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Dynamics of the Nitrergic System in Experimental Hypercholesterolemia

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Abstract

Hypercholesterolemia and atherosclerosis are still the main cause of mortality in Central Asia.

Study of the molecular mechanisms of endothelial dysfunction with changes in the nitroergic system in experimental hypercholesterolemia. The experiments were carried out on 28 Chinchilla rabbits with an average weight of 2.5-3.0 kg. The action of drugs was studied in dynamics: the initial 3-month condition and after one month of drug administration. The results obtained were compared with those of the control and intact groups. The activity of the enzyme nitrate reductase in the blood serum on the 30th day of the introduction of exogenous cholesterol increases only 1.15 times, then on the 60th and 90th days of the introduction - 1.3 and 1.76 times. In the dynamics of hypercholesterolemia and atherosclerosis in the blood serum, there are noticeable disturbances in the NO-ergic system.

***Keywords*:** *hypercholesterolemia, atherosclerosis, nitrate reductase, endothelin, endothelial dysfunction, NO-ergic system.*

Introduction

Endothelial dysfunction is an early stage in the development of atherosclerosis and is characterized by impaired endothelium-dependent vascular relaxation. It is known that the cause of endothelial dysfunction is a decrease in the biological activity of NO, the main mediator secreted by endothelial cells [1, 2].

It has now been established that the vascular endothelium plays an extremely important role in the activity of the cardiovascular system. The classical ideas about it as an anatomical barrier that prevents the penetration of blood into the walls of blood vessels have significantly expanded [3, 15]. It turned out that the vascular endothelium is a powerful metabolic system that supports vascular hemostasis by performing a number of important functions: modulating vascular tone, regulating the transport of dissolved substances into the cells of the vascular wall, the growth of these cells, the formation of an extracellular matrix, protecting vessels from the possible adverse effects of circulating blood cells and substances, regulation of chemotactic, proliferative, inflammatory and reparative processes in response to local damage [4, 5, 6]. These functions of the vascular endothelium are carried out by the synthesis and release of a number of biologically active compounds in response to mechanical and humoral stimuli. Vasodilator substances produced by the vascular endothelium include NO, prostacyclin (PGI_2), various hyperpolarizing factors and C-natriuretic peptide, vasoconstrictor substances include endothelin-1 (ET-1), angiotensin II, thromboxane A_2 and reactive oxygen species. Endothelial modulators of inflammation are NO, intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VSFM-1), E-selectin, and nuclear factor kappa B (NF-xB). Modulation of endothelial hemostasis is carried out by isolating such compounds as plasminogen activator, tissue factor inhibitor, von Willebrand factor, NO, PGI_2, TxA2, plasminogen activator inhibitor-1, and fibrinogen. The endothelium also takes an active part in the regulation of mitogenesis, angiogenesis, vascular wall permeability, and fluid balance [7].

It is known that the cause of endothelial dysfunction

is a decrease in the biological activity of NO, the main mediator secreted by endothelial cells. NO modulates a number of physiological processes in the body: it inhibits platelet adhesion and aggregation, proliferation and migration of vascular smooth muscle cells, plays a key role in the interaction of endothelial cells and circulating leukocytes, and also affects the permeability of endothelial cells to lipoproteins and other atherogenic macromolecules. NO is synthesized from L-arginine under the influence of 3 isoforms of NO synthase: 2 constitutive - endothelial (eNOS) and neuronal (nNOS), one inducible (macrophage, iNOS). They carry out the incorporation of molecular oxygen to the nitrogen atom in the terminal guanidine group of L-arginine [8].

eNOS is localized in the alveoli (lacuna-like microsites 50–1000 nm in size) of plasma membrane endothelial cells, where it is associated with caveolin. In this state, eNOS activity is sharply reduced. Under the influence of a number of receptor-dependent stimuli (acetylcholine, bradykinin, thrombin, ADP, glutamate, substance P, etc.), which increase the calcium concentration in endothelial cells, eNOS is released, it is activated by calcium-calmodulin, L-arginine is oxidized and a small amount is synthesized. (picomoles) NO. The formation of NO is also increased by receptor-independent agonists (Ca^{2+} - ionophores, Ca^{2+}-ATP), stretching of the vessel wall, displacement of blood relative to endothelial cells (the so-called shear stress) and some other factors [9].

