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PATHOGENETIC FEATURES OF THE NITRIC OXIDE SYSTEM STATE IN THE LEFT VENTRICULAR MYOCARDIUM OF THE RATS WITH EXPERIMENTAL ARTERIAL HYPERTENSION

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Abstract

The aim was to determine the pathogenetic features of the NO system in the myocardium of the left ventricle in the rats with endocrine-salt hypertension.

Material and methods. The experiment was conducted on 20 male rats 220-290 g weight, 6–10 months old which were divided into 2 experimental groups: 1st – the control group (10 intact normotensive male Wistar rats); 2nd – 10 male Wistar rats with endocrine-salt arterial hypertension. Systolic and diastolic BP levels were measured in all the rats using a system of non-invasive arterial pressure measurement BP-2000. The objects of study in the experimental animals were blood plasma in which the nitrotyrosine level was measured, and

the fragment of left ventricle, which was divided into two parts, one of which was homogenized using a Silent Crusher S homogenizer (Heidolph, Germany), in which nitrites level was determined. Concentration of immunoreactive material to NOS isoforms was detected with immunofluorescence method. The study of NOS mRNA isoforms expression in the left ventricular myocardium homogenates was carried out using a real-time polymerase chain reaction

Results. In the experiment, an increase in all 3 isoforms and their mRNA was obtained. In the study, a decrease in nitrites concentration and a significant increase in nitrotyrosine levels, indicates the development of nitroso-oxidative stress.

Conclusions. The development of experimental endocrine-salt hypertension in the Wistar rats leads to a stable increase in mean blood pressure by 65 % compared to control. Endocrine-salt arterial hypertension in Wistar rats is characterized by an increased mRNA content of all three isoforms of nNOS, eNOS and iNOS by 2.7, 2.8 and 5.7 times, respectively, compared to the control; increased expression of immunoreactive material to isoforms in transverse fibers by 14.3 %, 16.2 % and 18.5 %, respectively; in longitudinal fibers IRM to nNOS was higher by 8.3 %, to iNOS - by 8. 5%, but to eNOS it was lower by 7.6 %. At the same time, nitrites level decreased by 11.7 % and nitrotyrosine concentration was significantly higher, exceeding the control value by 88.5 %.

Key words: nitric oxide synthase; NOS isoforms; myocardium; left ventricle; heart; Wistar rats; endocrine-salt arterial hypertension; secondary hypertension.

Introduction

According to the WHO, every third adult in the world suffers from arterial hypertension (AH). Secondary hypertension accounts for 5-10 % of all identified causes of persistent hypertension, among them hypercorticism and hyperaldosteronism lead to the development of AH in 70-85 % of patients over 40 years of age [1]. It should be noted that in patients with hypercorticism combination of high cortisol levels with increased secretion of mineralocorticoids is observed.

The pathogenesis of persistent hypertension caused by steroid hyperproduction is primarily related to their effects on glucocorticoid (GRs) and mineralocorticoid renal receptors (MRs), that leads to increased sodium reabsorption, water retention and potassium excretion. Cortisol usually interacts with the MRs receptor as an antagonist in the sense that it binds to it but does not activate it. Under conditions of pathology, especially in the case of superoxide formation, it becomes an agonist of MRs, mimicking the action of aldosterone [2].

A change in the effect may occur due to a defect in 11-beta-hydroxysteroid dehydrogenase, NADH deficiency and c-terminal-binding protein, the role of which is to maintain cortisone in the receptor inactive state [3]. Sodium intake levels, which influences the severity of the pathogenetic relationships in cardiovascular disease are also important for the development of hypertension [4].

Such a multifactorial pathogenesis of this form of secondary hypertension is a chronic stress and a factor of significant overload of the cardiovascular system, which alters its functional state and structure. Left ventricular myocardium is of particular scientific interest because it is the first to change its geometry in conditions of long-term increase of blood pressure. This change is termed as pathological myocardial remodeling (PRM) [5]. In the case of endocrine hypertension, PRM is not only the result of pressure and volume overload, but also the direct damaging effects of steroids, which cause dystrophic changes in cardiomyocytes with the development of fibrosis due to activation of GRs and MRs receptors in the myocardium [6, 7]. Moreover, the ability of exogenous steroids to induce myocardial remodeling has been demonstrated even with its single administration [8].

