA (Kynurenic acid) production in the rat brain slices

Treatment (mM)		KYNA content (% of control)
Control		100
Veratridine	0.001	64**
	0.005	32***
	0.05	15***
4-AP	0.01	93
	0.1	72**
	0.5	71**
NMDA	0.1	97
Kainate	0.1	91
AMPA	0.1	85
Quisqualate	0.001	98
	0.01	54**
	0.1	34***
t-ACPD	0.05	97
	0.1	72*
L-Glutamate	0.1	95
	1.0	49**
	5.0	13***

*p<0.5, **p<0.01, ***p<0.001 vs control (Student t-test).

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THE INCREASE IN CYCLIC NUCLEOTIDE LEVEL DECREASES THE CONTRACTILE RESPONSE OF GASTRIC SMOOTH MUSCLE STRIPS TO GALANIN

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We found that NE; dib cAMP and papaverine are not competitive antagonists of pGal in rat stomach fundus. They do not cause statistically significant changes of EC_{50}, while changing the slopes and E_{max} of respective concentration-response curves.

Keywords: Galanin – cyclic nucleotide level – gastric smooth muscle

Porcine galanin (Gal) a 29 amino-acid peptide was isolated from porcine intestine extracts by Tatemoto et al., [1]. Gal and its receptors are widely distributed in the central and peripheral nervous systems, on the urogenital and gastrointestinal (GI) tracts of several mammalian species including man. Gal can exert many different biological actions, including regulation of GI motility, influences gastric acid secretion, modulates the pituitary-hypothalamus axis or inhibits pancreatic endocrine secretion. There is evidence of specific Gal binding sites in intestinal and gastric muscle and Gal and some of its analogues cause concentration dependent contractions in vitro, little information has been published on the mechanism of action of Gal. The aims of this study was: to investigate the intracellular mechanism of Gal action in rat gastric fundus muscle. (Katsoulis et al. [2]).

Methods

Albino-Wistar rats were killed by a blow on the head. Abdominal cavity was opened, stomach excised, the fundus cut off, and longitudinal strips were prepared according to Vane [3]. Strips were suspended in organ baths containing Tyrode's solution at 37 °C bubbled with O_2/CO_2 (95:5), and kept at resting tension of 2.0 g. Responses of the stomach strips to the tested agents were recorded isotonically with transducers connected to a line recorder. Tissues were allowed to equilibrate for 60 min before beginning of the experiment. Buffer was changed every 5 min, except for the contact time of tested agent with the tissue, lasting up to 20 min. Gal or Gal analogues were added in increasing concentrations, directly into the organ baths by a cumulative additions, until maximum contraction occurred. Contact time of a given peptide with muscle strip which allowed a development of maximum contraction ranged from 15 to 20 min. 3 min after the maximum contraction was reached the tissue was washed out at a rate of 2.5 ml/s for 2–3 min until the length of the strip returned to the basal level. Then the strip was left to equilibrate for 20 min (buffer changed every 5 min). Cumulative concentration-response curves for Gal were constructed in the absence (control) and presence of increasing concentrations of: norephinephrine (NE), papaverine hydrochloride, N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate monosodium salt (dib cAMP). The contact times of all tested agents with the

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muscle strips were 3 min. Results are expressed as a percentage of the maximum response induced by each peptide. Potency (EC_{50}), and the slope of the concentration-response curve are expressed as means with respective confidence limits. Maximal contractile effects of pGal in presence and absence of the tested agents (E_{max}) are expressed as means ± SEM. Efficacy, EC_{50}, and E_{max} were compared using and the Bonferroni post ANOVA test. The slopes of concentration response curves were calculated and their statistical significance was measured using the PCS, version 4 (Tallarida and Murray [4]). A p value of less than 0.05 was taken to indicate a significant difference.

Results and discussion

NE 30 or 60 or 100 and 300 nM; papaverine 10 or 100 nM and 10 μM; Dib cAMP 10, 100 and 300 μM impaired E_{max} elicited by pGal 1 nM–1 μM in a concentration-dependent manner, without significant changes in EC_{50}. NE (an adenylate cyclase activator); papaverine (increases the intracellular level of cyclic AMP and is a non-specific muscle relaxant) and a more specific agent dib cAMP inhibited the contraction to Gal in a concentration-dependent manner, as expected from the known action of cyclic nucleotides in smooth muscle relaxation. There is much evidence that Gal controls cyclic adenosine monophosphate levels in the pancreatic β-cells [5]. Whether Gal acts by decreasing the level of cAMP in rat gastric fundus muscle cells, or the cyclic nucleotides just counteract the action of Gal in smooth muscle, is not known. We found that NE; dib cAMP and papaverine are not competitive antagonists of pGal in rat stomach fundus. They do not cause statistically significant changes of EC_{50}, while changing the slopes and E_{max} of respective concentration-response curves.

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DESENSITISATION TO SUBSTANCE P DECREASES THE CONTRACTILE EFFECT OF M15 AND GAL (1–14)-(Abu 8) SCY-I ON THE RAT GASTRIC FUNDUS

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Tachyphylaxis to SP decreased the effect of M15 and Gal(1–14)-[Abu^8]SCY-I on gastric smooth muscles, without effect on the action of Gal. These findings support our initial hypothesis: the action of M15 and Gal(1–14)-[Abu^8]SCY-I on the smooth muscles may not only be due to their agonist activity at Gal receptors, but may result from a subsequent stimulation of receptors for SP and perhaps for other tachykinins as well, however a possibility that Gal analogues release endogenous SP can not be excluded. Further studies involving a tachykinin antagonist (spantide) are in progress at the moment. [1].

Keywords: galanin, substance-P, gastric fundus

Galanin (Gal), a 29-amino acid peptide, occurs in the brain, spinal cord, urogenital and gastrointestinal (GI) tracts of several mammalians including man. The effects of Gal may be quite different depending on the organ and the species studied, resulting in contraction, relaxation or modulation of the effects of other peptides and neurotransmitters on GI smooth muscle-therefore implying an important role in regulation of GI motility. [2] Because of the lack of specific inhibitors in GI tract it is not known which effects of Gal are physiological and which are pharmacological. In the process of assessing the possible usefulness of chimeric Gal analogues for defying the physiological role of Gal in regulation of GI motility we tackled several problems: a comparison of the effects of Gal and its analogues on strips of rat isolated gastric fundus; a pharmacodynamic characteristics of interaction of Gal and Gal analogues with Gal receptor. We investigated over 13 analogues. For the sake of clarity and brevity of the experimental protocol we divided Gal congeners into three broad categories: Gal and Gal fragment containing 15 N-terminal amino acids, family of galantide (M15) consisting of 13 N-terminal amino acids coupled to a fragment of substance P (SP). Chimeric peptides formed from the N-terminal part of Gal and a modified molecule of scyliorhinin-I (SCY-I). [3] We established all Gal analogues display agonistic properties in preparations of gastric smooth muscles. It is worth emphasizing the sensitivity of different sets of receptors to the action of Gal analogues for example. M15 is a selective antagonist at Gal CNS receptors and at the same time a full agonist at Gal receptors in GI muscle. SP and SCY-I belong to the family of tachykinins. Effects of M15 and Gal(1–14)-[Abu^8]SCY-I may therefore be partially produced by stimulating SP receptors or release of endogenous SP. A hypothesis was proposed: tachyphylaxis of gastric smooth muscle strips to the action of SP decreases the effects evoked by M15 and Gal(1–14)-[Abu^8]SCY-I without a change in Gal action.

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Materials and methods

Male Albino-Wistar rats (weighing 180–250 g) were killed by a blow on the head. Abdominal cavity was opened using a midline incision, stomach excised and longitudinal strips of the fundus were prepared according to Vane. [4] Strips were suspended in organ baths (volume 15 ml) containing Tyrode's solution at 37 °C bubbled with carbogen and kept at resting tension of 2.0 g. Responses of the stomach strips to the tested agents were recorded isotonically. Tissues were allowed to equilibrate for 60 min before beginning of the experiment. Experiments were begun when a reproducible contractile responses to carbachol (10–30 nM) were obtained. Gal or its analogues were added in increasing concentrations, directly into the organ bath by cumulative additions, until maximum contractile response occurred, a response which could not be further increased by a higher concentration of peptide. Contact time of a given peptide with muscle strip allowing for a development of maximum contraction ranged from 15 to 20 min. 3 min after the maximum contraction was reached the tissue was washed out at a rate of 2.5 ml/s for 2–3 min until the length of the strip returned to the basal level. Then the strip was left to equilibrate for 20 min (buffer changed every 5 min). Second part of experiments was conducted in the same experimental set as described above. Concentrations of Gal, M15 and Gal(1–14)-[Abu^8]SCY-1 established to have caused maximal contractions, were added to the organ bath 3 min after the maximum contraction was reached the tissue was washed as described above. A fast, progressive diminution of the contractile response of the longitudinal gastric smooth muscle strips to the action of SP was obtained as described by Holzer-Petsche [5]. Briefly: 500 nM of SP were administered to the organ bath for 1 min. Then 5000 nM of SP were applied for 5 min. Four minutes after the wash out, the test concentration was added again with the same contact time as before. One minute later the concentration of Gal, M15 of Gal(1–14)-[Abu^8]SCY-I evoking a maximal contractile response in controls were added to the organ bath. Results are expressed as a percentage of the maximum response induced by each peptide. Efficacy, potency (EC_{50}), and the slope of the concentration-response curve are expressed as means with respective confidence limits. Efficacy is expressed as a percentage of the maximum contractile effect of Gal. Efficacy, EC_{50}, and E_{max} were compared using ANOVA and the Bonferroni post ANOVA test. [6] The slopes of dose-response curves were calculated and their statistical significance was measured. Relative potencies of Gal or Gal analogues were determined as the ratio of equi-effective concentrations of each peptide from their respective graded concentration-response relations. The Hill's coefficient was calculated to examine whether the Gal or Gal analogue-receptor interaction follows classical receptor theory. Contractile response produced by Gal, M15, Gal(1–14)-[Abu^8]SCY-I and the test concentration of SP were measured before (controls) and after tachyphylaxis to SP. Results were expressed as a percentage of respective controls. Values are compared using a two-tailed Wilcoxan signed rank test for pairs. $P<0.05$ was taken to indicate a significant difference.

Results and discussion

Gal, M15 and pGal(1–14)-[Abu^8]SCY-I evoked concentration-dependent contractions of rat gastric fundus strips. EC_{50} of Gal was 13.7 nM and Hill's coefficient 1.03. EC_{50} of M15 equaled 70.1 nM and Hill's coefficient was 0.73. EC_{50} of Gal(1–14)-[Abu^8]SCY-I was 187 nM and Hill's coefficient was 1.56. The concentration-response curves of M15 and Gal(1–14)-[Abu^8]SCY-I are slightly to the right but are almost parallel to that one of Gal and their slopes are not significantly different. The efficacy and EC_{50} of M15 and Gal(1–14)-[Abu^8]SCY-I differed significantly from those of Gal. Our study supports previous findings suggesting that Gal contracts rat fundus by a direct interaction with Gal receptors of the type found in the smooth muscle cells. Hill's coefficient for Gal is close to 1 indicating an interaction of one Gal molecule with one receptor as in classical receptor theory. Both analogues also caused contraction but their Hill's coefficients differ significantly from 1, so that one drug molecule does not seem to interact with one receptor. This might indicate activation of more than one type

of receptor, negative or positive receptor cooperativity or multiple-step reaction of analogues with the receptor. The relative potencies on the rat gastric fundus were: Gal>M15>Gal(1–14)-[Abu^{8}]SCY-I. We showed that the significant loss of potency is achieved with the substitutions of amino acids in C-terminal part of Gal molecule by SP or modified SCY-I. It is not known if this is due to the conformational changes of Gal molecule. The cumulative concentration-response curves for Gal are similar to those with non-cumulative addition of peptide, and this does not corroborate the former concern about possible tachyphylaxis to Gal. Simpler and quicker experiments are therefore possible with Gal on rat gastric fundus.

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EFFICACY AND TOLERANCE OF 400 MG BEZAFIBRATE IN DIABETIC AND HYPERLIPIDAEMIC PATIENTS

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The authors report the results of an open clinical study using 400 mg Bezafibrate once a day. Among 25 diabetic patients (type II.) underwent a 4 weeks period with nutritional advice. Average changes from inclusion levels were –24% for total cholesterol, –56% for triglicerides, +11.9% for HDL-cholesterol, –19% for plama fibrinogen. In conclusion, bezafibrate at a daily dose of 400 mg had significant lipid-modifying properties but also exhibited a beneficial effect on other related risk factors such as fibrinogen reduction.

Keywords: bezafibrate, lipoproteins, fibrinogen, risk factors

Coronary artery disease (CAD) is the leading cause of death in most developed countries. The association between blood lipid levels and CAD has been well established through a number of important studies (1–4). The number of available lipid-lowering drugs is still rather limited, and those on the market differ in their effects on the various lipoprotein fractions. The introduction of a new agent in this therapeutic category is of special interest to clinicians, not only of its specific effects on serum lipids, but of its clinical administration, safety, and side effects.

