The Significance of Nitric Oxide as A Signaling Molecule In Regulation of Subclinical Inflammation and Apoptosis in Hypertensive Elderly Patients

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Abstract: Nitric oxide (NO) is a universal regulator of different processes, including vascular tone, hemostasis and apoptosis. One of the important functions of NO as a signaling molecule is determined by a high capacity for diffusion through the cell membrane to synthesize it and penetrate into target cells by controlling their metabolism and realizing intercellular interactions.

For the purpose of estimation in elderly persons due to for determine the effect of nitric oxide and its products on the process of subclinical inflammation and apoptosis of endothelial cells in the development of endothelial dysfunction as a target organ in arterial hypertension 66 patients of elderly age with arterial hypertension (AH) of II stage and 24 persons of similar age without cardiovascular diseases were examined. The degree of endothelial dysfunction was examined by dopplerography of brachial artery, the endotheliocytemia level was estimated by the Hladovec and Rossmann method, the concentration of nitric oxide - in the Griess reaction, the levels of C-reactive protein, primary inflammatory mediator of TNF-α, apoptosis markers and nitrotyrosine - were determined by the immune enzyme method.

It has been established that the synthesis of basal nitric oxide depends on the activity of endothelial constitutive NO - synthase and maintains the tone of the vessel in a state of light relaxation. In the progression of endothelial dysfunction (ED) a number of phases were identified: a compensation phase with increased secretory activity of endothelial cells, an intermediate phase when the balance is disrupted due to changes in the secretion process of production and inactivation of endothelial factors and a decompensation phase as a result of structural and metabolic disorders of endothelium resulting in its functional failure, death and desquamation. Regulatory effect of NO on subclinical inflammation and apoptosis intensity was confirmed by its strong inverse correlation with the level of TNF-α and caspase-3. It proved the change in the NO concentration, its auto- and paracrine effect on the formation of involutive changes in the vascular wall, its regulatory effect on the processes of subclinical inflammation and apoptosis of endothelial cells in the formation of hypertension, as well as its progression during aging.

Keywords: Ageing, arterial hypertension, endothelial dysfunction, nitric oxide, subclinical inflammation, apoptosis.

1. INTRODUCTION

Nitric oxide (NO) performs the role of a universal regulator of numerous processes including vascular tone, hemostasis, apoptosis and others. The polyfunctionality of its effects may lead to opposite results including the activation or inhibition of oxidation-reduction reactions, vasodilation or vasoconstriction, the induction of apoptotic cell death or anti-apoptotic and pro-inflammatory influence. One of the important functions of NO as a signaling molecule is determined by a high capacity for diffusion through the cell membrane to synthesize it and penetrate into target cells by controlling their metabolism, realizing intercellular interactions, so acting as a neuro-transmitter.

Changes in the production of nitric oxide and its metabolites play an important role in the modulation of oxidative and nitrosative stress, subclinical inflammation, cell death, the desquamation of endothelial cells, the development of endothelial dysfunction (ED) underlying the heart – vascular continuum [1].

Despite the fact that the first role of NO has been set in the development of cardiovascular disease most of today's ideas about the pathogenetic mechanisms of damage to the heart and blood vessels often do not attach due importance to the fact of participation of nitric oxide metabolites that serve as signaling molecules. Endothelial cells contain a certain amount of nitric oxide continually expanding its metabolic cycle. NO production is induced by three NO-synthases (NOS) [2]: the two constitutive ones – endothelial and neuronal operating under normal conditions in intact vascular wall and the third one – inducible, contained in the plasma membrane of endothelial cells and macrophages and activated only under the influence of the damage effects.

The synthesis of basal level of nitric oxide in physiological conditions depends on the activity of endothelial constitutive NO – synthase and maintains the tone of the vessel in a state of light relaxation [3].

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Age-related development of oxidative stress is associated with the activation of inducible NO – synthase. In pathological processes the synthesis of NO increases by 1000 times. The aging of the vascular endothelium is a phase process, having different effects on NO signaling [4].

