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The Role of Nitric Oxide in the Changes of Blood Vessels Acetylcholine Induced Dilation in the Conditions of Hyperhomocysteinemia

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Abstract

The increase of Homocysteine in blood serum, according to some authors is considered to be a prognostic marker of lethal outcome. However, this issue is not fully clarified and requires further study. We considered it expedient to experimentally investigate the role of NO in the change in vasodilatory activity of Acetylcholine in the arterioles of animals with Hyperhomocysteinemia. To achieve this goal we found it necessary to study how the vasodilatation of normal and deendothelized segments of arterioles of control animals and animals with Hyperhomocysteinemia changed in response to Acetylcholine impact in the conditions of inhibition of Nitric Oxide Synthase (NOS) activity. The moderate Hyperhomocysteinemia was induced in male laboratory rats by addition of L-Methionine in drinking water. For inhibition of nonselelective NOS activity L-NAME has been used. Experiments were carried out on control and deendothelised preparations of first row arterioles of gracilis muscle of rats. Received results confirmed, that Hyperhomocysteinemia is associated with the decrease in dilation induced by Acetylcholine. Presumably, this change should be related with the disruption of arterial vasomotor reactions due to deficiency of NO and appears to be an important earlier stage in the development of vascular diseases related to Hyperhomocysteinemia.

Key words

Hyperhomocysteinemia, Acetylcholine, Nitric Oxide, Arterial preparation, L-Methionine, L-NAME.

1. Introduction

Over the years the diagnostics of cardiac ischemic diseases was based on th e determination of lipid metabolism and the indices of hemostasis. Then some data have been appeared in the literature that the development of atherosclerotic and thrombotic processes in the myocardium is due to “new risk-factor” – the increase of Homocysteine in blood serum, which according to some authors is considered to be a prognostic marker of lethal outcome. However, this issue is not fully clarified and requires further study.

Today it has already been established that a significant congenital defect in Homocysteine metabolism evokes a cardiovascular coronary damage in patients. But despite there are many *in vitro* and *in vivo* researches [3], regarding this problem, the mechanism of this damage development is still unknown.

Based on the above and taking into account a significant role of Nitric Oxide (NO) in the vasomotorics of blood vessels, we considered it expedient to experimentally investigate the role of NO in the change in vasodilatory activity of acetylcholine in the arterioles of animals with Homocysteinemia.

To achieve this goal we found it necessary to study how the vasodilatation of normal and deendothelized segments of arterioles of control animals and animals with Hyperhomocysteinemia changed in response to Acetylcholine impact in the conditions of inhibition of Nitric Oxide Synthase (NOS).

2. Methodical approach

The induction of moderate Hyperhomocysteinemia was performed in male laboratory rats (weighing 120-160 g, n=12) by addition of L-Methionine in drinking water (daily 1g/L) within 4 weeks. The amount of daily consumed water for each animal was defined by the volume of average consumed water in norm. The

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control animals (n=12) usually received water *ad libidum*. At the beginning of a four-week period and its end the animal's mass was determined. Blood sample was taken from the hip artery, which was centrifuged during 20 minutes at 3000g (at the temperature of 4°C). The isolated serum before the analysis was stored at -20°C. A total content of Homocysteine was determined by the use of chromatographic method by means of fluorometric determination.

One of the objective methods for the analysis of blood vessel smooth muscles appeared to be the measurement of constriction parameters of blood vessels isolated preparations by means of mechanotronic converters [1]. This method makes it possible to measure a degree of increase or decrease in the tone of blood vessels in conditions of different impacts. As a result of such a methodical approach it is possible to analyze some mechanisms of smooth muscle regulation without the interference of centrogenic neurohumoral signals in it. It also gives the experimenter the inexhaustible means for studying the effect of sequential or combined action of various biologically active substances on smooth muscle reactivity.

