BIPHASIC EFFECT OF ENDOTHELIN-1 ON CORONARY VASCULAR RESISTANCE IN ANESTHETIZED RATS WITH AN INTACT CHEST

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KEY WORDS: heart; autoperfusion of the coronary vessels; endothelin-1; nisoldipine; blocker of endogenous NO synthesis

Endothelin is one of the most effective vasoconstrictor endogenous peptides and was isolated from the supernatant of a culture of porcine aortic endothelial cells in 1988 [17]. This peptide causes constriction of both arteries and veins in rats [12], cats [11], dogs [7, 10], rabbits [13], guinea pigs [5], pigs [4], and man [2]. Expression of the endothelin gene arises under the influence of thrombin and catecholamines [17], indicating its possible involvement in the regulation of vascular tone in the course of various adaptive reactions. The effect of endothelin on coronary vascular tone has been studied in experiments in vitro – on isolated preparations of the coronary arteries [2] and the isolated perfused heart [16]. In experiments in vivo, the coronary blood flow was recorded under open chest conditions [4, 9]. In that case the sucking action of the negative pressure is lost, and this introduces significant changes into the activity of the cardiovascular system.

In the investigation described below we studied the effects of endothelin on coronary vascular resistance of anesthetized rats with an intact chest, and also the role of voltage-dependent Ca channels and of endogenous NO in these reactions.

EXPERIMENTAL METHOD

Experiments were carried out on male Sprague-Dawley rats weighing 250-300 g, anesthetized with pentobarbital (50 mg/kg, intraperitoneally). After tracheotomy polyethylene catheters (PE-10 and PE-50 respectively, from "Clay Adams," USA) were introduced into the femoral artery and jugular vein to record the systemic blood pressure and to inject drugs. The coronary arteries of the rat heart were perfused by the method suggested in [15], our modification. The method is based on introduction of a glass cannula of a special shape (Fig. 1A, external diameter 1.00 mm, internal 0.58 mm) into the right or left coronary artery. The cannula was 3.5 cm long. Blood for autoperfusion of the coronary arteries was taken from the left carotid artery of the animal, into which a PE-50 polyethylene catheter was inserted (Fig. 1, II). The perfusion system consisted of a silicone tube, into the wall of which the crystal of a Doppler flowmeter had been implanted (III), in order to record the blood flow rate. The silicone tube was connected successively to two 3-way cocks: one for intraarterial injection of substances (IV), the other for measuring perfusion pressure (V). After heparinization of the animal (1000 U/kg, intravenously) the perfusion system was filled with blood through a catheter introduced into the left carotid artery, and a glass catheter was introduced through the right carotid artery into the region of the ascending aorta. The catheter was carefully maneuvered into the region of the ostium and passed into one of the coronary arteries, as shown by the appearance of a blood flow in the perfusion

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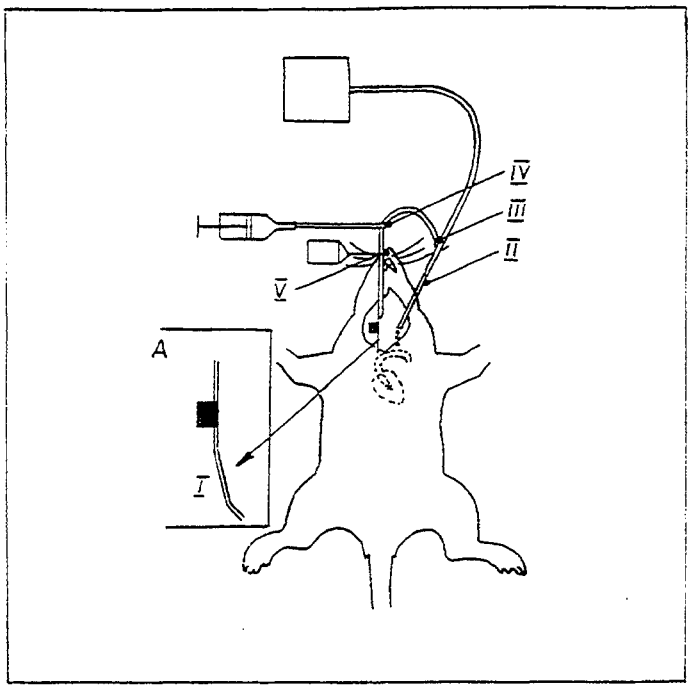