Endothelial dysfunction in chronic exposure to damaging factors develops gradually. In the progression of ED identified a number of phases: a compensation phase with increased secretory activity of endothelial cells, an intermediate phase when the balance is disrupted due to changes in the secretion process of production and inactivation of endothelial factors and a decompensation phase as a result of structural and metabolic disorders of endothelium resulting in its functional failure, death and desquamation, inhibition of regeneration [4]. The researchers determine the effect of nitric oxide and its active metabolites in the expression of endothelial cell apoptosis [5].

Despite the huge amount of scientific research on the assessment of changes in NO against the background of various diseases, its regulatory role in the mechanisms of subclinical inflammation and apoptosis in combination of hypertension and involutive changes of the vascular wall remains underexplored.

2. THE PURPOSE OF RESEARCH

The purpose of research is to determine the effect of nitric oxide and the products of its peroxidation on the process of subclinical inflammation and apoptosis of endothelial cells in the development of endothelial dysfunction as a target organ in arterial hypertension of elderly patients.

The study involved 66 patients with stage II hypertension (mean age 66.3 ± 0.5 years, mostly women). The average systolic blood pressure (SBP) of the patients was - 163.3 ± 6.5mm Hg., diastolic BP - 93.5 ± 5.8mm Hg., pulse pressure - 71 ± 4.1mm Hg. The comparison group included 24 patients of similar age without heart disease. Before including in the study patients signed an informed consent to participate in it.

3. MATERIALS AND METHODS

The diagnosis of hypertension and its degree were established on the basis of their diagnostic criteria according to the recommendations of the European Society of Hypertension and the European Society of Cardiology (2013). The degree of endothelial dysfunction was determined by the level of changes in vessel diameter in reactive hyperemia during dopplerography of brachial artery using a 7.5MHz linear transducer ultrasound machine «LOGJQ 7" (Japan). Measurements were carried out by the straight-line method proposed DS Celermajer and colleagues (1992). The concentration of nitrites in blood serum was investigated by Griess reaction.

Determination of the activity nitrotyrosine was performed by ELISA using a set of Hycult biotech (Netherlands). Its principle is based on a solid phase "sandwich" immunoassay method.

Endotheliocytemia level in blood was determined by the Hladovec and Rossmann method (1973) [6]. The principle of this method is based on isolation of endothelial cells along with platelets followed by precipitation of adenosine dyphosphate. The counting the number of endothelial cells was carried out in two grids of Goryaev's chamber. Contents of subclinical inflammation marker - C - reactive protein (CRP) was assessed by a highly sensitive immunoturbidimetric method using automatic biochemical analyzer "Vitalab Flexor E" (The Netherlands) using the set of reagents «Byo-Systems» (Spain) at a wavelength of 340nm. The content of primary inflammatory mediator of TNF-α was determined by ELISA using a monoclonal antibody to TNF-α.

The activity of caspase-3 in serum was evaluated by ELISA using an enzyme-linked immunosorbent analyzer Human Caspase-3 of Bender MedSystems GmbH (Austria).

4. STATISTICAL ANALYSIS

Data are expressed as means ± standard errors. Statistical analysis was performed using parametric methods, the significance of differences was assessed by Student's t - test, the strength and direction of correlations between parameters were estimated by Pearson.

5. RESULTS AND DISCUSSION

Aging gradually leads to the development of functional changes of vascular tone and subsequently the structure of the vascular wall. Endothelial NOS provides in physiological conditions a sufficient synthesis of the basal level of nitric oxide to maintain vascular homeostasis and the regulation of blood

pressure. Research of the endothelial– dependent vasodilation (EDVD) allows to estimate the functional state of the brachial artery wall Lyons D. *et al.* (1997) [7]. Found that endothelial vasotonic disorders were revealed in physiologically aging people without cardiovascular disease. The data of levels NO and markers of subclinical inflammation and apoptosis associated with the formation of ED at physiological aging are shown in Table **1**.