Fibrates have been widely used in the treatment of hyperlipidaemic patients. These agents primarily lower triglycerides and increase HDL-cholesterol, and has fibrinogen-lowering effect. Clinical and epidemiological studies suggest that several haemostaseological factors (e.g. fibrinogen, plasma viscosity, ect) might play an important role in atherosclerosis.

Patients and methods

Selection criteria

- both males (12 pts) and females (13 pts)
- aged 18–70 years, median age 42 years
- all of them suffering from NIDAM
- previously applied lipid lowering drugs were stopped

Exclusion criteria

- patients who did not give written consent or who were suspect of a high probability of non-compliance with the protocol
- with a history of pancreatitis or cholestasis
- with chronic alcoholism
- pregnancy

Protocol

Patients were put on a low fat diet during a 4 week period. While still on the diet, the patient received 400 mg bezafibrate once a day during a 3 month period. Clinical visits were scheduled at inclusion, 1 and 3 months later when, in addition to medical examination, blood samples were taken for serum lipids and hemorheology.

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Results and discussion

Three of the 25 patients (2 female, 1 male) were dropped out because of the side effects (mild gastrointestinal complaints i.e. meteorism, epigastric distress) of drug.

The results of study showed that bezafibrate produces moderate reduction in total plasma cholesterol. Plasma triglyceride levels were reduced by about 50 percent, while HDL-cholesterol was increased by 11.9 percent and plasma fibrinogen level decreased by 19 percent.

Mean percent changes from baseline in lipoprotein levels are presented in Table I. Table II presents the haemostasiological factors.

Table I

Effect of bezafibrate (400 mg once a day during a 3 months) on the level of serum lipids

	total-chol.	HDL-chol.	LDL-chol	triglycerides
0.week mmol/l ±2SD	8.97 ±3.04	1.55 ±0.40	4.08 ±2.44	7.36 ±4.70
12.week mmol/l ±2SD	6.89 ±2.84	1.85 ±0.60	3.57 ±1.95	3.24 ±2.72
p< changes %	0.02 24%	NS 12%	0.04 12%	0.01 56%

Table II

Effect of bezafibrate (400 mg once a day during a 3 months) on haemostaseological parameters

	atherogen index tot-chol/HDL	fibrinogen mg/dl	blood-viscosity mPas	plasma viscosity mPas
0.week ±2SD	5.78	264 ±35	5.51 ±0.50	1.54 ±0.1
12.week	3.72	214 ±32	4.69 ±0.5	1.37 ±0.1
p< changes %	0.03 35%	0.02 19%	0.01 15%	0.01 11%

It is generally accepted that for each decrease of 1% of the serum total cholesterol, a 2% decrease in CAD incidence is observed.

Our study show that bezafibrate was well tolerated, efficacious and safe for men and women with hypertriglyceridaemia with or without hypercholesterinaemia. Not any disadvantageous side effects were observed. The results show that bezafibrate reduced total cholesterol within 4 weeks of starting treatment and maintained these reductions throughout the study. It also significantly reduced triglycerides and LDL cholesterol. Besides 12% increase of HDL cholesterol could be observed as well as 19% decrease of plasma fibrinogen level. On the basis of these results bezafibrate had not only significant lipid-modifying properties, but also exhibited a beneficial effect on other presumed risk factors such as elevated fibrinogen level.

We are confirmed that more attention should be paid to elevated levels of fibrinogen and triglycerides in CAD and their analysis should be included in multiple parameter approaches to therapy.

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TWO ANIMAL MODELS OF ANXIETY – DIFFERENT SENSITIVITY FOR ANXIOLYTIC ACTION OF $GABA_A$ RECEPTOR COMPLEX LIGANDS

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It can be postulated that an interaction between serotonergic and GABA-ergic system and anxiolytic properties of drugs inhibiting 5-HT turnover (5-HT synthesis enzyme inhibitors, neurotoxines) may be indicated by similarity of action of serotonin depletion and of GABA/BDZ partial agonists.

Keywords: Benzodiazepine-$GABA_A$ (BDZ-$GABA_A$) receptor complex, anxiety

Benzodiazepine-$GABA_A$ (BDZ-$GABA_A$) receptor complex agonists clinical relevance in anxiety states is well established. The adverse effects of these compounds however, stimulates the search for more specific way of regulation of GABA-ergic system. This goal may be achieved by benzodiazepine receptor ligands with partial agonistic properties or selective receptor subtypes agonists [3, 10]. In this case, unfortunately, the reduction of adverse effects may be also accompanied by reduction of anxiolytic potency. Thus, it seemed interesting to examine the effects of some selected BDZ-$GABA_A$ ligands in the pre-clinical behavioral models of anxiety to find out the differences in their anxiolytic profiles. In addition, the influence of inhibiting 5-HT transmission was compared to the effects of BDZ-$GABA_A$ receptor ligands.

Methods

Open field test (OFT). During 10 minutes observation of unhabituated rat behavior the following parameters were scored: General activity defined as the number of photobeams interruption. Central entries defined as the number of entries to the central part of the open field. Time in central sector defined as the total time (sec) spent in central part of the open field.

Vogels test (VT). During 15 min long session, water deprived and prehabituated rats were exposed to conflict situation in which an attempt to drink could be punished by an electroshock delivered in 4 sec trains separated by 5 sec long periods of free drinking. The amount of water intake (ml) during the test session was considered a measure of the conflict behavior.

Drugs. Drugs were injected i.p. 15–30 min before experiment dissolved in appropriate vehicles (0.9% NaCl, 1% Tween 80 or 45% 2-Hydroxypropyl-β-cyclodextrin).

Results (See table 1)

Full agonists at the BDZ-$GABA_A$ complex, midazolam and diazepam had anxiolytic profile in the OFT and VT. Partial agonists bretazenil, RO 19-8022 (quinazoline derivative) and abecarnil (which is actually a full agonist at BDZ1 rec. subtype and partial at other receptor subunit composition [6], failed to show clear anxiolytic profile in the OFT but they appeared anxiolytics in the VT. Zolpidem and alpidem, alpha 1 subunit specific ligands differing in their pharmacological profiles (full agonist vs. partial agonist respectively) differently acted in VT (where zolpidem but not alpidem showed anticonflict activity) and did not produce anxiolytic

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effect in the OFT. In case of β-CCM, an inverse agonist at BDZ rec., anxiogenic-like profile of action could be observed in OFT and no "pro-conflict" effect was recorded in the VT. Flumazenil, an antagonist at BDZ rec. did not produce any effect in both tests. In our study anxiolytic effectiveness of *p*-CPA (p-chlorophenylalanine, 5-HT synthesis inhibitor) and 5,7 DHT (5,7 dihydroxytryptamine, 5-HT neurotoxine) was revealed only in the Vogel's test. In the OFT paradigm these two compounds remained behaviourally inactive.

Table 1

Profile of action of compounds and their OFT (open field test) and VT (Vogels test) anxiolytic effects with increasing potency (-, 0, +)

Profile of action	Compound	OFT anxiolytic effect	VT anxiolytic effect
BDZ-$GABA_A$ full agonist	Diazepam	++	+++
	Midazolam	++	+++
type 1 selective	Zolpidem	0	++
BDZ-$GABA_A$ partial agonist	Bretazenil	0	+++
	Quinazoline	0	+++
	Abecarnil	0	+++
type 1 selective	Alpidem	0	0
BDZ-$GABA_A$ inverse agonist	β-CCM	--	0
BDZ-$GABA_A$ antagonist	Flumazenil	0	0
5-HT synthesis inhibitor	*p*-CPA	0	++
5-HT neurotoxine	5,7 DHT	0	++

Discussion

Since many years the Open Field Test (OFT) and Vogel's test (VT) [5] have been well established as standard procedures sensitive to the anxiolytic properties of drugs. It seems interesting however, that compounds effective in one model appear to be ineffective in the other paradigm. Both tests have been found equally sensitive for full agonists at BDZ-$GABA_A$ rec. complex (midazolam, diazepam). All tested compounds with partial agonistic properties however, irrespectively of their affinities to the receptor complex subunits or chemical structure remained inactive in OFT test. On the other hand, Vogel's test inadvertently revealed their anticonflict properties. The explanation of this discrepancy cannot be based on the lack of sensitivity since doses of full agonists active in this test were even 100 fold lower than ones active in VT. Moreover OFT confirmed anxiolytic potencies of non-GABA-ergic drugs such as 5-HT1A agonists [9] or competitive and non-competitive NMDA antagonists [4]. It can be postulated that differences in the profiles of anxiolytic action in both tests may depend on different rec. affinities or intrinsic activities of BDZ-$GABA_A$ receptor complex ligands. It is also possible that the effect may be mediated by different brain structures [1, 7]. Apparently, the Vogel's test and the Open Field Test model two distinct types of anxiety (conflict vs. neophobic reaction). Moreover, the OFT seems to be very sensitive to the sedative effect of drugs acting via BDZ-$GABA_A$ [2]. Aforementioned types of anxiety reactions may differ in neurochemical organization characterized by GABA, 5-HT and possibly NMDA systems involvement [8]. An interaction between serotonergic and GABA-ergic system and anxiolytic properties of drugs inhibiting 5-HT turnover (5-HT synthesis enzyme inhibitors, neurotoxines) may be indicated by similarity of serotonin depletion and of GABA/BDZ partial agonists.

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OPIOID RECEPTOR AFFINITY AND ANALGESIC ACTIVITY OF O- AND C-GLYCOSYLATED OPIOID PEPTIDES

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O- and C-glycosylation of the mu-agonist dermorphin reduced neither its μ receptor affinity in binding assay nor its agonist potency in guinea-pig ileum assay (GPI). O- and C-glycosylation of the delta-agonist deltorphin reduced its δ-receptor affinity and its agonist potency in mouse vas deferens assay (MVD). O- and C-glycosylated dermorphin, administered i.c.v. and s.c., produced long-lasting antinociception in mice and rats. The ratio between i.c.v. and s.c. antinociceptive ED50 demonstrates facilitated transport into the CNS only for the galactosil peptide. Acetylation significantly reduced penetration of glycopeptides into the CNS indicating that facilitated transport into the CNS exists, but does not depend on the glucose transporter (GLUT-1).

Keywords: Glycosyldermorphin, glycosyldertolphin, receptor affinity, CNS penetration

Peripheral administration of glycopeptide analogues of stable enkephalins produces analgesia in mice (1). Glycosylated peptides could enter the brain from the periphery by being transported through the endothelial barrier by the glucose transporter GLUT-1. We studied here receptor affinity, functional biological activity on isolated tissues and in vivo analgesic potency of glycopeptide analogues of dermorphin and deltorphin. To investigate whether the glucose-transporter has a role in glyco-peptide penetration through the blood-brain barrier, we compared the analgesia produced by peripheral administration of glycopeptides with that produced by their acetylated analogues in which hydroxyl groups of the sugar are not available for the glucose transporter.

Methods

The O-glucosylated and C-galactosylated analogs of dermorphin and deltorphin were prepared by solid-phase synthesis starting from the appropriate N^{α}-Fmoc-glycosyl-aminoacids. Binding of dermorphin, deltorphin and their glycosylated analogs to δ- and μ-sites was assayed on crude membrane preparations from rat brain. The δ-binding sites were selectively labeled with [^{3}H][D-Ala^2, Asp^4]deltorphin (0.3 nM), the μ-sites with [^{3}H]DAMGO (0.5 nM). The inhibition constant (K_i) of the various peptides was calculated from competitive binding curves. "In vitro" biological activity was studied on mouse vas deferens (δ-receptors) and on guinea pig ileum (μ-receptors) preparations stimulated with bipolar rectangular pulses of supramaximal voltage. The analgesic activity of glycosylated dermorphin analogs was evaluated after intracerebroventricular (i.c.v.) and subcutaneous (s.c.) administration in rats (Male Sprague-Dawley rats, 240–260 g) and mice (CD1 Swiss mice, 30–35 g). Antinociception was measured by the tail-flick test and expressed as percentage maximum possible effect (% MPE).

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Results and discussion

Glycosylation of deltorphin decreased the affinity for the delta opioid receptors dramatically (90 to 600 times) confirming that the C-terminal domain, in the deltorphin molecule, is essential for delta opioid receptor binding (2). Glycosylation of the C-terminal aminoacid of dermorphin slightly affected μ opioid receptor affinity (twice for glucosyl and galactosyl derivatives, and 7–10 times for the corresponding acetylated analogs). The biological activity in functional assays for the μ and δ receptors paralleled the changes in binding affinity. Both by peripheral and central administration glycopeptide analogues of dermorphin were about twice as potent as dermorphin. Acetylation of sugars reduced the antinociceptive potency, especially by the peripheral route: by this route they were about four times less potent than the glycodermorphins. The ratio between ED50 calculated for i.c.v. and s.c. routes provided an indirect measure of entry into the brain and demonstrated significantly increased transport into the CNS only for the galactosyl peptide.