Fig. 1. Plan of experiment. A: I) glass cannula whose end is introduced into one of the coronary arteries; II) polyethylene catheter inserted into left carotid artery; III) crystal of doppler flowmeter; IV) 3-way cock for injecting drugs at constant velocity; V) 3-way cock for measuring perfusion pressure.

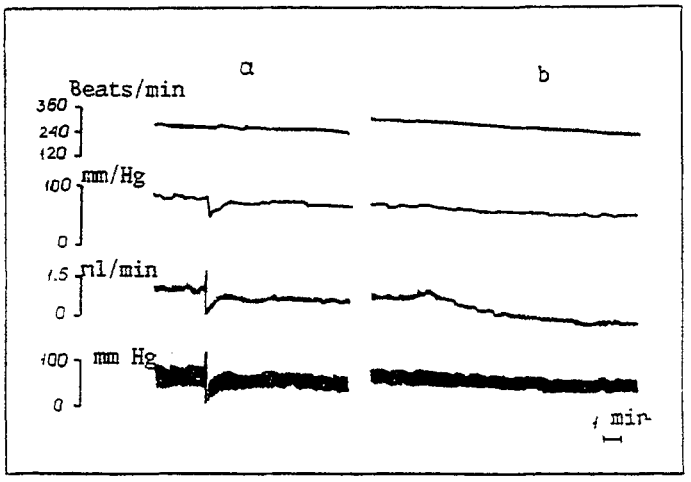

Fig. 2. Effect of endothelin-1 on coronary blood flow when injected by bolus method in a dose of 10 pmoles/kg (a) and infusion of 3.3 pmoles/kg/min for 3 min (b). From top to bottom: heart rate (in beats/min); perfusion pressure (in mm Hg), blood flow in perfusion system (in ml/min), and systemic blood pressure (in mm Hg).

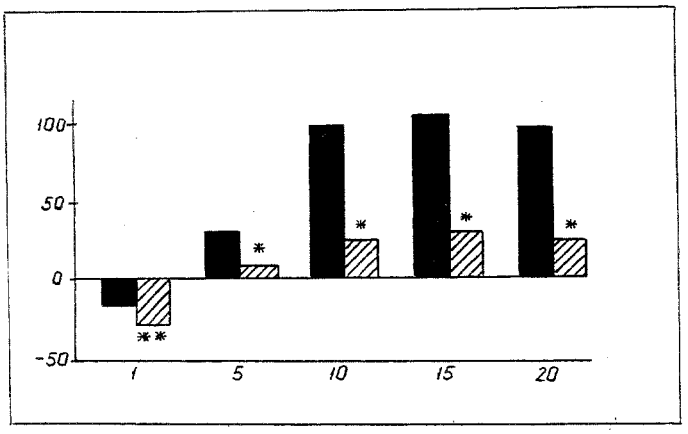

Fig. 3. Effect of nisoldipine (10 μg/kg) on reaction of coronary vessels of rat heart to injection of endothelin-1. Abscissa, time of development of effect (in min); ordinate, change in coronary resistance (in percent). Black columns – effect of endothelin-1 before injection of nisoldipine; obliquely shaded columns – after injection of blocker of voltage-gated Ca-channels.