Table 1: Indicators of Endothelium-dependent Vasodilation, NO, Markers of Inflammation, Apoptosis and Endotheliocytemia in Healthy Elderly People

The Degree of ED	0 n=10	1 n=4	2 n=10
	1	2	3
EDVD, %	24.3 ± 1.7	9.2 ± 0.8 P_{1-2}<0.01	6.8 ± 0.6 P_{1-3}<0.001
NO, mkmol/l	4.83 ± 0.18	4.44 ± 0.1	4.32 ± 0.18
Hs C-reactive protein, g/l	0.52 ± 0.06	0.78 ± 0.07	0.94 ± 0.09 p_{1-3}=0.0007
TNF-alpha, pg/ml	3.8 ± 0.5	4.2 ± 0.7	4.7 ± 0.8
Caspase-3, ng/ml	4.9 ± 0.8	6.7 ± 1.1	7.8 ± 1.8
The level of endotheliocytemia, 10^9cells /l	0.4 ± 0.02	0.58 ± 0.02	0.74 ± 0.03

Vasotonic mild dysfunction of the vascular wall (ED 1-2degree) was detected in 58.3% of elderly people with normal blood pressure. Signs of ED 1 degree were found in 4 (16.7%), 2 degree - in 10 people (41.7%). The remaining 10 patients (41.7%) had no signs of vasotonic endothelial dysfunction.

In elderly patients without hypertension we observed a gradual decline in production of NO to 4.44 ± 0.1mmol/ l at the I degree of ED and up to 4.32 ± 0.18mmol/ l at the II degree of ED.

Violations of endothelial-dependent vasodilation in healthy aging individuals without hypertension indicate their involutive character, as evidenced by the weakening of the brachial artery constriction after the injection of L-NMMA - basal production inhibitor NO [8].

Aging patients with mild forms of ED should be included in the risk group for cardiovascular disease, since endothelial dysfunction is considered as one of the earliest and initial stage lesions of the vascular wall as a target organ, that allows to recognize it as a reasonable call to early diagnostics and prevention of vasotonic disorders [9].

The concentration of the subclinical inflammation markers did not go beyond the range typical for healthy middle-aged people. Endotheliocytemia level of healthy elderly men was low and served as an indicator of intensity in the elimination process of dead cells as a result of physiological apoptosis.

Table 2: Indicators of Endothelium-dependent Vasodilation, NO, Markers of Inflammation, Apoptosis and Endotheliocytemia in Elderly Hypertensive Patients

The Degree of ED	0 n=22	1 n=16	2 n=20	3 n=4	4 n=4
	1	2	3	4	5
EDVD, %	14.6 ± 2.1	8.7 ± 0.6 P_{1-2}=0.025	6.2 ± 0.4 P_{1-3}=0.001	2.3 ± 0.6 P_{3-4}=0.001	1.5 ± 0.4
NO, mkmol/l	4.8 ± 0.2	4.9 ± 0.25	4.2 ± 0.3	4.0 ± 0.2	5.9 ± 0.25 p_{1-5}=0.032
Hs C-reactive protein, g/l	0.85 ± 0.1	1.0 ± 0.1	1.4 ± 0.1 p_{1-3}=0.001	1.5 ± 0.1 p_{1-4}=0.01	1.7 ± 0.2 p_{1-4}=0.002
TNF-alpha, pg/ml	4.9 ± 0.7	5.1 ± 0.7	6.1 ± 0.8	7.6 ± 0.9	9.3 ± 1.1
Caspase-3, ng/ml	5.6 ± 0.8	7.2 ± 1.1	8.1 ± 1.3	9.6 ± 1.4	10.5 ± 1.4 p_{1-5}=0.02
The level of endotheliocytemia, 10^9cells/l	1.2 ± 0.02	1.33 ± 0.02 p_{1-2}=0.001	1.5 ± 0.02 p_{1-3}=0.0003 p_{2-3}=0.001	1.65 ± 0.03 p_{1-4}=0.0001 p_{2-4}=0.0001	1.78 ± 0.05 p_{1-5}=0.0001 p_{2-5}=0.0001 p_{3-5}=0.001

Chronic hypertension is a widespread pathological process, its duration and severity have a direct impact on the nature of disorders of vascular endothelium. The results of the study of EDVD parameters, the levels of NO, the inflammation and apoptosis markers, endotheliocytemia in elderly hypertensive patients are given in Table **2**.