These results are difficult to explain in terms of utilisation of the glucose transporter since galactose has three times less affinity than glucose for GLUT-1. As expected, acetylation significantly reduced penetration of glycopeptides into the CNS. These results indicate that a facilitated transport into the CNS exists, but does not depend on the glucose transporter.

Table 1

μ and δ receptor affinities and biological activities of O- and C-linked glycopeptide dermorphin and deltorphin analogues

Peptide	Ki, nM		IC_{50}, nM	
	μ [3H]DAGO	δ [3H]DELT-I		MVD
Dermorphin	1.2±0.2	1120±41	1.3±0.5	17±3
[(βGlc)Ser7]dermorphin	2.4±0.3	1230	3.9±0.32	40±5
[(βGlc(Ac)4)Ser7]dermorphin	7.9±0.9	1400	8.8±0.9	18.1±2.9
[(αGal)Ala7]dermorphin	2.5	2500	6.3±0.8	55±4.5
[(αGal(Ac)4)Ala7]dermorphin	11.2±1.5	>5000	8.4±0.9	65±7.1
Deltorphin	1980±220	0.8±0.05	1239±203	0.2±0.03
[βGlc]deltorphin	879±100	64±5.5	>5000	6.1±1.1
[βGlc(Ac)4]deltorphin	1915±210	77±8	>5000	15.1±1.2
[(αGal)Ala7]deltorphin	1900	250±30	>5000	30±4
[(αGal(Ac)4)Ala7]dermorphin	2900	475±50	>5000	50±6

Glycopeptides and their acetylated analogues produced a longer-lasting effect than dermorphin. This is probably due to the protection against carboxypeptidase activity afforded by glycosylation of C-terminal aminoacid (3). Acetylation of hydroxyl groups of glycopeptides further prolonged analgesia. This effect was particularly evident when the peptides were injected s.c.

Table 2
Analgesia and relative CNS penetration of dermorphin analogues

Peptide	Analgesia (ED_{50})		ED_{50} ratio	CNS entry
	i.c.v. (nmol)	s.c. (nmol)	i.c.v./s.c.	
Dermorphin	0.030	2500	1.20×10^{-5}	1
[(βGlc)Ser^7]dermorphin	0.021	1400	1.50×10^{-5}	1.25
[(βGlc$(Ac)_4$)Ser^7]dermorphin	0.035	4500	7.80×10^{-6}	0.61*
[(αGal)Ala^7]dermorphin	0.023	1300	1.80×10^{-5}	1.50*
[(αGal$(Ac)_4$)Ser^7]dermorphin	0.040	7000	5.70×10^{-6}	0.48*

In conclusion, glucosyl-dermorphin crosses the BBB at the some rate as dermorphin. In contrast, galactosyl-peptides appear to have facilitated access to the CNS by means of non-glucose transporter. Glycosylation may increase the half-life of peptides and prolong their biological effects. Site-directed glycosylation can be used to reveal binding domains in the peptide sequence.

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PHARMACOLOGICAL ACTIVITY OF FLUOXETINE

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Some SSRIs like fluvoxamine and zimeldine have already been investigated for their memory improving activity in humans and animals, with positive results. The purpose of this paper is to observe some activities of fluoxetine the known antidepressant on some control neuron system functions [3].

Keywords: fluoxetine, anxiolytic activity, locomotor activity, memory, rats

Methods

The experiments were carried out on male Wistar rats (180–200 g) housed in groups 8–10 with free access to food and water. Fluoxetine 5 mg/kg was suspended in 0.5% solution of CMC and administered p.o. 60 min before the anxiolytic and locomotor activity test.

In memory assays Fluoxetine was given 24 h before the test. MK 801 – an NMDA – receptor antagonist (0.2 mg/kg i.p.) was given 30 min before the locomotor activity test. Scopolamine hydrobromicum was administered 0.5 mg/kg s.c. 25 min before test. Memory was tested using the Hill's [2] method, the anxiolytic activity was investigated using Crowley's "Two compartment exploratory test" [1] and locomotor activity was estimated in the PAN-Licence Activity Meter.

Statistical analysis. The statistical significant of the results of the anxiolytic, locomotor and memory trials were evaluated according to the 2-tailed student "t" test [4].

Results

It was shown, that fluoxetine has a high anxiolytic activity after the first administration. After that however, week by week the anxiolytic effect became smaller till 28 days, when it was no longer present (Fig. 1). The drug also by itself had no influence on locomotor activity, but it enhanced significantly the hyperactivity induced by MK 801 (Fig. 2). In memory experiments (Hill test) fluoxetine favoured the performance of memory task (exception: fluoxetine + scopolamine, single administration) until 14 days there were no signs of tolerance (Fig. 3).

Concluding

Mk 801 induced locomotor hyperactivity was potentiated by fluoxetine. Fluoxetine increase the Mk 801 – induced locomotor activity via activation of 5-HT receptors.

The effects mentioned above may participate in the antidepressant activity of the drug.

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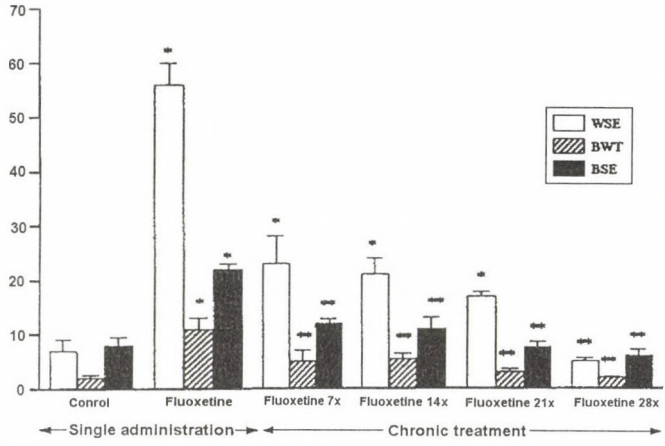

Fig. 1. The effects of single and chronic administration of fluoxetine on the rats in the "two compartment exploratory test of Crowley"
* Statistically valid differences in relation to the control group for $p<0.05$
** Statistically valid differences in relation to the Fluoxetine (single administration group) for $p<0.05$

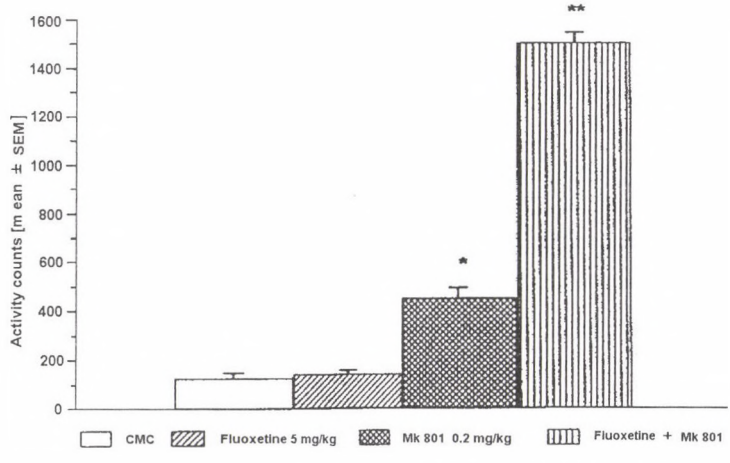

Fig. 2. Locomotor hyperactivity induced by Mk-801 0.2 mg/kg
* Statistically valid differences in relation to the control group for $p<0.05$
** Statistically valid differences in relation to the Mk-801 group for $p<0.05$

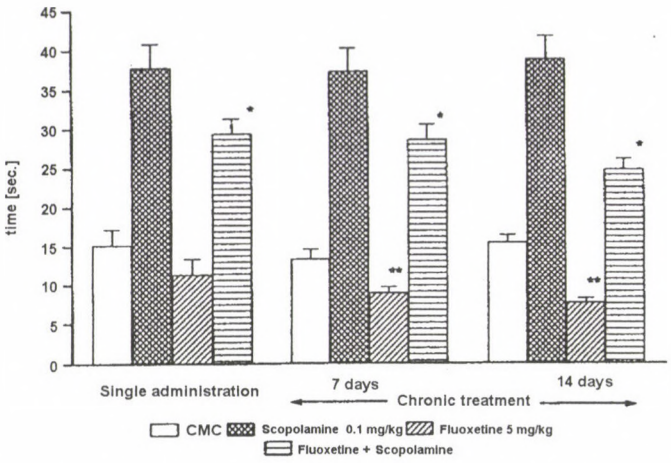

Fig. 3. Influence of fluoxetine 5 mg/kg p.o. (before experiment) single and chronic administration on memory deficit caused by scopolamine in the Hill's test
* Statistically valid differences in relation to the scopolamine group for $p<0.05$
** Statistically valid differences in relation to the CMC group for $p<0.05$

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CENTRAL SEROTONERGIC SYSTEM AND MECHANISM OF ANXIOLYTIC ACTION

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The results clearly indicate that the hippocampus, rather than nucleus accumbens is involved in mediating anxiolytic-like effects of the 5-HT1A receptor agonists. Furthermore, hippocampal postsynaptic 5-HT1A receptors may account for the anti-emotional influence of this groups of drugs. As far as the 5-HT3 receptor antagonists are concerned, it was more difficult to localize their central anti-anxiety like action. More clear and unequirocal effects could be observed after intra-accumbens, rather than after intra-hippocampal injections of tropisetron and ondansetron.

Keywords: Central serotonergic system, anxiolytic action, benzodiazepines

It is believed that brain serotonergic systems (5-HT) significantly contribute to the physiology and psychopathology of emotional processes. The arguments in favor of this concept have recently been summarized by Schreiber and de Vry [2, 7]: (i) serotonin depletion usually coincides with disinhibition of punished behaviour (ii) the 5-HT1A receptor agonists are already proved to be effective anxiolytic drugs in the clinic; (iii) antidepressant drugs selectively inhibiting serotonin reuptake (SSRI) attenuate psychotic anxiety and fear in endogenous depression.

To further elucidate the problem of involvement of serotonin in emotional control, in the series of papers (8–10) we concentrated upon three topics: (i) what are the qualitative and quantitative differences between benzodiazepines, the 5-HT1A receptor agonists, and the 5-HT3 receptor antagonists in some preclinical tests predictive of anxiolytic activity (ii) the role of some limbic structures (the hippocampus and nucleus accumbens) in the anti-anxiety like action of benzodiazepines, the 5-HT1A receptor agonists, and the 5-HT3 receptor antagonists (iii) what is the role of pre-versus post-synaptic parts of the central serotoninergic system in the effects of selected serotoninergic drugs.

Methods

A model reaction employed was the change in rat behaviour in the Vogel, and in the open field tests [8]. In the first test, thirsty rats were given mild electric shocks when drinking. The amount of drunk water was taken as an index of animals' conflict behaviour. In the open field, more ethologically-oriented "anxious" behaviour related to thighmotaxis, i.e. to the inborn neophobic reaction, was measured in rats. Here, the number of entries into the central part of a large and previously unknown arena, as well as time spent in the central sector, was registered via closed-circuit TV and taken as an index of rat timidity.

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Results

The data showed similar sensitivity of both employed tests for benzodiazepine derivatives and various classes of serotonergic agents. Different serotonergic agonists and antagonists (buspirone, gepirone, ipsapirone, ritenserin, ondansetron, tropisetron) appeared to have similar anxiolytic-like profile to this of the benzodiazepines. Tropisetron was characterized by the most advantageous spectrum of activity with very potent action devoid of any clear-cut general inhibitory properties. The imidazobenzodiazepine midazolam was highly effective in disinhibiting rat fear-controlled behavior in both tests in a selective way, after intra-hippocampal administration. The hippocampus appeared also important for mediating anti-fear-like effects of the 5-HT1A receptor agonists. The intra-hippocampal microinjections of 8-OH-DPAT in the Vogel test, and 8-OH-DPAT and buspirone in the open field test, potently disinhibited examined parameters of rat behaviour suppressed by fear. After intra-accumbens administration, 8-OH-DPAT and the 5-HT3 receptor antagonists showed anxiolytic-like potency in the Vogel test. It appeared that the potent and selective depletion of hippocampal and forebrain levels of serotonin (5,7-DHT), below the detection limits, had no influence on the anti-neophobic action of the 5-HT1A receptor agonist (8-OHDA), and the 5-HT3 receptor antagonist (tropisetron). The drug, however, tended to increase rat motor activity in lesioned animals.