The experiments were carried out on first row arterioles of gracilis muscle of rats (diameter 130-180 mkm). At the end of 4th week after receiving L-Methionine under condition of Pentobarbital Sodium (50 mg/kg) a Systemic Arterial Pressure of a rat was measured and a blood sample was taken for the measurement of Homocysteine content. Then the isolation of gracilis muscle from surrounding tissues was performed. The mentioned muscle was cut and placed into the Ringer-Heilit solution at the temperature of 0-4°C. In binocular microscope a 1.5-2 mm length segment of intramuscular arteriole of the first row was isolated, which by means of a special auxiliary instrument was placed in a small basin of Ringer-Heilit flow solution chamber. In this basin the preparation was placed on two small hooks of tensometric mechanotron (Fig. 1). One hook was rigidly fixed to the rod of 6 MXIC type mechanotron**.** The preparation was stretched and a constant stretching value was chosen according to the results of testing of arterial smooth muscle compression. The testing was performed using standard solutions, containing potassium in the concentration of 80mol and was an average of 5.1mN. Before the measurement for reaching equilibrium condition the preparation stayed in Ringer Solution for 1.5 hours at the temperature of 37°C.

The electric signal received from mechanotron was transmitted to amplifiers. The calibration of mechanotron was performed in milli Newtons (mN). For this the horizontal rod was loaded by standard small weights and the deviation from an initial level was recorded. The value of preparation stretch is usually normalized according to the maximum contractile response (100%) of the preparation to the action of hyperpotassium (800 mMol/L) solution.

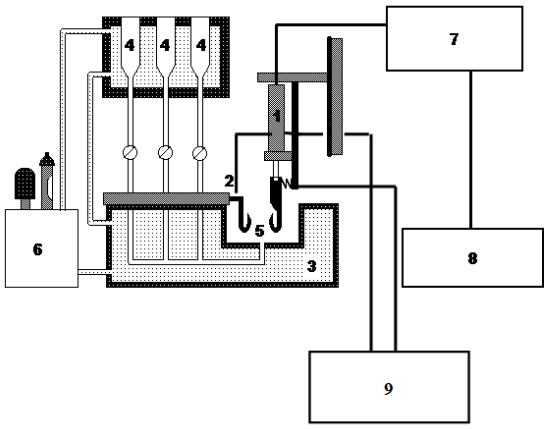

Figure 1. The block-scheme of the device: 1 – mechanotron; 2 – the mechanism of stretching and calibration; 3 – thermostated chamber; 4 – the flasks with Krebs solution; 5 – the working chamber; 6 – ultrathermostat; 7 – the block of amplifiers; 8 – recorder; 9 – electric stimulator.

The preparation of solutions, control of pH and temperature

The Ringer-Heilit flow solution was used as a nutrient solution, the content of which was the following (mmol/L): NaCl – 118.0; KCl – 4.7; $NaHCO_3$ – 14.9; KH_2PO_4 – 1.18; $MgSO_4.7H_20$ – 1.17; $CaCl_2;2H_2O$ – 2.5; Glucose – 11.0.

The experiments were carried out under pH control, the measurement of which was directly performed before each impact by means of pH-meter (or ionometer). Solution pH change was allowed within 7.35-7.45.

During the experiment the permanence of solution temperature was performed by means of ultrathermostat at 37±0.5°C level, which pumped the warmed water into special flasks with water and temperature-controlled chamber, which were united in a common, continuous, flowing system (Fig. 1).

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The protocol of experimens

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, Bethesda, 1985) and after receiving the approval from the Ethics Committee of I. Beritashvili Center for Experimental Biomedicine.

Dilated reactions were studied on normal and deendothelized segments of arterioles. By means of special instruments made from soft (lime) tree, the removal of endothelial layer from the preparation was performed, the perfectness of it was examined on endothelium-dependent (Acetylcholine, 10^{-6} M) and endothelium-independent (Sodium Nitroprusside – 10^{-6} M) dilated agents using reaction test.

Peak-reaction of the arterioles of control animals and animals with Hyperhomocysteinemia was studied in the solution surrounding the segments at cumulative dose of Acetylcholine (10^{-10} – 10^{-5} Mol/L) and Sodium Nitroprusside (10^{-10} – 10^{-5} mMol/L). Then during 30 minutes the segments were incubated with L-NAME (10^{-4} Mol/L) solution (nonselective inhibitor of NOS) and the segment reaction to the injection of above doses of Acetylcholine and Sodium Nitroprusside was again measured.