system. The catheter was fixed in this position and left until the end of the experiment. After the experiment, to determine the zone of perfusion of the heart, a solution of Evans' Blue dye was injected through the glass catheter. In the course of the experiment the systemic blood pressure, heart rate, and pressure and blood flow in the perfusion system were recorded. The resistance of the coronary vessels during autoperfusion was calculated as the ratio of pressure to blood flow, after deduction of the intrinsic resistance created by the tubes and catheters of the perfusion loop. In a series of six experiments, in four cases the catheter was inserted into the left coronary artery, and in two cases into the right coronary artery. No significant differences in the results were found, and they are therefore given in the form of mean values for six experiments. Endothelin-1 (from "Sigma" USA) was injected into the coronary artery by the bolus method in a dose 10 pmoles/kg body weight. During infusion of the preparation it was injected over a period of 3 min in a dose of 3.3 pmoles/min/kg, or 5 min in a dose of 2 pmoles/min/kg. The Ca-channel blocker nisoldipine (from "Miles," USA) was injected intravenously in dose of 10 μg/kg, and the blocker of endogenous NO synthesis N-nitro-L-arginine methyl ester (L-NAME) also was injected intravenously in a dose of 3.4 mg/kg. The results were subjected to statistical analysis by Student's t test.

EXPERIMENTAL RESULTS

The blood flow during autoperfusion of the coronary artery of the rat heart in six experiments averaged 1.37 ± 0.32 ml/min. Bolus injections of endothelin-1 in a dose of 10 pmoles/kg caused a marked increase in the resistance of the coronary vessels in the course of 60-90 min. This effect was accompanied by the fall of the systemic blood pressure during the first minute of development of the reaction, which masked the true reaction of the coronary vessels in this period of time (Fig. 2a). For a more selective study of the response of the coronary vessels to endothelin, in the next experiments the preparation was infused, when no change in systemic blood pressure was observed.

In response to infusion of endothelin-1 in a dose of 3.3 pmoles/min/kg for 3 min or 2 pmoles/min/kg for 5 min a biphasic reaction of the coronary vessels appeared (Fig. 2b). During the first 3 min the coronary blood flow increased but the resistance of the vessels of the heart fell by 18% (from 56.6 ± 4.1 to 46.2 ± 2.8 mm Hg/ml/min), but later it rose. The maximal increase in resistance was observed at the 15th minute of development of the effect, when

the resistance increased by 107% (from 56.6 ± 4.1 to 110.5 ± 6.8 mm Hg/ml/min) compared with initially (Fig. 3). During the next 60-130 min the resistance of the coronary vessels remained raised, and only in one of six experiments did the resistance of the coronary system return to normal toward the end of the second hour after infusion of endothelin-1. The systemic blood pressure and heart rate were unchanged during the 120 min after infusion of endothelin-1.

At the time of intravenous injection of nisoldipine (10 μg/kg) a significant fall of the systemic blood pressure was observed, but toward the 30th-40th minute after bolus injection, when blockade of Ca-channels reached its peak [6], the systemic blood pressure did not differ significantly from normal (113 ± 24 and 112 ± 20 mm Hg respectively) whereas the heart rate fell from 344 ± 34 to 326 ± 31 beats/min.

The dilator phase of the effect of endothelin increased significantly 40 min after injection of nisoldipine (10 μg/kg), whereas the constrictor effect was largely blocked. On average of 6 experiments the resistance of the coronary vessels decreased by 38% (from 84.2 ± 4.0 to 58.4 ± 2.7 mm Hg/ml/min) during the first minute of endothelin infusion against the background of the action of nisoldipine, compared with 18% before its injection. The differences are statistically significant, $p < 0.05$ (Fig. 3). The maximal increase of resistance caused by endothelin against the background of the action of nisoldipine was 28% (from 84.2 ± 4.0 to 106.5 ± 12.1 mm Hg/ml/min) compared with 107% before its injection ($p < 0.01$).