Discussion

The present data add more arguments for the concept that activation or inhibition of different postsynaptic 5-HT receptor subtypes may have functionally opposite meaning. As far as anxiolytic effect is considered, stimulation of the 5-HT1A- and inhibition of the 5-HT3-postsynaptic receptors within the hippocampus yield potent anxiolytic activity. Anxiolytic effect induced both by stimulation of the 5-HT1A receptors and inhibition of the 5-HT3 receptors most probably appears to be related to the hyperpolarization of postsynaptic neurons and consequently, neuronal inhibition of selected parts of the limbic system [5, 6, 14]. Such mechanism accords with the concept that an increase in the intensity of anxiety is related to an enhancement of the hippocampal functions, controlling emotional input [3]. Conceivably, depolarization of postsynaptic neurons, i.e. neuronal excitation induced by stimulation of the

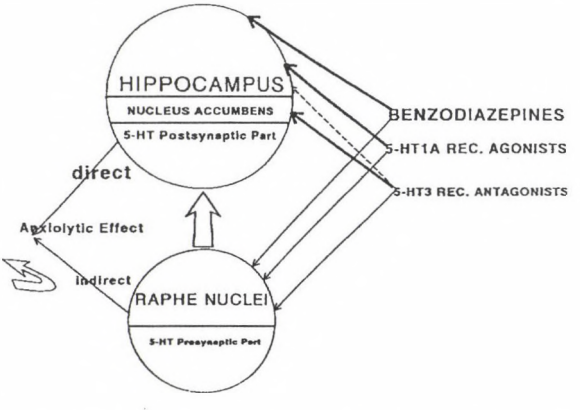

Fig. 1. A schema showing hypothetical organization of central serotonergic system involved in the anxiolytic effects of different compounds. For explanations see the text.

hippocampal non 5-HT1A receptors, may result in an anxiogenic response [13]. Furthermore, it is well recognized that all known classes of anxiolytic drugs, including barbiturates, benzodiazepine derivatives, 5-HT1A receptor agonists and 5-HT3 receptor antagonists, exert a clear-cut anti-epileptic influence, in different models of a seizure activity [12]. This fact probably is a reflection of more general inhibition of the temporal lobe function, a phenomenon closely related to the anxiolytic activity as well [6]. It is also conceivable that stimulation by the 5-HT1A receptor agonists of the 5-HT1A autoreceptors abundantly represented within the raphe nuclei (a negative feedback mechanism regulating 5-HT neurons activity), would cause a temporary functional lesion of the serotonergic system resulting in a nonspecific enhancement of fear-suppressed behaviours [1, 4]. Inhibition of the cell body firing rate of the raphe neurons appears to be an important element of anti-anxiety action of benzodiazepines and 5-HT1A receptor agonists. It is worth noting that the 5-HT3 receptor antagonists were also found to suppress the cell body firing in the dorsal raphe nucleus [11]. Thus, presynaptic mechanism of action of drugs acting upon 5-HT1A and 5-HT3 receptors can not be neglected. It is hypothesized that this dual mechanism of the 5-HT1A receptor agonists and the 5-HT3 receptor antagonists action cooperate synergistically in the processing of emotional functions (Fig. 1).

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INFLUENCE OF BILATERAL CLAMPING OF CAROTID ARTERIES ON THE SEIZURES SUSCEPTIBILITY AND CENTRAL ACTION OF AOAA IN MICE

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In the experiments carried out on Albino-Swiss mice it was found, that bilateral clamping of carotid arteries (BCCA) for 30 min produce the increase of GABA content in hippocampus, striatum and frontal cortex and decrease of the seizures susceptibility to bicuculline, investigated 7 days after surgery. Moreover, BCCA modulates the action of aminooxyacetic acid (AOAA): potentiates anticonvulsive effect of low doses of AOAA (33 mg/kg) and inhibits the convulsive action of high doses. Also the potentiation of the anticonvulsive effect of diazepam, phenobarbital and valproic acid (VPA) was observed in BCCA and AOAA (CD_{97}, 150 mg/kg) treated animals. Observed effects suggests that enhanced GABA-ergic activity and decrease of excitatory amino acid (EAA) system could be involved in these processes.

Keywords: bilateral clamping of carotid arteries (BCCA), seizure, aminooxyacetic acid (AOOA)

Oxygen deficit in brain leads to the increase concentration of Ca ions in neurones, enhances production of free radicals and increases release of excitatory amino acids. All these events result in cell damage and neuronal death (2, 3).

BCCA (Bilateral Clamping of Carotid Arteries) is a model of moderate ischemia, in which neuronal necrosis is not observed in rats and mice, however one can see increased lactate concentration and decreased pO2 in brain structures in 2/3 of forebrain, what indicates the decrease of cerebral blood flow (1, 6, 7). Aminooxyacetic acid (AOAA) is known as a GABA-T inhibitor (4), increasing GABA content in the brain and having anticonvulsive properties in different models of convulsions. But in higher doses, despite of the high GABA content, possess convulsive properties, dependent probably on the inhibition of the kynurenine transaminase (8). Changes in brain metabolism of kynurenine induced by AOAA caused probably the decrease of brain kynurenic acid, endogenous inhibitor of EAA receptors, and increase of quinolinic acid, enhancing seizures susceptibility and neurodegeneration. Intracerebral administration of AOAA induces neuropathological changes similar to changes observed after deep ischemia and in Huntington disease, dependent on the quinolinic acid (8).

The influence of BCCA on the activity of GABA-ergic system, seizure susceptibility and central action of AOAA was the subject of present studies.

Material and methods

The experiments were carried out on female Albino-Swiss mice, body weight 20–25 g. The animals were anaesthetised with pentobarbital (Nembutal, 60 mg/kg i.p.) and subjected to 30 min of bilateral clamping of carotid arteries (BCCA). The common carotid arteries were carefully isolated from the surrounding tissue and clamped by vessel clips. During anaesthesia and surgery the rectal temperature was kept at 37 °C. After the procedure clips were removed and circulation recovered. Sham operated control animals had their vessels prepared but not clamped. 7 days after surgery biochemical and behavioural experiments were performed. AOAA was injected i.p.

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at the doses 33 mg/kg (1/2 of CD_{50}), 66 mg/kg (CD_{50}) or 150 mg/kg (CD_{97}), 30 min before the experiment. ED_{50} for diazepam, phenobarbital and valproic acid (VPA) after the injection of 150 mg/kg of AOAA were calculated using the method of Lietchfield and Wilcoxon. The threshold for bicuculline after BCCA and injection of 33 mg/kg of AOAA was determined. GABA content in brain structures was determined by spectrofluorimetric method of Love et al. (5).

Results and discussion

BCCA did not affect spontaneous locomotor activity and motor coordination of mice 7 days after the surgery. In histological studies in light microscopy no neuronal necrosis was observed. BCCA increased the GABA content in brain cortex, striatum and hippocampus up to 7 days after surgery. Similar increase in GABA content was observed in AOAA treated animals. AOAA (1/2 of CD_{50}) administered to the animals 7 days after BCCA increased GABA content in brain structures to much higher level than in sham controls. BCCA potentiated anticonvulsive effect of small dose of AOAA (33 mg/kg) in bicuculline-induced seizures (Fig. 1). In addition BCCA enhanced anticonvulsive action of diazepam, phenobarbital and valproic acid in AOAA (CD_{97}) induced seizures (Fig. 2).

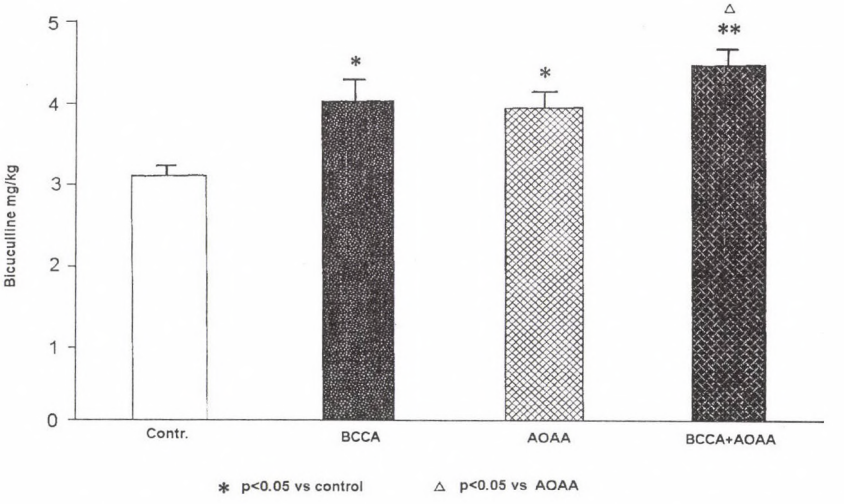

Fig. 1. The influence of BCCA on the bicuculline threshold 30 min after AOAA 33 mg/kg

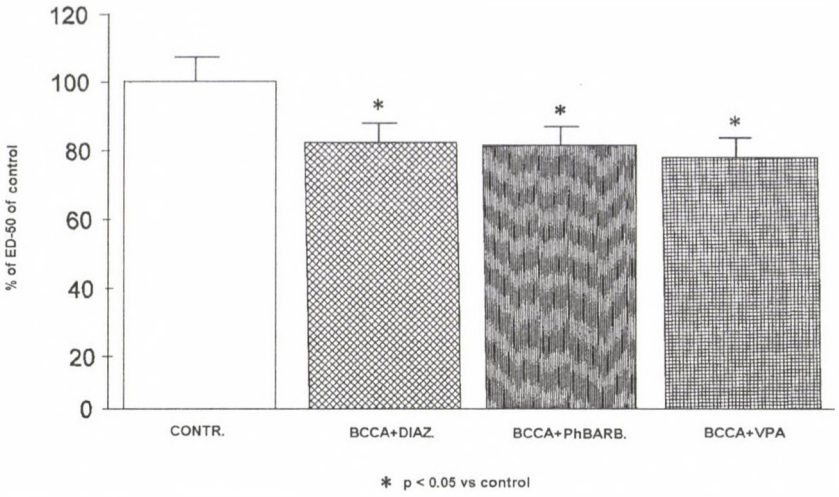

Fig. 2. The influence of diazepam, phenobarbital and VPA on the AOAA (150 mg/kg) induced seizures in BCCA mice

Obtained results indicate, that short-lasting oligemic hypoxia caused by BCCA produce the activation of GABA-ergic system, which could be responsible for decrease of seizure susceptibility. Synergic action of BCCA and AOAA (1/2 CD_{50}) support this hypothesis. Enhanced protective effect of such anticonvulsants as diazepam, phenobarbital and VPA against AOAA (CD_{97}) induced seizures suggest, that EAA system is also involved in the hypoxic functional changes. One can suppose, that short-lasting moderate oligemic hypoxia triggers a compensatory mechanism which could protect the organism against the next hypoxic or seizure episode.

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L-ARGININE – SUBSTRATE FOR NO SYNTHESIS – ITS BENEFICIAL EFFECTS IN THERAPY OF PATIENTS WITH PERIPHERAL ARTERIAL DISEASE: COMPARISON WITH PLACEBO-PRELIMINARY RESULTS

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Intravenous infusions of L-arginine (L-ARG) and placebo (saline) resulted in improvement of clinical assessments, statistically significant after L-ARG but not after saline. Results of laboratory estimations for platelet and fibrynolysis changed significantly following L-ARG infusions but not after infusions of placebo. These data indicate beneficial effects of L-ARG as a therapeutic agent in patients with peripheral arterial obliterative disease (PAOD). In these patients exogenous L-ARG can be converted to NO.

Keywords: L-arginine, endothelium derived relaxing factor, arteriosclerosis obliterans

Endothelium cell lining is important in the control of human vascular tone. It releases vasoactive substances such as nitric oxide (NO). EDRF/NO is synthesized directly from L-ARG or, indirectly, from peptides containing L-ARG by Ca^{2+}/calmodulin dependent NO synthase. Atherosclerosis evokes an imbalance between endothelial secretogogues. The endothelium of atherosclerotic arteries has a reduced capacity to release EDRF/NO (1). The atherosclerosis-induced impairment of endothelium-dependent vasorelaxation can be reversed by the treatment with L-ARG (5). It seems that in atherosclerosis conversion of endogenous L-ARG to NO is impaired and loading with exogenous L-ARG may overcome this block.

Patients and methods

Fourteen male patients aged 39–66 (average 54.4) who had suffered from PAOD were studied. All patients showed atherosclerotic lesions typical for stage II a and b according to the Fontaine's classification. All patients were hospitalised and received a 3 h i.v. infusion of saline for 7 consecutive days followed by infusions of 12.6 g (60 mmol) of L-arginine daily dissolved in 300 mL of saline for the next 7 days. The trial was conducted in accordance with the Declaration of Helsinki. Clinical estimations were performed before starting the 1st and after the 7th infusion of saline, and after the 7th infusion of L-ARG. An exercise test was done for the patients on a horizontal treadmill at the speed of 4 km/h. The pain free and maximal walking distance was estimated. The duration of claudication pain was registered. We measured the ankle-arm pressure ratio (AAPR) and arterial blood flow at rest in both calves. Blood samples for functional tests for fibrynolysis and platelets were taken before and after the 1st and 7th infusion of the placebo and L-ARG and 1 day after termination of the therapy. Euglobulin clot lysis time (ECLT) was estimated according to von Kaulla, the platelet aggregates ratio (PAR) was calculated using the Wu and Hoak's method and threshold proaggregatory concentrations for ADP and collagen were determined according to the Born's method. Statistical analysis was performed using the paired Student's t-test. Statistical differences were considered significant at $p<0.05$.