Nitric oxide is a regulatory oxygen molecule involved in the regulation of tissue metabolism in normal and various pathological conditions. Today it has been proven that it is involved in the regulation of vascular tone, inhibits platelet aggregation and their adhesion to the walls of blood vessels. Nitric oxide plays the role of a mediator in the development of physiological and pathological processes in the body. Relaxation of vascular smooth muscle elements under the action of nitroglycerin is explained by the release of NO during the metabolism of this drug. In patients with myocardial infarction, an inverse correlation was found between the level of nitrites/nitrates and risk factors for the development of left ventricular failure and the severe clinical course of myocardial infarction [10].

Aim. Study of the molecular mechanisms of endothelial dysfunction with changes in the nitroergic system in experimental hypercholesterolemia.

Materials and methods

The experiments were carried out on 28 Chinchilla rabbits with an average weight of 2.5-3.0 kg, kept on a standard diet. The model of experimental hypercholesterolemia in experimental animals was reproduced using the Anichkov method. Experimental hypercholesterolemia was caused by oral administration of dissolved cholesterol in sunflower oil in the ratio of 0.2 g per 1 kg of body weight daily for 3 months.

After 2 months from the start of the experiment, the rabbits were divided into the following groups:

group 1 - intact (3 rabbits), which were injected with vegetable oil daily at a rate of 1.0 ml/kg through the oral cavity;

group 2 model of experimental hypercholesterolemia with water intake - control (5 rabbits);

group 3 model of experimental hypercholesterolemia with gemfibrazil 100 mg/kg (5 rabbits);

group 4 model of experimental hypercholesterolemia with the intake of chitosan derivative No. 1 at 25 µg/kg (5 rabbits);

group 5 model of experimental hypercholesterolemia with the intake of chitosan derivative No. 2 at 50 µg/kg (5 rabbits);

Group 6 model of experimental hypercholesterolemia with heparin at 15 units/kg (5 rabbits).

The action of drugs was studied in dynamics: the initial 3-month condition and after one month of drug administration. The results obtained were compared with those of the control and intact groups.

Study of endothelial dysfunction (level of endothelin) by enzyme immunoassay using ELIZA reagent [14].

The method for determining the level of NO by the sum of metabolites of nitrites and nitrates (NO_2 and NO_3) was carried out according to the method described by Golikov P.P. et al., modified by Metelskaya V.A. et al., 2.5% phosphoric acid (Sigma, USA) and incubated for 10 min at room temperature. The absorption value was measured at a wavelength of 546 nm on an SF-46 spectrophotometer (Russia). Sodium nitrite (NaNO2) was used as a standard [13].

where k is the calculated coefficient, and E is the extinction index of the sample.

Method for determining the activity of nitric oxide synthase (e NOS). To 0.2 ml of the sample was added a reaction system containing 0.1M Tris- HCl buffer (pH=7.4), which also included $CaCl_2$ (10mm), 0.3ml of an aqueous solution of arginine (substrate eNOS) at a concentration of 80 µm and 0.1 ml of 10 mm aqueous solution of $NADPH_2$. Incubation was carried out in a water bath at 37°C for 20 min. The reaction was stopped by introducing 0.02ml of a 0.02% aqueous solution of sodium azide (NaCN) into the

cuvette, and the decrease in extinction at 340nm was recorded on an SF-46 spectrophotometer (Russia). Control samples were prepared similarly, but instead of $NADPH_2$, 0.1 ml of distilled water was added [12].