L-NAME, a blocker of synthesis of endogenous NO, was injected intravenously (3.4 mg/kg) 2.5-3 h after injection of nisoldipine, when the second phase of the effect of endothelin was restored. L-NAME caused an increase in systemic blood pressure by 43% from 103.8 ± 23.4 to 149 ± 26 ($n = 6$) mm Hg, which was maintained at this level for 90-120 min. The heart rate fell from 350.4 ± 41 to 336.6 ± 41.9 beats/min. Against the background of the action of L-NAME the resistance of the coronary vessels rose by 130% from 78.7 ± 12.8 to 180.9 ± 61 mm Hg/ml/min ($n = 6$).

Infusion of endothelin-1 5 min after injection of L-NAME, after stabilization of the blood pressure, caused death in 1 of 6 cases, total arrest of the blood flow in one case, and a biphasic effect in three cases. During the first 3 min of endothelin infusion the resistance of the coronary vessels fell by 31 ± 7%, an amount comparable with the effect of endothelin before injection of L-NAME, and in this series of experiments it amounted to 37 ± 18.7%. The phase of an increase of resistance of the coronary vessels in response to an injection of endothelin, and against the background of the action of L-NAME, increased both in magnitude and in duration. At the 15th minute after infusion of endothelin the resistance increased by 354% (131% in the control), and it remained at that level for 2-3 h.

A modification of the method of autoperfusion of the coronary vessels of the heart in a rat with natural ventilation of the lungs, which was published for the first time in [15], was thus suggested for use in the present investigation [15]. The use of an ultrasonic blood flow sensor to record coronary blood flow greatly simplifies the autoperfusion technique. The perfusion loop illustrated in Fig. 1, which stores 0.08-0.1 ml of blood, enables the responses of the coronary vessels to be investigated virtually without any blood loss or cooling of the circulating blood. The coronary blood flow recorded in the present study, when expressed per gram tissue, averages 4.2 ml/min/g, confirming data published in [15] and indicating a higher value of the coronary blood flow in animals with natural breathing compared with thoracotomized animals. A similar value of the coronary blood flow was recorded during investigations conducted by the method of labeled microspheres, in experiments in vivo on conscious animals, and this confirms that the technique used in the present investigation to study the coronary blood flow is adequate.

Intracorconary infusion of endothelin, unaccompanied by changes in systemic pressure, can shed light on the true reaction of the vascular system of the heart to exogenous administration of endothelin-1. The presence of a transient increase in blood flow during the first minute of infusion is evidently due to the action of lower concentrations of the peptide, which has also been demonstrated in experiments carried out on isolated preparations of the coronary vessels [5] and infusion of the coronary circulation in thoracotamized animals [3]. The absence of any effect of the blocker of endogenous NO synthesis on the dilator phase is evidence that this phase of endothelin is indepen-

dent of production of endothelium-dependent dilator factor. According to data in the literature, this phase in the coronary vessels of the guinea pig is abolished by indomethacin, an effect which is not observed in isolated preparations of the aorta [5]. Possibly the dilatation induced by endothelin in the coronary vessels of the rat also is mediated through activation of the cyclooxygenase pathway of arachidonic acid metabolism. However, the considerable potentiation of the constrictor phase of the effect of endothelin against the background of L-NAME indicates that endothelium-dependent dilator factor may be involved in the general vascular effect of this peptide. This mechanism may perhaps be activated simultaneously with the constrictor phase of the action of endothelin, and may counteract its appearance to some extent. Further evidence is given by results obtained on isolated preparations of the canine and porcine arteries, where the contractile effect of endothelin on de-endothelized preparations appeared at lower concentrations of the peptide than in the presence of endothelium, and it was more marked. Moreover, an inhibitory action of NO has been demonstrated on the appearance of the constrictor effect of endothelin [10]. The effect of an increase in the cyclic GMP concentration in segments of arterial vessels also is linked with the ability of endothelin to induce release of endothelium-dependent dilator factor. The cGMP concentration in isolated de-endothelized segments is unchanged by the action of endothelin. Thus endothelin evidently facilitates release of endothelium-dependent dilator factor from vascular endothelium. This effect is coupled with the intrinsic constrictor action of the peptide and is aimed at reducing it.