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Results

The seven days-therapy with L-ARG caused a subjective improvement in all patients. Both the infusions of placebo and of L-ARG resulted in increasing of the pain free and maximum walking distances, but only the improvement after infusions of L-ARG was significant. There were none statistically important changes in relapse time of pain relief after the maximal walking distance. In both limbs a statistically significant increase in AAPR and improvement of impaired blood flow was achieved after the infusions L-ARG, but not after the placebo infusions. The ECLT was shortened after the 1st infusion of L-ARG for about 27%, after the 7th infusion for about 30% and it remained shortened for about 17% after termination of the therapy. The 1st and the 7th infusion of L-ARG caused a rise in PAR. The susceptibility of platelets to ADP and collagen decreased after the 1st and 7th infusion of L-ARG.

Discussion

The short term treatment with L-ARG offered therapeutic benefits to patients with PAOD. The objective measurements such as an increase in AAPR and augmentation of blood flow may depend on vasodilation evoked by NO which is generated from infused L-ARG. The delayed onset of pain during the treadmill test may also be associated with the analgesic effect of L-ARG itself (3). We observed a transient activation of plasma fibrinolytic system. The mechanism of the L-ARG/NO-induced fibrinolysis is not clear. Most likely it arises neither from inhibiting of PAI release from platelets (4) nor from activating t-PA release from endothelial cells (2). In our trial the shortening of ECLT occurred in all patients, irrespectively of their degree of impairment of endothelial of platelet functions. Infusions of L-ARG decreased platelet susceptibility to ADP and collagen in a transient manner. The assumption on conversion of exogenous L-ARG to NO in our patients is supported indirectly by the fact that in PAOD patients both generators of NO, i.e. molsidomine (2) and L-ARG are more effective in suppressing of fibrinolysis and of spontaneous platelet aggregation then in anti-aggregatory action on stimulated platelets. Exogenous L-ARG seems to generate NO only in patients with atherosclerosis and this process is controlled by the efficacy of nitric oxide synthase. In contrast with classic NO donors the treatment with L-ARG cannot lead to overdosing of NO because only a limited capacity of NO synthase. These promising preliminary results call for further investigations in long term double blind cross-over placebo-controlled studies in patients with PAOD.

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PHARMACOKINETICS AND BIOAVAILABILITY OF STEREOISOMERIC ANALOGUES OF IFOSFAMIDE

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Analogues of ifosfamide (IPA): racemic bromofosfamide (±)-(R,S)-KM 135, racemic chlorobromofosfamide (±)-(R,S)-CBM 4a and levorotatory enantiomer of chlorobromofosfamide (-)-(S)-CBM 11 belong to the known group of oxazaphosphorines. Antitumor activity of those three selected compounds, investigated in the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wrocław against L1210 leukemia, Lewis lung carcinoma and B16 melanoma tumor model system showed cytostatic activity higher than the referential – ifosfamide [1]. These interesting data prompted us to conduct the preclinical pharmacokinetic studies in rats.

Keywords: analogues of ifosfamide – pharmacokinetic, bioavailability

Materials and methods

The tested and standard compounds were given i.v., i.p. and p.o. in equimolar doses of 300 mg/kg (0.981 mmol/kg) and 256 mg/kg (0.981 mmol/kg), respectively. The heparinized blood samples were taken in sterile conditions at 5, 10, 15, 20, 30, 45, 60, 90, 120, 180 minute intervals. Plasma samples were extracted with 4 ml ethyl acetate after the addition of 0.270 μmol/ml IPA as internal standard [2, 3]. The peak plasma concentration of these compounds were quantified by using GC-MS method. The pharmacokinetic parameters were calculated according to the accepted one – compartment open model.

Table 1

Pharmacokinetic parameters of the derivatives of ifosfamide after i.p. and p.o. administration at a dose of 300 mg/kg (0.981 mmol/kg)

Compound/dose 0.981 mmol/kg	Route of administration	$t_{0.5}$±SD min	AUC±SD μmol×min×ml^{-1}
(±)-(R,S)-CBM 4a	i.p.	77.09±2.75	84.44±1.17
	p.o.	79.39±4.56	48.30±2.96
(-)-(S)-CBM 11	i.p.	162.12±6.36	212.51±2.80
	p.o.	46.82±2.70	50.41±3.18
(±)-(R,S)-KM 135	i.p.	77.09±2.75	54.79±1.96
	p.o.	67.66±1.93	43.11±0.78
(±)-(R,S)-IPA	i.p.	118.71±5.95	158.37±3.69
	p.o.	40.23±2.68	47.00±2.30

$t_{0.5}$ – terminal elimination half-life; AUC – area under the blood concentration curve

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Table 2

Pharmacokinetic parameters and bioavailability of the racemic chlorobromofosfamide (±)-(R,S)-CBM 4a after i.v., i.p. and p.o. administration

CBM 4a/dose	Route of administration	$t_{0.5}$±SD min	AUC±SD μmol×min×ml^{-1}	$\frac{AUC_{p.o.}}{AUC_{i.v.}}$ ± SD
300 mg/kg (0.981 mmol/kg)	i.v.	75.04±3.34	125.99±4.49	0.38±0.02 (38±2)%
600 mg/kg (1.962 mmol/kg)	p.o.	78.24±2.11	94.72±0.73	–
2×300 mg/kg (2×0.981 mmol/kg)	p.o.	96.92±5.95	106.04±3.76	–

Bioavailability was calculated as the ratio of $AUC_{p.o.}/AUC_{i.v.}$

Results and discussion

The pharmacokinetic investigations showed considerable, stereoselective differences in the biotransformation of tested compounds, depending on the routes of administration. AUC of (±)-(R,S)-CBM 4a and (-)-(S)-CBM 11 after p.o. administration was similar and statistically different from AUC calculated for (±)-(R,S)-KM 135, throught the same route of application (Table 1, 2). Large differences were observed in the calculated values of AUC of three compounds following i.p. administration (84.4; 212.5; 54.8 μmol×min×ml^{-1}, respectively), also in comparison with a standard (158 μmol×min×ml^{-1}). Bioavailability estimated for (±)-(R,S)-CBM 4a after p.o. administration at a dose of 300 mg/kg (0.981 mmol/kg) reached 38%. The results indicate that further investigation with p.o. administration of stereoisomeric analogues of IPA might be undertaken, especially on fractionated dosage. Additionally, attention should be given to appropriate conditioning formulations of these compounds to obtain the therapeutically more efficacy.

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THE ROLE OF SUPRASPINAL MODULATION OF α-2-ADRENERGIC RECEPTORS ON NOCICEPTIVE PROCESS

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Our findings have shown that both supraspinal activation and blocade of α-2-adrenergic receptors change bioelectrical response of studied brain structures on the nociceptive stimulation, among other things by the modulation of hippocampal activity. The mechanism of this effect remains to be established.

Keywords: α-2-adrenergic, clonidine, yohimbine, nociception, lateral reticular formation, DTF.

Noradrenaline is known as a neurotransmitter involved in the pain modulation in CNS, especially by α-2-adrenergic receptors (1, 3, 4). High concentrations of α-2-adrenergic receptors are localised in many brain regions closely connected with pain transmission i.e. brainstem, cerebral cortex, hypothalamus, thalamus, hippocampus, amygdala, and dorsal horn of spinal cord (3, 6, 7). Recently, much experimental work has drawn attention to the role of α-2-adrenergic receptors of the medullary lateral reticular formation (LRF) in antinociception. Brainstem LRF is a structure with noradrenaline-containing spinally projecting neurons. The findings of recent studies suggest that LRF is involved in tonic descending inhibition of spinal nociception and it is also a relay for antinociceptive signals descending from the periaqueductal gray to the spinal cord (4, 5). There are many conflicting data referring to the effects of α-2-adrenergic agents applied supraspinally on the nociceptive process. Both α-2-adrenoreceptors agonists (e.g. clonidine, medethomidine) and antagonists (yohimbine, idazoxan) were observed to have opposed action (inhibition or facilitation) on the pain transmission (1, 3). The aim of the present study was to evaluate the influence of the activation and blocade of the α-2-adrenergic receptors in LRF on the nociceptive process. As a model of pain we used bioelectrical response of chosen brain structures to the nociceptive stimulation (NS).

Material and methods

The experiments were carried out on 28 male rabbits weighing 3.0–3.5 kg. The stainless steel electrodes were implanted bilaterally into: the motor-sensory cortex (MSC), ventro-postero-lateral thalamic nuclei (NVPL), hippocampus (HIP), and LRF where additionally a cannula was implanted. Three weeks after the surgery (the recovery and habituation period) we started NS on immobilized but conscious animals located in a sound-proof chamber. NS was performed by means of electrical pulses (60 Hz, 8 mA, 5 s) applied to the front paw using intracutaneous electrodes. Bioelectrical activity (BA) of the analysed brain structures was registered before and after NS in control animals and after administration of drugs. The drugs: clonidine (3 μl, conc. 5 mg/ml) and yohimbine (3 μl, conc. 7 mg/ml) were applied locally into LRF 5 minutes before NS. The 10 second EEG recordings before and after NS were analysed by means of autoregressive method (AR) for multichannel model. Parameters achieved in AR made it possible to calculate the directed transfer functions (DTFs). DTFs were counted in the frequency

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range 0–50 Hz for all channels. DTF gives information on the frequency content and direction of BA flows between the studied brain structures (2).

Results and discussion

The results of DTF analysis are presented in the form of schemata demonstrating the significant differences of BA flows between the structures analysed (Fig. 1, 2).

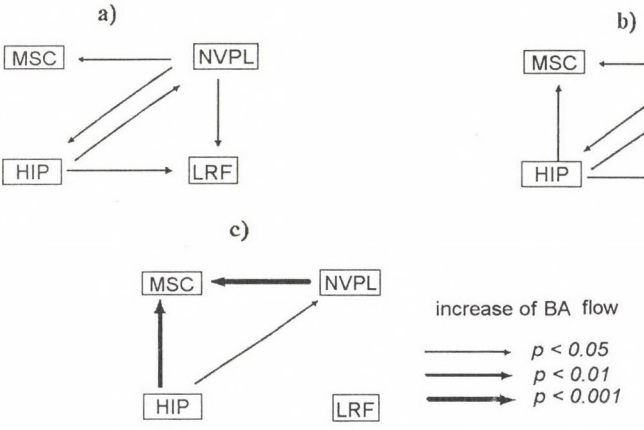

Fig. 1. Results of DTF analysis (scheme): a) – spontaneus BA, b) – BA after nociceptive stimulation, c) – statistically significant differences between a) and b).
MSC – motor-sensory cortex, NVPL – ventro-postero-lateral thalamic nuclei, HIP – hippocampus, LRF – lateral reticular formation, BA – bioelectrical activity.

DTF analysis revealed the following changes in BA after NS: the increase of BA flow: from NVPL and from HIP to MSC, and from HIP to NVPL (Fig. 1c). In the case of spontaneous activity microinjection of both clonidine and yohimbine decreased the BA flow from NVPL to HIP (Fig. 2a, c). Additionally clonidine caused the increase of BA flow from HIP to LRF (Fig. 2a). After NS yohimbine suppressed BA of HIP (the decrease of BA flow from HIP to NVPL and to LRF – Fig. 2d), whereas clonidine increased the BA flow from HIP to LRF (Fig. 2b). These results are in agreement with the findings that the activity of HIP is under tonic inhibition of noradrenaline released from neurons linking HIP with many noradrenergic regions in brainstem. Probably, there is a connection between dentate gyrus of HIP and reticular formation of brainstem via raphe nuclei. NS activating noradrenergic system may influence HIP activity and in this way it may change an animal behaviour (7).

In conclusion, our findings have shown that both supraspinal activation and blocade of α-2-adrenergic receptors change bioelectrical response of studied brain structures on the nociceptive stimulation, among other things by the modulation of hippocampal activity. The mechanism of this effect remains to be established.