All the above-said substances were administered in flowing solution chamber (Fig. 1), were the target arteriole segment was placed. After the recorded reaction a pure solution of Ringer-Heilit was administered into the system.

Statistical analysis of obtained results

The results obtained were expressed by their mean values and standard error. The statistical analysis was conducted by the use of ANOVA package. The statistical significance was examined by Student's t-test. $P<0.05$ was considered to be statistically significant.

3. The results

The reaction of arterioles to acetylcholine

The indices of control animals and animals on the diet of L-Methionine at the end of 4th week are given in the Table below.

Table 1

The indices	Control animals (n=12)	Animals on the diet of L-Methionine (n=12)
Homocysteine (mcMol/L)	7.3±0.5	22.2±2.6*
Body mass (g)	341±12	349±14
Systemic arterial pressure (mm Hg)	95±4	99±6

***as compared to control the difference is statistically reliable, P<0.01.**

Acetylcholine (10^{-9} – 10^{-6}Mol/L) induced a dose-dependent dilation of arteriole, which was significantly more expressed in blood vessels of control animals, than in arteriole of Hyperhomocysteinemia group animals (Fig.2).

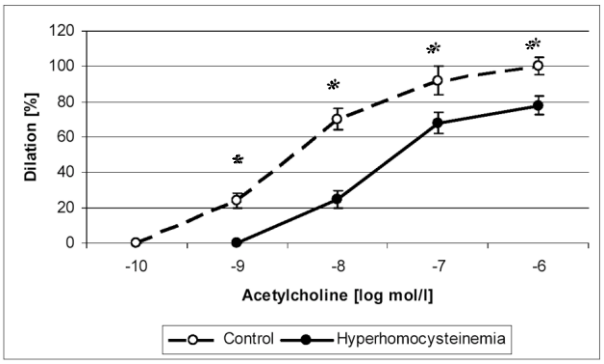

Figure 2. The effect of cumulative dose of Acetylcholine on dilation of isolated arterioles of control and Hyperhomocysteinemia-induced animals. M±SE – are presented. * – statistically significant difference with control data (P<0.05), n=8.

After 30 min lavage of blood vessel with Ringer-Heilit solution at the background of the administration of L-NAME – a nonselective inhibitor of Nitric Oxide Synthases the conduction of the same test induced a significant decrease in dilation degree of arteriole in control animals (Fig. 3), while acetylcholine dilation remained unchangable in Hyperhomocysteinemia group of animals (Fig. 4).

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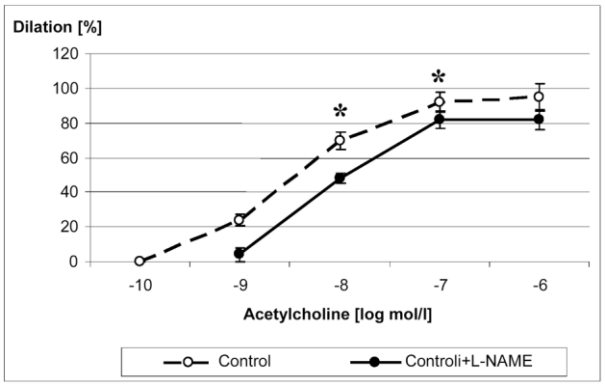

Figure 3. The effect of cumulative dose of Acetylcholine on dilation of isolated arterioles in control animals against the background of L-NAME – nonselective inhibitor of NOS and without it. M±SE are presented. * – statistically significant difference with control data (P<0.05), n=6.