Another, but no less important, etiological factor of PRM is inflammatory reaction with the generation of numerous free radicals (O2-, OH-, ONOO-) that results in nitroso-oxidative stress. Steroids induce the production of superoxide through MRs receptor-mediated activation of NADPH oxidase, thereby contributing to the development of endothelial and myocardial dysfunction followed by inflammation and apoptosis [9, 10]. It has been shown that superoxide plays an important physiological role influencing cell signaling pathways and activation of local immunity, otherwise its high content can rapidly react with nitric oxide (NO) to form peroxynitrite or convert to hydrogen peroxide to form hydroxyl radicals [11]. This interaction reduces the bioavailability of NO, breaking adaptation-compensatory changes [12]. Interestingly, nitric oxide synthase (NOS), especially endothelial (eNOS), can switch from NO-producing, cardioprotective isoform to the factor of damage due to its «uncoupling» and superoxide production [13].

This relationship suggests the pathogenetic importance of the nitric oxide system in the development of PRM. This assumption may be evidenced by a decrease in the severity of structural changes in the heart in the rats with DOCA-salt hypertension with chronic administration of L-arginine, which is a substrate for the NO formation [14]. In the previous studies, we have proven an important cardioprotective role of the NO system in physiological remodeling in the rats exposed to intermittent hypoxia and pathological effects in PRM in the rats with essential hypertension (SHR) [15, 16]. A study of NO system state in the rats with

secondary hypertension will enable an analysis of its changes and their significance for myocardial remodeling depending on the nature of the impact and etiopathogenesis of hypertension.

Therefore, **the aim** of our study was to establish the pathogenetic features of the NO system in the myocardium of the left ventricle in the rats with endocrine-salt hypertension (ESAH).

Materials and methods. The experiment was conducted on 20 male rats 220-290 g weight, 6–10 months old which were divided into 2 experimental groups: 1st – the control group (10 intact normotensive male Wistar rats); 2nd – 10 male Wistar rats, which were 2 times a day intraperitoneally injected with prednisolone (7 a.m. – 2 mg/kg, 8 p.m. – 4 mg/kg) with simultaneous forced drinking of 5 ml of 2.3% NaCl solution for 30 days [17].

The experimental part of the study was carried out exactly in accordance with the National "Common Ethical Principles of Animal Experiments" (Ukraine, 2001), in agreement with the Directive 2010/63EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes [18]. The protocol of the study is agreed with the local ethics committee (from March 1, 2018).

The experiment was conducted on the basis of the Training Medical Laboratory Center of the Zaporizhzhia State Medical University (Certificate of technical qualification 033/18 of 12.26.2018, valid until 25.12 2023). All devices used for study are certified and underwent annual metrological control (Laboratory of Experimental Pathophysiology, License 2CK2 YMK2 T6PB SG5N SJLS4).

Systolic and diastolic blood pressure (BP) were measured in all the rats using a system of non-invasive arterial pressure measurement BP-2000 (Visitech Systems, USA). The first measurement of BP was carried out at the time of groups formation, and then on the 1st, 5th, 10th, 15th, 21st and 30th days of the experiment. After a series of BP measurements (at least 7-10 registrations with intervals of 1.5-2 minutes), mean blood pressure (mBP) was obtained. It was calculated automatically in accordance with the manufacturer's instructions by the formula: mBP =(systolic BP+(2*diastolic BP))/3

Animals were euthanized via rapid decapitation under thiopental anesthesia (45 mg/kg body weight, intraperitoneally). The objects of study in the experimental animals were blood plasma (nitrotyrosine level was determined) and the fragment of the left ventricle of the heart. The left ventricle fragment was divided into two parts, one of them was homogenized using a Silent Crusher S homogenizer (Heidolph, Germany), then nitrites level was detected, the second fragment was fixed in paraplast blocks after standard histological preparation and then

serially sectioned into 5 μm-thick slices using a rotary microtome Microm-325 (MicromCorp, Germany).

The level of nitrites in homogenate of the left ventricular myocardium was measured by the biochemical Griess nitrite test on the Libra S 32 PC spectrophotometer [19]. The blood plasma nitrotyrosine concentration in the rats was measured with an immunoassay according to the instructions for the reagent set (Hycultbiotech, HK501 – Nitrotyrosine).

Concentration of immunoreactive material to NOS isoforms was determined with immunofluorescence method in accordance with the protocol of immunohistochemical study and manufacturer's instructions [20].