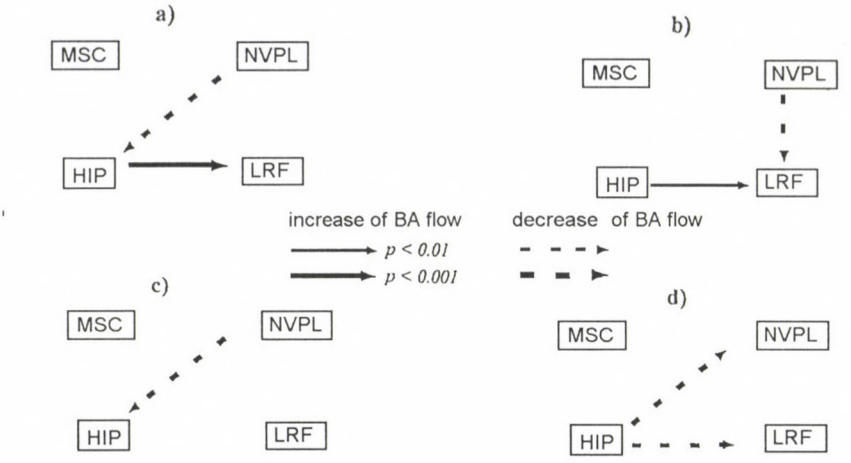

Fig. 2. Results of DTF analysis (scheme): statistically significant differences in the layout of BA flows between the analysed brain structures: a) – comparison of the spontaneous BA before and after clonidine administration, b) – comparison of BA after nociceptive stimulation before and after clonidine administration, c) – comparison of the spontaneous BA before and after yohimbine administration, d) – comparison of BA after nociceptive stimulation before and after yohimbine administration.
MSC – motor-sensory cortex, NVPL – ventro-postero-lateral thalamic nuclei, HIP – hippocampus, LRF – lateral reticular formation, BA – bioelectrical activity.

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ZINC AND CADMIUM IONS DIFFERENTLY MODULATES A1 ADENOSINE RECEPTORS

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The results suggest that: 1) Zinc ions may chelate the histidines critical for the agonist binding preventing hydrogen bonds between nonprotonated nitrogen atom of His-251 and the exocyclic N^6-H in CHA or CCPA molecule and between His-278 and –OH of the ribose ring. This mechanism can explain the reduction in the number of binding sites without changing the affinity. 2) Cadmium ions may oxidize cysteine SH-groups. The redox reaction between Cd^{2+} and receptor thiols may result in binding of the metal into stable (di)thiol-cadmium complexes rather than in the formation of disulfide and liberation of the reduced metal. This mechanism can justify the conformational modifications of the receptor molecule producing the decrease in affinity.

Keywords: zinc, cadmium, adenosine receptors

Divalent cations can modulate in opposite way the agonist binding to A1 adenosine receptors (A1AR). Whereas the increasing effect on binding of Mg^{2+} is related to the well known action on G-protein, the mechanism of decreasing effects on agonist binding of Zn^{2+}, Cu^{2+} and Cd^{2+} is unknown. Recently studies have suggested that these cations decreased binding apparently by acting on some molecular domains of A1AR (1, 2). They can modulate other receptor types, either by oxidizing essential sulfhydryl groups in opiate receptors (3), or by chelating histidine residues in $GABA_A$ receptors (4). The two histidine residue (251 and 278 in α-helices VI and VII) of A1AR have been recently shown to be essential for agonist binding (5). Cysteine residues, localized in the extracellular, transmembranal and intracellular domains, could be important for the receptor structure (6).

Methods

The present study investigates on the inhibitory mechanism of Zn^{2+} and Cd^{2+} on agonist binding to A1AR in membrane preparations of the rat cerebral cortex. Zinc and cadmium ions form complexes with amino acid and peptides. The stability constants of Zn complexes are greater than those of Cd when nitrogen (imidazol group of histidine) serve as ligand, but Cd binds more firmly to free sulfur groups (SH group of cysteine) (7).

The Zn^{2+} inhibition (IC_{50}=123.9±1.5 μM) of ^{3}H-CHA or ^{3}H-CCPA specific binding was counteracted by 1 mM histidine (IC_{50}=719.3±11.1 μM), whereas that of Cd^{2+} (IC_{50}=16.4± 2.4 μM) was abolished by 1 mM cysteine, but not by histidine. Moreover, the inhibitory effect of ions was present also after several washing of membranes.

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Results and discussion

Table 1

		Saturation isotherm binding				
		Control	Zn^{2+} 100 μM	DEP 2 mM	Cd^{2+} 100 μM	DTNB 100 μM
^{3}H-CHA	Kd	0.96±0.75	1.02±0.12			
	Bmax	0.42±0.013	0.31±0.02*			
^{3}H-CCPA	Kd	0.50±0.015		0.52±0.04		
	Bmax	0.43±0.010		0.04±0.01*		
	Kd	0.34±0.014			1.25±0.11*	0.73±0.04*
	Bmax	0.29±0.02			0.22±0.01	0.28±0.02

		Displacement experiments			
		Control	Zn^{2+} 100 μM	Control	Cd^{2+} 100 μM
R-PIA	Ki	0.78±0.16	1.14±0.19	1.29±0.2	5.94±0.9*
S-PIA	Ki	28.3±3.6	27.2±2.0	24.4±1.9	57.9±15.2*

In saturation experiments ranges 0.1–20 nM ^{3}H-CHA (N^6-cyclohexyl-[2,8-^{3}H]-adenosine) and 0.05–2.0 nM ^{3}H-CCPA (chloro- N^6-2-[cyclopentyl 2,3,4,5-^{3}H]-adenosine) were used. Incubations for 120 min or 180 min, respectively, were performed at 25 °C in Tris-HCl 50 mM, pH 7.4. 10 μM R-PIA was used for non-specific binding. The binding parameters were obtained by 3 different assays, each dose in triplicate. In displacement assays 1 nM ^{3}H-CHA or 0.5 nM ^{3}H-CCPA were used. *P<0.005 significant difference vs control was calculated by Student's t-test. Kd and Ki=nM; Bmax=pmol/mg protein

Saturation curves showed that Zn^{2+} did not alter the affinity, but reduced significantly the number of binding sites, whereas Cd^{2+} decreased the affinity without changing Bmax. The Ki values for R-PIA and S-PIA stereoisomers were not changed by Zn^{2+}; Cd^{2+} significantly decreased the affinity of stereoisomers changing, also, their potency ratio. These data suggest that the ions could act with different mechanisms at different sites on the receptor, probably Zn^{2+} at histidine and Cd^{2+} at cysteine residues.

It is possible that Zn^{2+} decreased the number of binding sites by chelating histidine residues essential for agonist binding. If the formation of coordination bonds with histidines can be the mechanism of Zn^{2+} actions, when: A) The pretreatment of rat cerebral cortex membranes with agonist should protect A1AR against zinc modification. The observed protection afforded by agonist could be due to the fact that agonist, added before Zn^{2+}, impedes the modification of histidines in the binding domain. B) A variation of pH of incubation buffer should change the inhibitory potency of Zn^{2+}, according to Henderson-Hasselbach equation. The pK_a of imidazol group of histidines is 6. At pH 5 the decreasing action was greatly reduced (7%) since the binding of Zn^{2+} to histidine was inhibited by [imidazole$^+$] 10 fold higher than [imidazole]. At pH 8 [imidazole] is 100 fold higher than [imidazole$^+$] and the chelating effect of zinc was greatly increased (75% binding inhibition). At pH 6 and 7.4 the inhibition was 32% and 57%, respectively. C) Diethylpyrocarbonate (DEP), a histidine-specific alkylating reagent could show a zinc-like effect. The DEP alkylation of the histidines leads to a dose-dependent inhibition of ^{3}H-CCPA binding (IC_{50}=532.5±0.9 μM). DEP 2 mM dramatically reduced the number of bindings sites, but did not alter the Kd value.

The data on the antagonism of cysteine of Cd^{2+} inhibition of ^{3}H-CCPA binding, and those on saturation and competition studies, suggest that Cd^{2+} could modify the conformational state of A1ARs. It is known that the oxidation-reduction state of SH groups plays an important

structural function related to receptor activity. Since Cd^{2+} has an oxidation potential related to its high affinity for SH groups, the mechanism of Cd^{2+} action could depend on a modification of thiol group of cysteines.

The SH-oxidating reagent, 5-5'dithio-bis-(2nitrobenzoic acid) (DTNB) behaved as Cd^{2+}. DTNB oxidation of cysteine thiol groups leads to a dose-dependent inhibition of ^{3}H-CCPA binding (IC_{50}=36.3±4.3 μM), and DTNB 100 μM significantly decreased ^{3}H-CCPA affinity without altering Bmax. Moreover, whereas DTNB 50 μM or Cd 10 μM reciprocally potentiated (60%) the inhibition of binding on membranes pretreated with Cd (20%) or DTNB (40%), the reducing agent dithiothreitol (DTT 1 mM) abolished cadmium and DTNB effects. DTT alone did not modify ^{3}H-CCPA binding in a range between 50 μM and 5 mM.

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PARACETAMOL INDUCED ACUT INTERSTITIAL NEPHRITIS SUPERIMPOSED ON MESANGIOCAPILLARY GLOMERULONEPHRITIS

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We report the case of a young alcoholic male whose first renal biopsy disclosed mesangiocapillary glomerulonephritis. One month later he took 1.5 g paracetamol to control the fever. Soon he got hospitalized due to toxicoderma, elevated liver and renal function tests. While the liver enzymes returned to normal, uremia developed. A repeated renal biopsy revealed severe interstitial inflammation, tubular atrophy. Haemodialysis was started and he got steroids (1 mg/kg body weight). He showed considerable recovery of renal function in some weeks. The case points to the possibility that paracetamol – even in therapeutic dosage – might result in hepatic and renal damage in alcoholics.

Keywords: Ethanol, paracetamol-toxicity, acut tubulointerstitial nephritis

Hepatic and renal toxicity of paracetamol overdosage is well-known (7) like the fact that ethanol enhances the toxicity of the drug (3,5). Scanty data report on reversible hepatic and renal failure appearing after therapeutic dose of paracetamol in alcohol-abusers (5,6). Renal damage might also occur without gross hepatocellular damage in alcoholics. We report the case of a young male with mesangiocapillary glomerulonephritis (MCGN) who showed a moderate hepatocellular damage and a sudden deterioration of renal function after taking 1.5 gr paracetamol.

Methods and Results

Case reportA 30 year old man was referred to our unit in October 1994 because of asymptomatic proteinuria, microscopic, dysmorph haematuria, high blood pressure, slightly impaired renal function. He was a heavy smoker and his alcohol intake has been 1 l wine/day for the last 10 years. His liver function tests were normal (after two weeks abstinence), his renal function and urinalysis was similar to the previous data. Renal biopsy disclosed MCGN with severe vascular damage. He was given an ACE and a Ca channel-blocker which – through controlling his BP optimally resulted in reducing the proteinuria and his se-creatinine value returned to normal.

One month later he took 1.5 g paracetamol to control the fever caused by an upper respiratory tract infection. Soon he got hospitalised with rash, fever, arthralgia, elevated liver and renal function values (Table 1).

In two weeks the liver function tests normalised, and oliguric renal failure, macroscopic haematuria, eosinophyluria, nephrotic range proteinuria developed. A severe systolic and diastolic high blood pressure without diurnal rhythm appeared.

The second renal biopsy showed additionally to the known MCGN more severe vascular changes and acut tubulointerstitial nephritis (ATIN) (Fig. 1).

He was started on haemodialysis, was given more aggressive antihypertensive medication and steroid treatment in a dose of 1 mg/body weight/day. The urine output increased, dialysis could be discontinued, the proteinuria, haematuria, renal function values returned by the end of March 1995 almost to the previous level. Steroid treatment was gradually tapered off. He is

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regularly followed-up, by now he is symptomfree, has moderately impaired renal function, is on antihypertensive medication, and on low protein diet (1 gr/body weight/day).

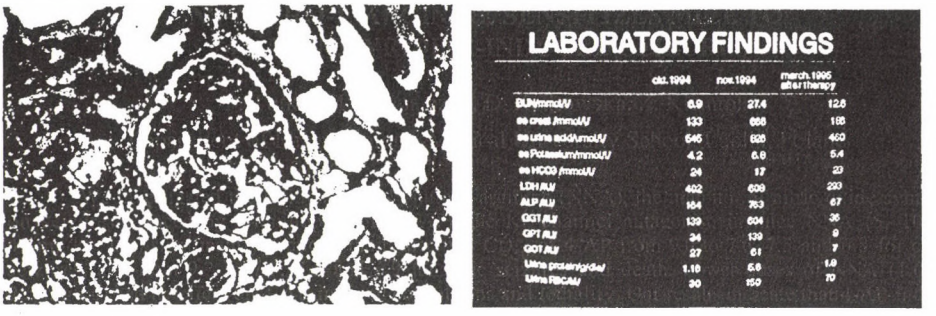

Fig. 1. Severe cellular infiltration and widening of the interstitium tubular atrophy, ATIN Light microscopy, haematoxylin-eosin stain, orig. magn. × 400

Discussion

The case points to the possibility,

1. That paracetamol– even in therapeutic dose – might result in hepatic and renal damage in alcoholics. Alcoholics should be informed about the potential hazard of the drug.
2. The sudden deterioration in renal function was due to the ATIN superposed on MCGN. The provoking drug was paracetamol (1, 4).
3. Early diagnosis, immediate withdrawal of the drug, steroid treatment might have kidney and life saving effect.