There are some interesting data on enhancement of the dilator phase of the effect of endothelin against the background of the action of nisoldipine, a blocker of dihydropyridine-sensitive calcium channels. It can be tentatively suggested that this effect is connected with the blocking action of nisoldipine on the endothelin-induced constrictor reaction, which promotes manifestation of the dilator action of this peptide.

The considerable (almost threefold) reduction of the constrictor effect of endothelin after intravenous injection of nisoldipine is evidence that voltage-dependent calcium channels are involved in the realization of this reaction of the coronary vessels in rats. Similar data have been obtained for human resistive arteries [8]. The remainder of the effect is evidently mediated through calcium inflow via other (possibly receptor-dependent) channels, as has been shown, for example, for the isolated preparation of the rabbit aorta [6]. A role of intracellular calcium in this effect likewise cannot be denied. It has been shown that the contractile effect of endothelin in isolated segments of porcine coronary arteries [9] and of the rat aorta [1] is independent, or only partly dependent, on the presence of calcium in the external medium. Thus, according to data in the literature and the results of the present investigation the mechanism of realization of the constrictor reaction to endothelin is rather complex. Depending on the species of animal and the type of vessel the contribution of voltage-dependent and receptor-dependent calcium channels, and also of intracellular calcium release to the increase in the intracellular calcium concentration under the influence of endothelin varies considerably and requires special investigation in each individual case.

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THYMOSIN-α_1, AN ENDOGENOUS MODULATOR OF THE α-THROMBIN RECOGNITION SITE

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Thrombin is the most important bioregulatory enzyme of the homeostasis system and of certain other linked systems [4]. Its physiological functions are largely determined by an additional recognition site of high-molecular-weight substrates, located on the surface of the molecule outside the active center zone [7]. This center is important for the manifestation of specific proteolytic functions and also of the hormonelike properties of α-thrombin during interaction with cells [5, 10]. A substrate locus complementary to the recognition site has been found in the 34-51 region of the Aα-chain of fibrinogen [4] and in the C-terminal fragment 48-65 of hirudin [9]. It can be tentatively suggested that ligands with an amino-acid sequence homologous with these loci will modulate the physiological functions of thrombin. Previously [3] a negatively charged cluster at the C-end of thymosin-α_1, largely homologous with the C-terminal fragment of hirudin, and also a tetrapeptide Arg(Lys)-Glu-Val-Val, homologous with one region of the Aα-chain of fibrinogen, were discovered on the basis of computer screening of regions complementary with the additional recognition site of high-molecular-weight substrates. Thymosin-α_1 inhibited the enzymic activity of α-thrombin relative to fibrinogen and amide and ester substrates. The mechanism of inhibition of activity of this enzyme has not been studied. The investigation described below was undertaken to shed light on this problem.

EXPERIMENTAL METHOD

Bovine α-thrombin was purified from a commercial preparation of USSR/CIS origin by the method in [3]. The clotting activity of the α-thrombin was 2500 NIH U/mg protein, and its esterase activity relative to benzoyl-arginine methyl ester 10 μmoles $\cdot$ min^{-1} $\cdot$ mg^{-1}. β/γ-Thrombin was obtained by restricted proteolysis of α-thrombin by immobilized trypsin [2]. The clotting activity of the β/γ-thrombin did not exceed 7 NIH U/mg protein, and its esterase activity relative to benzoyl-arginine-methyl ester was 9 μmoles $\cdot$ min^{-1} $\cdot$ mg^{-1}. The fibrinogen-clotting activity of α-thrombin was determined turbidimetrically [3]. The amidase activity of α- and β/γ-thrombin relative to H-D-Phe-Pip-Arg paranitroanilide was determined spectrophotometrically in accordance with recommendations of the firm "Kabi Diagnostica." The kinetic constants K_m and V_{max} of hydrolysis of the amide substrate were calculat-

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