To study nNOS and eNOS expression serial slices after procedure of deparaffinization and rehydration were incubated for 1 day at T = +4 °C with primary polyclonal rabbit anti-nNOS and anti-eNOS antibodies, respectively, (1: 200; Santa Cruz Biotechnology, Inc., USA). After rinsing with 0,1 M phosphate buffer (pH = 7.2) sections were incubated for 45 minutes in a humid chamber at T = + 37° C with the secondary FITC-conjugated rabbit anti-mouse antibodies (1: 200; Santa Cruz Biotechnology, Inc.). To determine the iNOS expression, the slices of the myocardium were incubated with monoclonal FITC-conjugated mouse antibodies to iNOS (1: 200; Santa Cruz Biotechnology, Inc.).

The sections were examined with ultraviolet microscopy (AxioScope microscope, Carl Zeiss, Germany) in AxioVision 40 V 4.8.2.0 software program (License No. 3005339) with an excitation wavelength 390 nm, using a filter 38HE with high emission (Carl Zeiss, Germany). Zones with statistically significant fluorescence were identified while analysing the images in the interactive mode. At least 100 fields of view from each series were subjected for study.

The study of NOS mRNA isoforms expression in the left ventricular myocardium homogenates was carried out using a real-time polymerase chain reaction (RT-PCR) in the Department of Molecular Genetic Researches of the Training Medical Laboratory of ZSMU [21].

All statistical calculations were performed by the Microsoft Excel 2016 table processor (Microsoft Corp., USA). For all parameters, the arithmetic mean (M), its dispersion and mean error (m) were calculated. To determine the reliability of differences between the results of research in the experimental and control groups of the rats, the Student's coefficient (t) was calculated, after that the probability of the difference between the samples (p) and the confidence interval of the mean according to the Student distribution tables were determined. Valid values for $pSt<0.05$ were considered statistically reliable [22].

Results of research. Analysis of BP in the rats showed that mean BP in the group of rats with ESAH significantly exceeded the control value by 65 % (Fig. 1A).

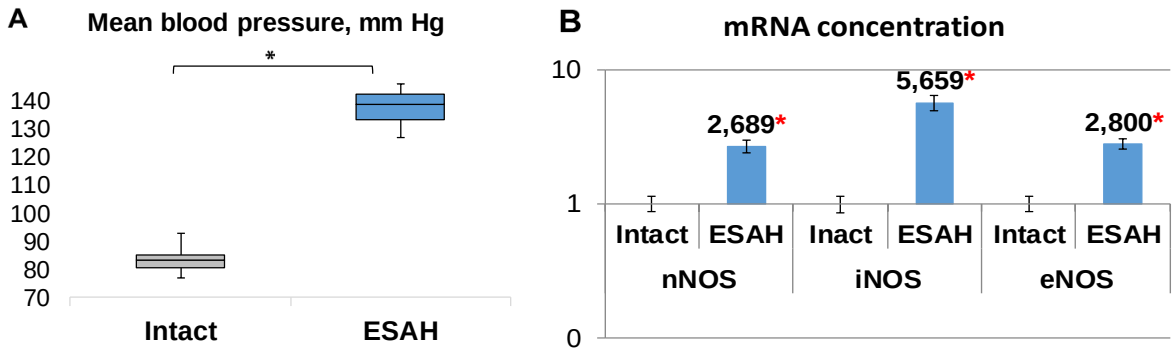

Fig. 1 – The mean blood pressure (A) and mRNA expression (B) of NOS isoforms (nNOS, iNOS and eNOS) in the left ventricular myocardium

Note 1. (*) – * reliability of the differences ESAH (pSt<0.05) to the corresponding indices of the control group.
Note 2. (A) – The data are presented as median, the 1st and the 3rd quartiles, min and max.
Note 3. (B) – The data are presented as M ± m.

The study of mRNA expression of NOS isoforms by PCR-RT showed that hypertension leads to a significant increase in mRNA to all three NOS isoforms with the highest values of iNOS mRNA (Fig.1B).

An immunofluorescent study of myocardial sections of the rats of the experimental groups showed that visually the immunoreactive material (IRM) to all three isoforms of NOS in the left ventricle had a diffuse distribution. Analysis of nNOS expression showed that in the group of the rats with ESAH compared with the control in the transverse fibers there was a significant increase in the content of IRM to nNOS by 14.3 %, while in the longitudinal fibers its content increased by 8.3 % (Fig. 2). It should be noted that the increase in the expression of IRM to nNOS was combined with its increase in left ventricular myocardial homogenates. The content of its mRNA significantly increased 2.7 times (Fig. 1B).