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N^G-NITRO-L-ARGININE SENSITIZES MICE TO 4-AMINOPYRIDINE-INDUCED SEIZURES

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We investigated the influence of N^G-nitro-L-arginine (NNA), the inhibitor of nitric oxide synthese, on seizures induced by 4-aminopyridine (4-AP), the K^+ channel antagonist, in mice NNA (5, 10 and 40 mg/kg, i.p.) significantly reduced the respectives CD_{50} of 4-AP from 9.0 to 7.6, 7.5 and 6.8 for clonic seizures, and from 9.2 to 7.7, 7.5 and 6.9 for tonic seizures and death. Lower doses of NNA (1.0 and 2.5 mg/kg) had no effect on 4-AP-induced convulsions and lethality. Our results indicate that 4-AP-induced seizures may be, at least in part, dependent on nitric oxide level.

Keywords: N^G-nitro-L-arginine (NNA), nitric oxide, seizure

Nitric oxide (NO) plays an important role in the central nervous system in many physiological and pathological processes, among them probably in epileptogenesis (3). Our previous studies have shown that the inhibition of NO synthesis exerts contrasting effects upon seizures induced by various convulsants (4).

It is widely accepted that kalium ions play an important role in seizure susceptibility. 4-Aminopyridine (4-AP), by blocking voltage kalium channels, produces clonic and tonic convulsions in rodents (5). The aim of this study was to determine an influence of N^G-nitro-L-arginine (NNA), a NO synthase inhibitor, on convulsions evoked by 4-AP.

Material and methods

Experiments were performed on male Swiss mice weighing 20–28 g. The experimental groups, consisting of 6–10 animals, were completed by means of a randomized schedule. NNA was given i.p. in doses of 1–40 mg/kg 30 min. before i.p. injection of 4-AP. The convulsant action of 4-AP was evaluated as the CD_{50} value (convulsive dose 50%, i.e. the dose of 4-AP producing the seizure response in 50% of mice) and lethality as the LD_{50} value (lethal dose 50%, i.e. the dose producing death in 50% of mice). Both, the calculation of CD_{50}s and LD_{50}s, as well as statistical analysis, were based upon method of Litchfield and Wilcoxon (1), modified in that the dose-effect curve was calculated on a computer.

Results and discussion

4-AP, the K^+ channel antagonist, when administered i.p. produced typical sequence of behavioural activation (hyperactivity to touch of loud noise, vocalization, blinking/eye closing, trembling) followed by clonic, tonic seizures and death. The CD_{50} values were 9.0 mg/kg for clonic and 9.2 mg/kg for tonic seizures. The LD_{50} value was 9.2 mg/kg.

NNA administered i.p. in doses of 5, 10, 40 mg/kg potentiated the convulsive effect of 4-AP towards clonic seizures, decreasing its CD_{50} from 9.0 mg/kg to 7.6, 7.5 and 6.8 mg/kg and for tonic seizures from 9.2 to 7.7, 7.5 and 6.9 mg/kg, respectively. The LD_{50} values were also significantly reduced (7.7, 7.5 and 6.9 mg/kg, respectively). Lower doses of NNA (1.0 and 2.5 mg/kg) had no effect on 4-AP-induced convulsions and lethality.

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NO seems to be involved in the mediation of seizure activity but a lot of contradictory data exist. There are reports on the proconvulsant action of NNA in kainate-induced (i.c.v. and i.p.) seizures (2, 4). Contrary to kainate, glutamate-induced convulsions were inhibited by pretreatment with NNA (4). On the other hand, in the majority of convulsive tests NNA had no modulatory effects (4).

In the present study, it was found that NNA, in doses known to decrease the level of NO, potentiated the convulsive and lethal activity of 4-AP. These results indicate that 4-AP-induced seizures may be, at least in part, dependent on NO level.

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YAWNING INDUCED BY APOMORPHINE, PHYSOSTIGMINE OR PILOCARPINE IS INHIBITED BY ELECTROCONVULSIVE SHOCK (ECS)

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Single and repeated ECS decrease sensitivity of dopamine D_2 and acetylcholine receptors as measured by yawning behavior. This reduced sensitivity is dependent from the number of applied shocks, since effects of repeated ECS disappear 5 days after the last shock but effect of single ECS disappear 24 h after the shock.

Keywords: ECS, dopamine autoreceptors, acetylcholine receptor, yawning

Repeated ECS induced several changes on rats behavior, such as increased amphetamine and nomifensine-induced locomotor activity or attenuated suppression of locomotor activity induced by low doses of apomorphine (1, 3, 5). These results indicate that ECS may produce development of postsynaptic dopamine receptor supersensitivity and all presynaptic dopamine receptor subsensitivity (2, 3).

In the present study, the effects of single and repeated ECS on the behavioral responses to low dose dopamine D_2-like (apomorphine) receptor agonist and acetylcholine (pilocarpine, physostigmine) receptor agonist were investigated.

Methods

Male Wistar rats of 200–250 g were housed in plastic cages with free access to food and tap water. ECS was administered (100 mA for 0.8 s) through ear-clip electrodes, either once or 5 times every second day during 10 days. No anaesthetic was used the current used was always observed to cause a typical tonic clonic seizures lasted for 15–20 s. Untreated animals were handled identically and ear clipped without any current being applied. Behavioral observations were performed 30 min and 24 h after single ECS or 30 min, 24 h, 3 and 5 days after five ECS.

Yawning behavior is characterised by a slow wide opening the mouth. Rats were placed in boxes. After 20 min adaptation period the rats were treated with apomorphine (0.075 mg/kg), pilocarpine (4 mg/kg) and physostigmine (0.2 mg/kg) and the number of yawns was counted during 60 min observation.

Results and discussion

Single ECS decreased the number of yawns caused by injection of apomorphine, pilocarpine and physostigmine of 30 min but not 24 h after the shock (Fig. 1, 2, 3). Five ECS also attenuated the responses of rats to apomorphine, pilocarpine and physostigmine administered 30 min, 24 h or 3 days after the final treatment (Fig. 1, 2, 3). There was no differences between responses of the handled controls and rats receiving repeated ECS and apomorphine, pilocarpine or physostigmine given 5 days after the last shock. In the present experiment, decreased sensitivity to apomorphine, pilocarpine and physostigmine was observed following single and repeated ECS. The effect of single ECS was short-lasting (30 min) but the effect of repeated ECS was

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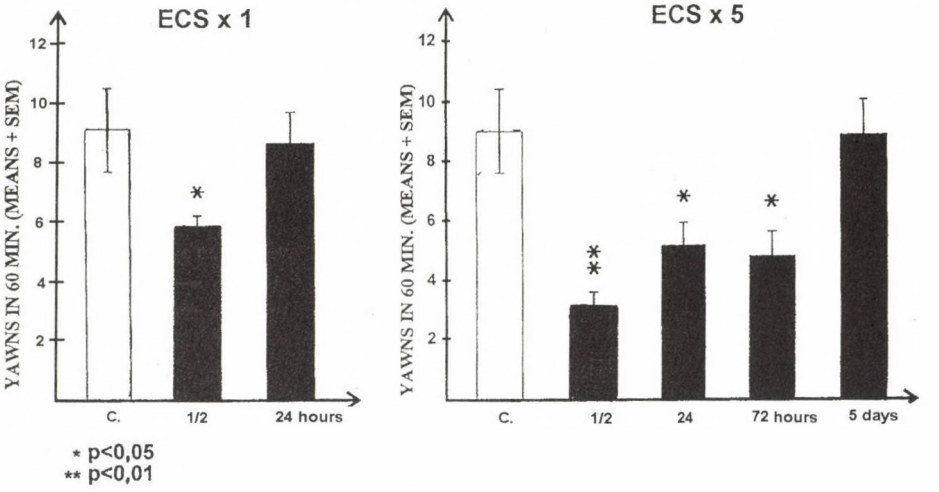

Fig. 1. Apomorphine-0,075 mg/kg

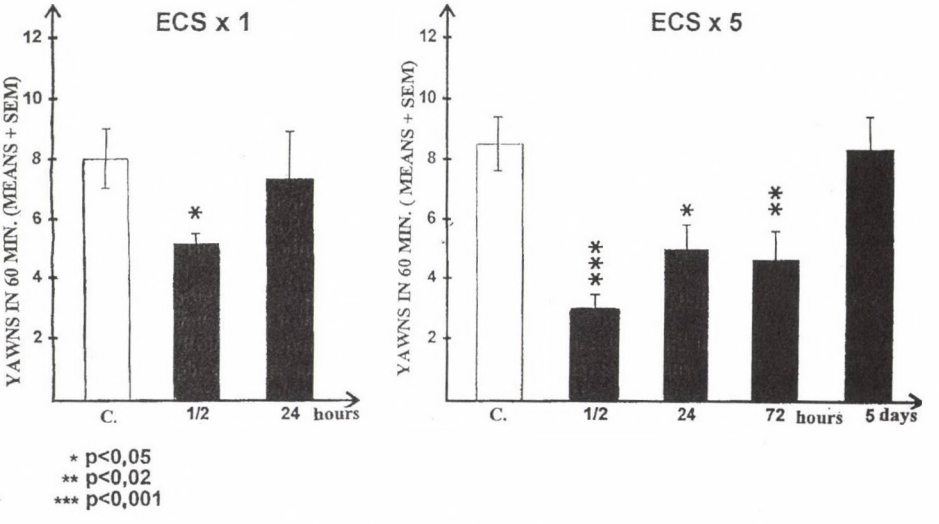

Fig. 2. Physostigmine-0,2 mg/kg

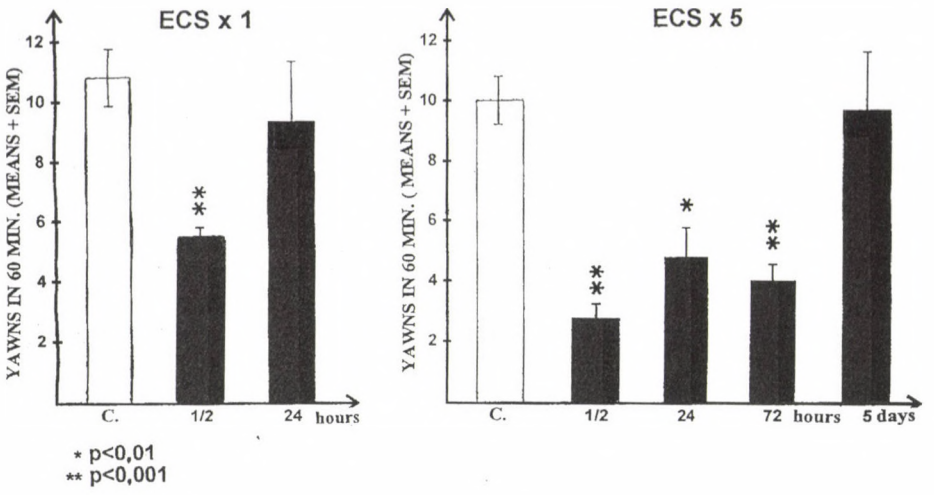

Fig. 3. Pilocarpine-4 mg/kg

observed 3 days but not 5 days following the last ECS. The ability of single and repeated ECS to attenuate apomorphine-induced yawning can be explained as a presynaptic subsensitivity of dopamine D_2 receptors.

The observation that single and repeated ECS also attenuate pilocarpine and physostigmine-induced yawning indicate decrease sensitivity of cholinergic muscarinic receptors. These results directly support the hypothesis that single and repeated ECS down-regulate dopamine D_2 and muscarinic receptors.

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Acta Physiologica Hungarica, Volume 84 (4), pp. 477–479 (1996)

THE ESTIMATION OF INTERACTIONS BETWEEN ARGININE-VASOPRESSIN (AVP) AND NMDA RECEPTORS IN MEMORY AND LEARNING PROCESSES

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Arginine-vasopressin (AVP) is a neuropeptide which facilitates learning and memory processes. We examinated the participation of NMDA receptors in beneficial effects of peptide. The results of our study show that noncompetitive antagonist of NMDA receptor – MK-801 impairs the effect of AVP on the consolidation of conditioned avoidance responses and antagonist of polyamines site – arcaine reduced advantageous effect of AVP on the retrieval of memory in passive avoidance situation.

Keywords: AVP, NMDA receptors, learning, memory, MK–801, AP-7, arcaine

Vasopressin is a neuropeptide physiologically involved in memory and learning processes independently on its hormonal function. It facilitates consolidation of acquired information and retrieval of memory in experimental animals (2). Although the behaviour-modulating action of vasopressin seems to be mediated via the V1 receptor subtype, the precise mechanisms of action of neuropeptide in learning and memory remain unclear. Its action was linked with noradrenergic mechanisms and β-adrenergic receptors, but last was found same interactions between vasopressin and glutaminergic system in the CNS.