Nitrate reductase activity was determined by the method of Vavilova T.P. and Petrovich Yu.A. To do this, 0.5ml of 5 10^{-2} phosphate buffer pH 6.5 containing 0.1ml of dithionite ($4.6 \cdot 10^{-3}$ Мв $95 \cdot 10^{-3}$ M $NaHCO_3$), 0.1ml 50mM NADPH, 0.1ml $NaNO_3$ ($1 \cdot 10^{-1}$M). The resulting mixture was incubated at 37°C in a water bath for 30 min. After incubation, the samples were vigorously shaken until complete discoloration, and the volume was adjusted to 2.0ml with distilled water. Then reagents for nitrites were added, including the Griess reagent [11].

The level of peroxynitrite ($ONOO^-$) was determined by the oxidation of hydroxylamine (NH_2O^-) formed peroxynitrite. The reaction was started by adding 0.2 ml of a 1.5% aqueous solution of hydroxylamine to 0.2 ml of the sample. 0.1 ml H_2O was added to a blank sample. The reaction was stopped after 10 minutes by adding 1.0 ml of a 4% ammonium molybdate solution. The intensity of the developed color was measured on an SF-46 at a wavelength of 410nm against a control sample.

The results obtained were compared with those of the control and intact groups. The digital material was processed by the method of variation statistics.

Results and discussion

The NO -ergic system plays the most significant role in the implementation of its functions by the vascular endothelium and the occurrence of its dysfunction. During the development of the pathology of the vascular system, intracellular signal transmission in the NO_x synthesis system with the participation of e NOS is disrupted. In the dynamics of experimental atherosclerosis, there is a significant decrease in the content of end products of nitric oxide, the severity of which corresponds to the progression of hypercholesterolemia. Thus, the content of nitric oxide, estimated by the amount of end products, on the 30th day of the experiment decreases by 1.29 times relative to the indices of intact rabbits. By the 60th day of cholesterol administration, the production of nitric oxide is even more inhibited, decreasing by 1.19 and 1.53 times relative to the values of the previous period and the values of intact rabbits. As the pathological process progresses, the content of nitric oxide decreases by 1.64 and 2.11 times, respectively, to the values of 30-day hypercholesterolemia and intact rabbits. Therefore, as experimental atherosclerosis progresses, endothelial nitric oxide production decreases.

Such changes in the level of nitric oxide in the blood serum may be due to inhibition of endothelial nitric oxide synthase. Indeed, the determination of eNOS activity showed its progressive decrease. So, if the activity of the enzyme on the 30th day of cholesterol administration decreased only 1.25 times, then on the 60th and 90th days of the experiment this decrease was 1.36 and 1.94 times, respectively, relative to the values of intact animals. In general, the shifts in the activity of the NOS enzyme that we have identified are consistent with shifts in the level of the product of the NO -ergic system, NO_x. At the same time, the lower the activity of the NOS enzyme, the lower the level of NO_x.

In contrast to the content of NO_x, in the blood serum of rabbits with hypercholesterolemia, an increase in the level of the product of the bioconversion of NO_x - $ONOO^-$. So, if the level of the latter on the 30th day of the introduction of exogenous cholesterol increases by 1.33 times relative to the values of intact rabbits, then on the 60th and 90th days of the experiment - by 1.93 and 2.47 times, respectively.

Consequently, the development of hypercholesterolemia and atherosclerosis is accompanied by an increase in the product of NO_x bioconversion - peroxynitrite ($ONOO^-$), the strongest oxidizing agent that has a negative effect on cellular structures.

Taking into account that under conditions of hypercholesterolemia the serum level of $ONOO^-$ is noticeably higher than in intact rabbits, the activity of another enzyme of the NO-ergic system involved in the bioconversion of nitric oxide - nitrate reductase. As can be seen from the above data, with hypercholesterolemia there are marked changes in activity and enzyme a NR. At the same time, if the activity of the enzyme nitrate reductase in the blood serum on the 30th day of the introduction of exogenous cholesterol increases only 1.15 times, then on the 60th and 90th days of the introduction - 1.3 and 1.76 times. Consequently, hypercholesterolemia and atherosclerosis are accompanied by activation of enzymes involved in the biotransformation of nitric oxide. The obtained data on the activity of the enzyme nitrate reductase are consistent with the data obtained in relation to the level in $ONOO^-$ in blood serum. At the same time, the higher the activity of nitrate reductase, the higher the level of peroxynitrite.