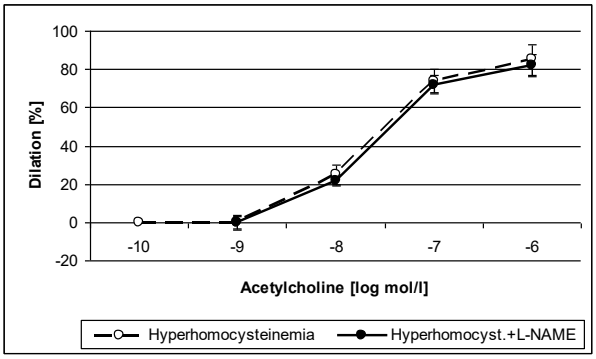

Figure 4. The effect of cumulative dose of Acetylcholine on dilation of isolated arterioles in animals with Hyperhomocysteinemia against the background of L-NAME and without it. M±SE are presented. * – statistically significant difference with control data (P<0.05), n=6.

The reaction of arterioles to Sodium Nitroprusside

Sodium Nitroprusside induced the similar dose-dependent dilation of arterioles in both control and Hyperhomocysteinemia group animals. The nonselective inhibitor of NOS did not actually change either the quality or the character of the arterioles dilation (Fig. 5) isolated from both control animals and animals with Hyperhomocysteinemia.

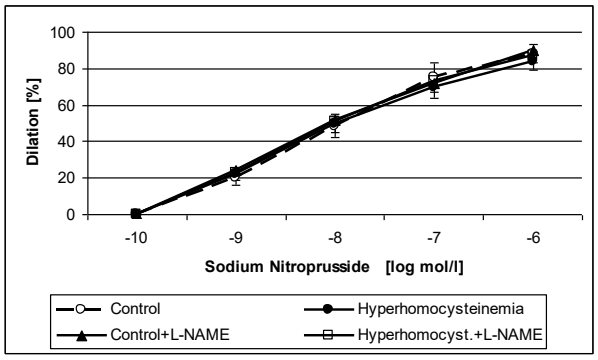

Figure 5. The effect of Sodium Nitroprusside on dilation of isolated arterioles of animals with Hyperhomocysteinemia on the background of L-NAME and without it. The differences are statistically nonsignificant; (n=8).

4. Discussion

Proceeding from the data published in literature [4], the increase of homocysrteine in plasma appears to be an independent risk-factor of atherothrombotic disease. Generally, a moderate Hyperhomocysteinemia is rather spread among the population and is characterized of about 30% of the population, who suffers from coronary, cerebrovascular and atherothrombotic disease [4]. As a rule, the reason of a high concentration of Homocysteine in plasma appears to be the insufficiency of the vitamins necesary for Homocysteine metabolism At the same time, it should be taken into account that in elderly people, pregnant women, nicotine consumers, alcoholics and hormonal contraceptive consumers the need for folic acid is increased.

There are several considerations how it is possible that increased concentration of Homocysteine in blood plasma should induce atherotrombotic diseases in cardiovascular system. In particular, it is believed that a damaged effect of Hyperhomocysteinemia may be expressed by the change in the morphology of blood vessels wall, increased activity of thrombocytes and in the stimulatioin of cell proliferation in smooth muscles [5] In some experiments carried out using histological methods the morphological changes in human and animal blood vessels endothelium induced by Hyperhomocysteinemia were revealed. There are some other studies where during diet-induced hypoerHomo-cysteinemia the functrional changes in the endothelium have been observed, for example, the reduction of endothelium-dependent relaxation of common carotid

arteries or the reduction of increased acetylcholine-induced circulation of limbs [4, 8]. According to these investigations, one of the reasons inducing the above-said changes is the fact that a high concentration Homocysteine induces the disorder of the function of vascular endothelium, although the impact of increased concentration of Homocysteine on the function of micro blood vessels is not established.

A number of experimental models were developed to study the intimate mechanisms of damage impact of Hyperhomocysteinemia on the cardiovascular system [5].

In our investigation in drinking water of the animals Methionine (precursor of Homocysteine) was added. As a result of it the concentration of Homocysteine in plasma increased almost threefold and reached the level, which in humans was connected to a high risk of blood vessels disease.

Generally, the increase of Methionine (or any other amino acid) in plasma concentration is not associated with a harmful impact on the blood vessels. But there are quite a lot of evidences that an increased concentration of Homocysteine appears to be a significant reason for the injury of blood vessels endothelium. In a number of investigations it has been shown that some risk-factors, which induce endothelial dysfunction, should not be considered as a valid cause of atherosclerosis development [6].