The study of iNOS expression in the myocardium of the rats with ESAH showed that there was an increase in the content of IRM to iNOS by 18.5 %, compared with the control in the transverse fibers, in longitudinal - by 8.5 % (Fig.2). At the same time the content of iNOS mRNA in homogenates increased in 5.7 times (Fig.1B).

The analysis of eNOS expression in the group of the rats with ESAH showed that there was a significant increase in the content of IRM to eNOS by 16.2 % compared with the

control in the transverse fibres of the myocardium, while in the longitudinal fibers – a significant decrease in content by 7.6 % (Fig.2). The expression of eNOS mRNA in ESAH rats was significantly higher than the control values by 2.8 times (Fig.1B).

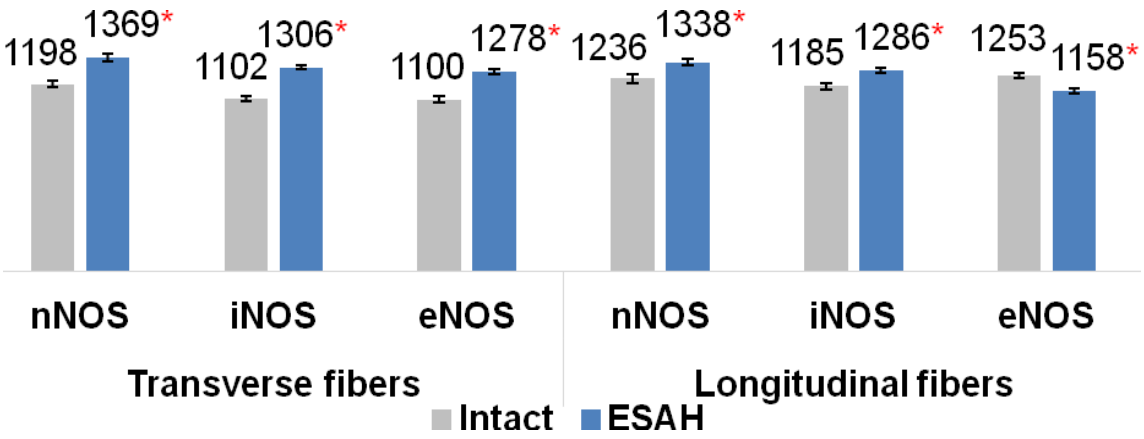

Figure 2 – The content of immunoreactive material to NOS isoforms in the left ventricular myocardium of rats, Units of fluorescence

Note 1. (*) – reliability of the differences (pSt<0,05) to the corresponding indices of the control group.
Note 2. The data are presented as M ± m.

In addition, the detection of NO terminal metabolites in the rats with ESAH showed that nitrites level was significantly lower by 11.7 %, while the concentration of nitrotyrosine was significantly higher by 88.5 % compared to control animals. (Fig. 3A-B).

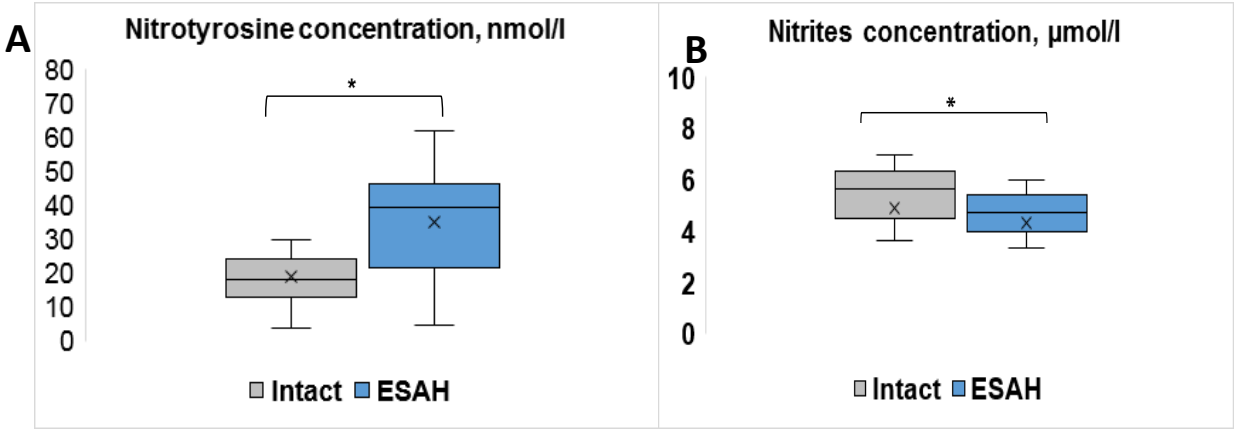

Figure 3 – Concentration of nitrotyrosine (A) in blood plasma and nitrites (B) in left ventricular myocardium homogenates of the experimental groups

Note 1. (*) – *: reliability of the differences (p<0.05)
Note 2. The data are presented as median, the 1st and the 3rd quartiles, min and max.