The aim of our studies was the estimation of participation of ionotropic NMDA receptors in action of vasopressin.

Methods

The experiments were carried on the male Wistar rats of the body weight of 160–180 g. The animals were kept in the light-dark cycle room 12:12 h. All behavioural tests were performed at the same hours of day between 8.00 and 14.00. We used the following drugs in our studies: Arginine-vasopressin (AVP), noncompetitive antagonist of NMDA receptor – dizocilpine (MK–801), competitive antagonist – 2-amino-7-phosphonoheptanoic acid (AP-7) and antagonist of polyamines site – arcaine. All antagonists in the doses of 5 nmole and AVP in the dose of 1 μg were injected into lateral cerebral ventricle left or right (i.c.v.). The antagonists were administrated 15 min before Arg-vasopressin.

The motor and exploratory activity of rats were quantified in the open field test. After 1 min of adaptation in "open field", the crossing of squares, rearings and bar approaches were counted for 5 min.

The retrieval of memory was evaluated in the passive avoidance situation. Retention of the passive avoidance responses was tested 24 h after electric footshock. The antagonists were injected 30 min before the retention trial and AVP 15 min before one.

Conditioning avoidance responses (CARs) consisted of five, 20-trial session daily. The drugs were injected on 5-th day immediately after trial session. The consolidation of CARs was evaluated on 12-th and 19-th days and expressed as per cent of the number of (+)CARs in tested groups. The control group received i.c.v. 0.9% NaCl.

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The results of experiments were evaluated statistically by analysis of variance (ANOVA) followed by Newman-Keuls test for open field test and CARs and by Mann-Whitney U test for passive avoidance situation.

Results and discussion

In used doses no compound change the motor and exploratory activity of rats in open field test. The antagonist of polyamines site – arcaine significantly reduced the beneficial effect of AVP on the retrieval of memory in passive avoidance situation (Fig. 1). AP-7 decreased the action of AVP, but not significantly. MK-801 not impairs the effect of AVP on the recall memory. No antagonist impairs the retrieval of memory in passive avoidance behaviour, when it was given alone.

MK-801 reduced the advantageous effect of AVP on the consolidation of conditioned avoidance responses (CARs) estimated on 12-th day (Fig. 2). AP-7 has not effect on the action of AVP in this test.

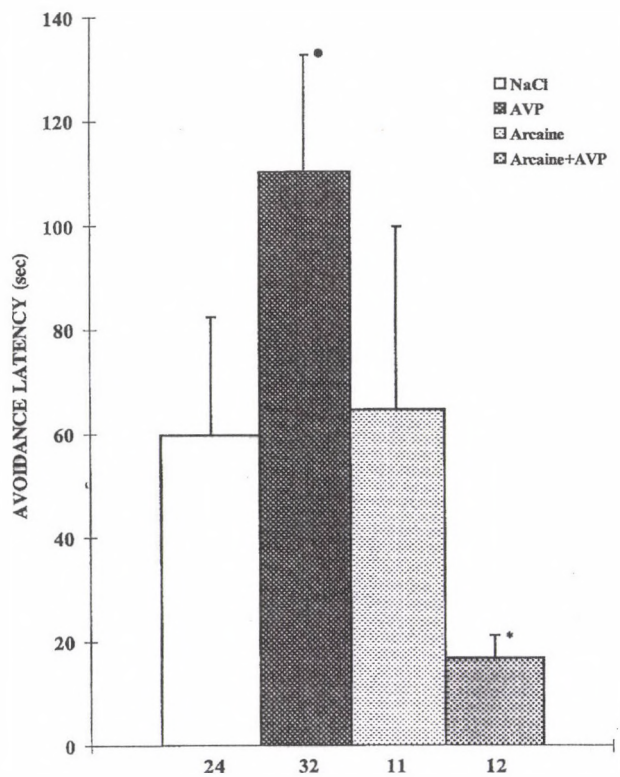

Fig. 1. The effect of AVP, arcaine and AVP+arcaine on the retrieval of memory in passive avoidance situtation in rats. Columns represents mean ± SEM of "n" subjects. •$p<0.02$ AVP vs control, *$p<0.05$ AVP vs AVP+arcaine. (Mann-Whitney test).

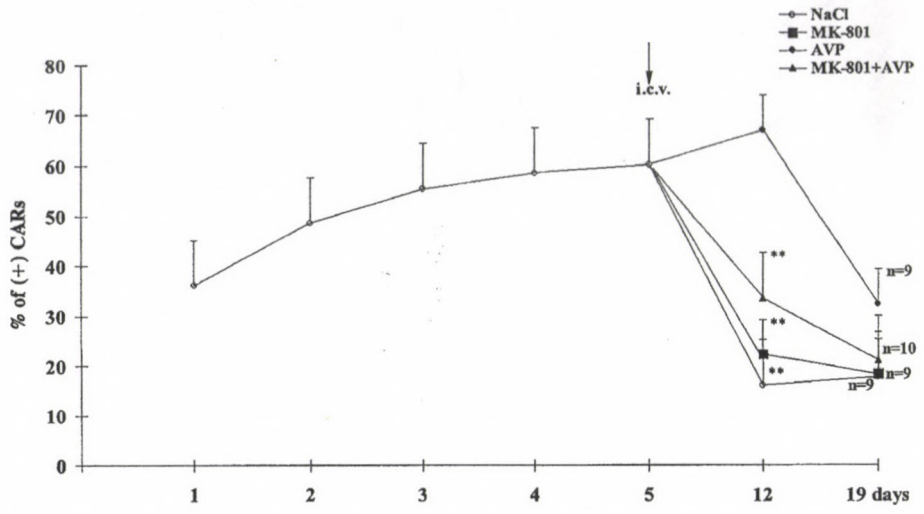

Fig. 2. The effect of AVP, MK-801 and AVP+MK-801 on the consolidation of conditioned avoidance responses (CARs) in rats. Each point represents mean ± SEM of "n" subjects. **p<0.01 AVP vs control, MK-801 and AVP+MK-801. (Newman-Keuls test).

These results suggest that excitatory amino acids and NMDA receptors may participate in the beneficial effects of vasopressin on the learning and memory processes. Data from literature indicate same interactions between AVP and glutaminergic system. AVP facilitates consolidation of information. There are evidence that NMDA receptors are involved in memory consolidation. Glutamate is the main neurotransmitter in hippocampus and cortex. Vasopressinergic neurones project to a number of brain regions engaged in memory and learning processes – hippocampus, septum. Browning et al (1) reported that AVP prolongs the maintenance of glutamate-induced LTP in the lateral septum. There are evidence indicating a role of the NMDA receptors in the induction and AVP in the maintenance of LTP in this structure (3). MK-801, AP-7 and arcaine exert different effect on action of AVP in performed tests. We are going to examinate, which another sites of NMDA receptors have the influence on the AVP action and which is the mechanism of these interactions.

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Acta Physiologica Hungarica, Volume 84 (4), pp. 481–482 (1996)

THE INFLUENCE OF 1S,3R-ACPD ON CENTRAL DOPAMINERGIC TRANSMISSION AND RECOGNITION MEMORY

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1S,3R-ACPD improved learning and memory in a passive avoidance situation. We examined its influence on recognition memory and dopaminergic transmission. The results of our study shown that 1S,3R-ACPD had not influence on recognition memory and diminished dopaminergic transmission.

Keywords: 1S,3R-ACPD, metabotropic glutamate receptors, learning, memory, dopaminergic transmission

The activation of metabotropic glutamate receptors (mGluRs) in the brain is known to produce profound effects on neurotransmission. MGluRs are not directly associated with ion fluxes as ionotropic receptors. Instead, they active second messenger-mediated, biochemical signaling cascades via GTP-binding proteins. At present, there are eight mGluRs, termed mGluR1–mGluR8. Group I mGluRs comprise mGluR1 and mGluR5 are linked to stimulation of phospholipase C. Group II (mGluR2 and mGluR3) and group III (mGluR4, 6, 7 and 8) are linked to inhibition of adenylate cyclase. 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), is an agonist of the first and second group of metabotropic glutamate receptors, which are localizated presynaptically (3).

We have previously shown that 1S,3R-ACPD improved learning and memory in passive avoidance situation. Several reports suggest that the positive effect on memory processes may be mediated by central dopaminergic system. 1S,3R-ACPD have been shown to produce presynaptic depression of inhibitory transmission in the striatum, olfactory bulb, neocortex, and thalamus (2). The aim of the present study was to determine the influence of 1S,3R-ACPD on the central dopaminergic transmission and recognition memory.

The effect of 1S,3R-ACPD on locomotor activity, apomorphine stereotypy and haloperidol induced catalepsy was observed. Some date indicated that dopamine play important role in memory object recognition. We have also study the effect of 1S,3R-ACPD in object recognition test.

Methods

All experiments were performed on male Wistar rats (160–180 g) prepared to the standard surgical procedure. After 48 h they were injected intracerebroventricularly (icv) with 50, 100 and 200 nmole 1S,3R-ACPD per rat in the volume 10 µl, immediately after apomorphine, 30 min after haloperidol, 30 min before learning session, immediately after it, and 30 min before testing the discrimination in the object recognition test.

Locomotor and exploratory activity of rats were measured in an "open field" apparatus as a number of crossing, rearings, and bar approaches made during 5 min starting 20 min after icv injection of the compound. Stereotypy behaviour was produced by an ip injection of 2 mg/kg of apomorphine hydrochloride administered in a volume of 1 ml/kg. Haloperidol catalepsy was evoked by haloperidol (1 mg/kg ip), an antagonist of dopamine receptor.

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Object recognition test was done in a plastic box 62×38×20 cm. The object to be discriminated were made of glass or porcelain and existed in duplicates. The procedure was similar to the described by Ennaceur and Delacour (2) and may be summarized as follows. All rats were submitted to two habituation sessions, with 1 h interval, whereby they were allowed for 3 min exploration of the apparatus. 24 h later testing began. The experimental session was made of two trials, each 3 min long. In the first trial (T1), rats were exposed to two identical objects A1 and A2. In the second trial (T2) performed 60 min later, rats were exposed to two objects, one of which was a duplicate of the familiar object A (A'), and a new object B. From rat to rat, the role (familiar or new object) as well as the relative position of the two objects were counterbalanced and randomly permuted during trial T2. The basic measure was the time spent by rat in exploring objects during T1 and T2 trials. Exploration of an object was defined as touching it with the nose. From this measure the following variables were defined: A = the time spent in exploring objects A1 and A2 in T1; A' and B = the time spent in exploring, respectively, the duplicate of familiar and the new object in T2. Object recognition was measured by the variable B–A' and total exploration in T2 by B–A'. Moreover as B–A' may be biased by difference in overall levels of exploration, the variable B–A'/B+A' was also computed.

Statistical comparisons were made by analysis of variance (ANOVA) followed by Newman-Keuls test. All ratings of stereotyped and catalepsy behaviour for each rat were summed up first, and overall group means were then calculated. In all comparisons between groups a probability of 0.05 or less was considered significant.

Results and discussion

1S,3R-ACPD did not change any of observed behaviour (crossing of squares, rearings and bar approaches) in the "open field" test in the comparison with control animals. Tested compound significantly decreased apomorphine stereotypy at the icv doses of 100 ($p<0.01$ vs control), and 200 nmole per rat ($p<0.05$ vs control). This effect was the most pronounced between 15 and 35 min of observation. The icv dose of 200 nmole 1S,3R-ACPD significantly decreased haloperidol induced catalepsy ($p<0.01$ vs control group). The most pronounced differences in comparison to the control group were in the 120 min.

Object recognition test: the time spent in exploring object A in T1 was comparable in all groups. Object recognition memory measured by the difference B–A' was not significantly between groups. The total time spent exploring objects B and A in T2 (B+A') was similar in all groups. Noteworthy, changes of the variable B–A'/B+A' in different groups were in the same direction as changes of the variable B–A'. This shown that object recognition scores were not biased by differences in overall levels of exploration.

These results indicated that 1S,3R-ACPD, an agonist of mGluRs, which in our previous study improved memory motivated affectively, had not influence on recognition memory and diminished dopaminergic transmission. May be the disinhibitory effect mediated by 1S,3R-ACPD play role in observed effects.

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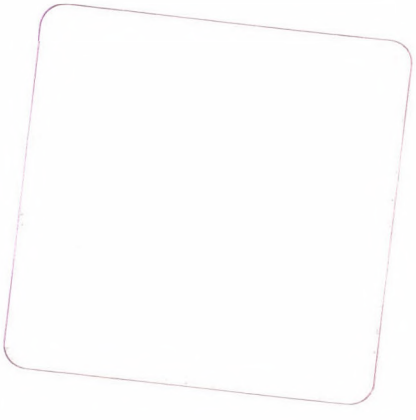

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