As the circulation of skeleton muscle appears to be a main component of hemodynamic impedance in peripheral circulation, its disorder during Hyperhomocysteinemia may be considered as a significant component in the development of peripheral vascular disease. For this purpose, we have studied the changes in endothelium vasoregulatory function in blood vessels of normal and Methionine-diet induced Hyperhomocysteinemia animals.

The tests of arterioles reaction to acetylcholine has been conducted, which, as is known, induces the generation of Nitric Oxide. As was shown the vasodilation of blood vessels skeleton muscle in control animals decreased, as compared with the arteriole reaction of Hyperhomocysteinemia animals. Moreover, the inhibition of NO production with L-NAME decreased acetylcholine-induced dilation of isolated arteriole only in control animals, while in the animals with Hyperhomocysteinemia the administration of L-NAME did not reveal any significant effect.

As it turned out, in Hyperhomocysteinemia conditions the sensitivity of blood vessels smooth muscles to NO did not change, as the reaction of arteriole to NO was not changed at the administration of its donor sodium nitroprusside. This result corroborates that during Hyperhomocysteinemia only the disorder of bioresource of endothelial NO takes place.

Earlier it has been shown that the process of release of endothelium-dependent smooth muscles relaxation factor (or that is the same nitrogen oxide) disturbs at Hyperhomocysteinemia [11]. Our results agree with these data and extend them at arterioles level.

Nitric Oxide synthase dysfunction causes the change in the action of endothelium-dependent dilators on those small-caliber crown arteries and arterioles, which do not suffer from atherosclerosis disorders [15]. At the same time it is known that the increase of Homocysteine concentration in plasma is accompanied by the increase of triglycerides [10]. All this should be added that hypertension – a known risk factor for atherosclerosis development also worsens the condition of the disordered endothelial function in arterioles [9].

The summarization of the above gives us an opportunity to assume that endothelium dysfunction of blood vessels appears one of the earlier components of those complex phenomena, which take place in the process of blood vessels disease development.

As Nitric Oxide along with vasodilatatory function has a significant role in the implementation of anticoagulation and antithrombotic reactions, the endothelium dysfunction should be considered as an important connecting link between Hyperhomocysteinemia and atherothrombic diseases.

It should necessary be taken into account that in Hyperhomocysteinemia conditions a deficit of Nitric Oxide may become the cause of proliferation of smooth muscles, while in turn, endothelial dysfunction promotes adhesion of leukocytes and platelets on blood vessels walls.

The analysis of literary data gives us the opportunity to consider the formation of excessive reactive metabolites of oxygen as one of the causes of violation of release or action of Nitric Oxide [2].

It has been shown that at auto-oxidation of sulfhydryl groups of biological thiols, a hydrogen peroxide is generated [13]. As is known, Methionine has no groups of free thiols and therefore Hyperhomocysteinemia has a leading role in the development of above-said endothelial disorders. The produced excess amount of free oxygen radicals reacts with Nitric Oxide and at the expense of peroxinitrate

production reduces its resources. Also it should be mentioned that Homocysteine reduces the amount of intracellular glutathione and glutathione peroxidase, on which the elimination of oxygen free radicals depends [7]. Homocysteine toxic effect on endothelial cell cultures due to the free radicals has been described as early as in 1980 [14]. The main thing was that the endothelial damage induced by Hyperhomocysteinemia had an irreversible character, as after using the scavengers of oxygen free radicals it was not possible to recover the reaction of arterioles due to the NO [12].

5. Conclusion

The mechanisms of endothelial damage induced by Hyperhomocysteinemia undoubtedly need further research, but our experimental material allows us to conclude that micro blood vessels suffer from a significant influence of Hyperhomocysteinemia (which accordingly is reflected on tissue blood supply).

Thus, the increase of Homocysteine concentration due to the diet, shown in our experiments, is associated with the decrease in dilation induced by acetylcholine. Presumably, this change should be related with the disruption of arterial vasomotor reactions due to NO and appears to be an important earlier stage in the development of vascular diseases related to Hyperhomocysteinemia.

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