From the data obtained, it becomes obvious that with hypercholesterolemia and atherosclerosis, noticeable disturbances occur in the NO-ergic blood system. If

we take into account the fact that NO in the blood serum is primarily involved in the implementation of the mechanisms for maintaining the functional activity of the vascular endothelium, then the genesis of the development of hypertension in the studied pathologies becomes clear. This is confirmed by a decrease in the blood serum level of experimental animals NO_x due to the inhibition of the activity of the enzyme e NOS in them. And an increase in the level of peroxynitrite indicates the implementation of the negative role of the NO-ergic system and indicates the pathological role of these disorders in the genesis of the onset and progression of atherosclerosis.

Our analysis of the ratio of components of the NO-ergic system of blood serum in experimental animals confirms this assumption. The ratio of products of the NO-ergic system of blood serum NO_x and $ONOO^-$ is approximately 111.2: 1, that is, the normal level of NO_x is 111 times higher than the level of peroxynitrite. This ratio is disturbed in animals with hypercholesterolemia due to a noticeable increase in the specific gravity of peroxynitrite, a product of the bioconversion of nitric oxide. At the same time, the ratio of NO_x: $ONOO^-$ becomes lower compared to intact rabbits: 1.72; 2.96, and 5.22 times. An almost similar dynamic is observed in the ratio of the ratio of NOS: nitrate reductase enzymes. And here, the violation of the ratio NOS: nitrate reductase occurs due to the predominant increase in the activity of the enzyme nitrate reductase, which is involved in the transformations of nitric oxide.

To confirm the assumption that, in experimental hypercholesterolemia, disturbances in the NO-ergic system of blood serum are predominantly pathological in nature, and are not a compensatory process, we also analyzed the ratio NOS: NO_x and nitrate reductase: $ONOO^-$ according to the "substrate ratio enzyme" principle. NOS: NO_x ratio in the dynamics of hypercholesterolemia compared with the intact group does not differ significantly, but the ratio of nitrate reductase: $ONOO^-$ markedly impaired.

Consequently, in the dynamics of experimental hypercholesterolemia and atherosclerosis, the violation of the NOx: $ONOO^-$ and NOS: nitrate reductase ratios is primarily based on shifts in the nitrate reductase: $ONOO^-$ ratio, and not in the NOS:NO_x ratio, and $ONOO^-$ is a signaling molecule that performs cellular - destructive processes.

With the progression of hypercholesterolemia and hyperbetalipoproteinemia, the production of nitric oxide and the activity of its synthase in endotheliocytes are suppressed, the content of its active radicals progressively increases. This is also facilitated by an increase in the activity of nitrate reductase.

Conclusions

Experimental hypercholesterolemia is manifested by endothelial dysfunction. This is due to interrelated changes in the level of CRP, endothelin-1, and homocysteine, the severity of which depended on the duration of the experiment and the concentration of cholesterol in low-density lipoprotein, which leads to atherogenesis, disruption of the integrity of the vascular endothelium and its dysfunction. In the dynamics of hypercholesterolemia and atherosclerosis in the blood serum, there are noticeable disturbances in the NO -ergic system. These disorders are characterized by NO $_x$ deficiency due to low NOS activity, as well as the accumulation of peroxynitrite, a nitric oxide bioconversion product, due to an increase in nitrator reductase activity and, apparently, the failure of the antioxidant defense system. Undoubtedly, the definitions of nitric oxide in the blood serum are accompanied by defective functioning of mechanisms aimed at regulating the functional activity of not only the vascular endothelium but also blood cells, contributing to the launch of the corresponding endothelial mechanisms on the feedback principle, which negatively affects the course and outcome of the studied pathology. This circumstance requires taking into account the violations we have identified in the choice of strategy and tactics for the treatment of hypercholesterolemia and atherosclerosis.

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