The symptoms of endothelial dysfunction in patients with hypertension were more pronounced and identified in 66.7% cases. The signs of ED 1 degree were revealed in 16 (24.2%) persons; grade 2–in 20 patients (30.3%), grade 3–in 4 (6.1%) and grade 4–in 4 persons (6.1%).

The revealed changes of NO concentration in patients with hypertension were different. In patients with ED I degree against the background of hypertension there was a slight increase in its level in the blood to 4.9 ± 0.25mmol/ l, probably due to the activation of inducible NO-synthase, that can be regarded as a compensatory response of the vascular wall despite its structural damages caused by the combination of involutive changes and chronic hypertension. Further progression of ED has led to decrease of adaptive responses and the gradual reduction of NO synthesis to 4.2 ± 0.3mmol/ l at II degree and up to 4.0 ± 0.2mmol/l at III degree of ED ($p<0.05$).

The maximum rise in the concentration of NO - 5.9 ± 0.25mmol/l noted in elderly hypertensive patients with ED 4 degree is no reason to consider it as compensatory. It can be caused by nitrogen oxides in the bloodstream as a result of release from dead cells of the endothelium. In addition, increasing the concentration of nitric oxide in the blood enhances nitrosylation reactions with the formation of peroxynitrite – one of the strongest cellular poisons for endothelial cells [10]. The study in hypertensive patients with high degrees of ED has revealed a significant increase in the level of nitrotyrosine – 2.42 ± 0.2nM compared to the same indicator of healthy elderly people – 1.8 ± 0.1($p = 0.04$).

Excessive entrance of toxic products of nitric oxide into the blood stream was accompanied by a significant activation of inflammatory responses and was confirmed by high concentrations of its markers – Hs CRP – 1.7 ± 0.2 g/l and TNF-alpha – 9.3 ± 1.1pg/ml ($p<0.01$). The dynamics of NO negatively correlated with changes in TNF-alpha ($r = – 0.51$, $p<0.01$) suppressing the activity of the latter.

The process of apoptotic death of endothelial cells in patients with hypertension and 4th degree of ED was significantly accelerated. The values of serum concentration of its integrated marker - caspase -3-10.5 ± 1.4ng/ml was significantly higher than the level of same parameter -4.9 ± 0.8ng/ml in healthy individuals ($p<0.05$). The regulatory effect of NO on apoptosis intensity was confirmed by its strong inverse correlation with the level of caspase-3 ($r = - 0.85$, $p<0.001$), which in turn is directly correlated with the marker of subclinical inflammation - Hs CRP ($r = 0.35$, $p<0.05$).

The apoptotic death of endothelial cells was verified by increased values of desquamation with a high degree of endothelial dysfunction – 1.78 ± 0.05×10^9 cells/ l ($p<0.001$).

The modulating influence of nitrosative stress on the intensity of apoptosis of endothelial cells was confirmed by a direct correlation between the product of nitrite peroxidation – nitrotyrosine and the level of caspase-3 ($r = 0.36$, $p<0.01$) and endoteliocytemia index ($r = 0.38$, $p<0.01$). These facts are further evidence of the negative role of the toxic derivatives of NO in the vascular intimal cells lesion.

CONCLUSION

The study has demonstrated the signal role of nitric oxide metabolites and products of its peroxidation in the regulation of vascular tone during aging. It proved the change in NO concentration, its auto- and paracrine effect on the formation of involutive changes in the vascular wall and its regulatory effect on the processes of subclinical inflammation and apoptosis of endothelial cells in the formation of hypertension, and the induction of its progression during aging.

CONFLICT OF INTEREST

The authors have no financial interests or any conflicts related to the material in the manuscript.

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