Analysis and discussion of results. It is known that hypertension includes several etiopathogenetic levels of its development, so the study of nitric oxide system in secondary endocrine hypertension is very important. The model of hypertension we used results not only in a constant increase in BP, but also includes the development of hypercorticism, hyperaldosteronism, hypernatremia, disturbs circadian rhythm of hormone production, which leads to increased circulating blood volume, peripheral vascular resistance, endothelial dysfunction and formation of AH with specific pathological myocardium remodeling that was also observed in our previous studies [23].

The assumptions about the relationship between changes in the NO system state with the hypertension development are based on its physiological properties and pathological manifestations. It has been proven that the nitric oxide system is a universal limiting system that is one of the first to respond to stress of any origin [24]. NOS-deficient models of hypertension demonstrated the development of hypertension in normotensive rats, that was accompanied by metabolic changes, increased vasoconstriction with reduced vasodilation, arterial wall thickening, myocardial fibrosis, and activation of the sympatho-adrenal and renin-angiotensin-aldosterone systems [25]. Therefore, the cardioprotective role of nitric oxide system in cardiovascular diseases is beyond doubt, which is possible only with normal feedback control of NOS expression, the presence of reaction substrates and NO bioavailability.

The present study found an increase in mRNA and IRM levels to constitutive NOS isoforms (nNOS and eNOS). It is difficult to study the role of each isoform due to inability to determine which of the NOS form produced NO. Moreover, these two isoforms are feedback-linked and in the case of one's deactivation the other will hyperproduce NO to maintain its constant levels [26]. This suggests their cardioprotective increase, which is confirmed by the results obtained by Zhang Y., (2014) who proved that nNOS is the main isoform that supports vital functions in the myocardium, namely, it corrects contractility, rhythm regulation and microcirculation [27].

Despite the fact that the actions of nNOS and eNOS are aimed at performing the same functions, there is a big difference between them, primarily localization features. Thus, nNOS is localized in the sarcoplasmic reticulum, sarcolemma, and partially is a cytosolic protein, it has a high ability to translocate, which expands its area of influence, that is especially important due to the short lifespan of the NO molecule [28]. At the same time eNOS is localized mainly in the caveolae and is a mediator of mechanical stress, stimulating the release of intracellular calcium from ryanodine receptors, that allows this isoform to respond

quickly to minimal changes in the composition of the intercellular environment and basal circulation [29]. Unlike other isoforms, nNOS is also localized in the intramural ganglia and fibers allowing it to participate in sympathetic transmission, enhancing the positive lusitropic effect in early hypertension [30]. It is also known that nNOS controls the activity of cardiac oxidases and NADPH oxidase, which modulates the levels of reactive oxygen species and peroxynitrite. nNOS-dependent regulation of ion channel activity and intracellular Ca2+ - processing proteins can be mediated by post-transcriptional modification, such as NO-dependent S-nitrosylation and ROS-dependent oxidation [31]. Thus, the type of the final NO metabolite determines the subsequent post-transcriptional modifications. It can explain the increase in nNOS expression in ESAH rats, which is aimed to improve myocyte relaxation.

In addition to the vasodilating effect, increased relaxation and contraction of the myocardium, eNOS is attributed to the regulation of cardiomyocyte growth, hypertrophy and heart failure development. In this case, if the first effects are mediated by regulation of phosphorylation / dephosphorylation after the translational activity of the eNOS-NO pathway, the latter due to factors such as phosphoinositide 3-kinase-serine (PI3K) and protein kinase (Akt) [32].

As for the iNOS function, the authors have different thoughts, because this isoform shas been considered a source of free radicals for many years. Recently, the evidence of its involvement in the physiological mechanisms of myocardial function has been demonstrated in an ischemia-reperfusion model. It is hypothesized that, in fact, iNOS may have the ability to protect myocardium against the reperfusion injury caused by ischemia-induced late preconditioning, along with antioxidant and vasodilatory effects [33].

The increase in mRNA content for nNOS and eNOS in the myocardium was about 1: 1, while iNOS mRNA content increased by 5.7 times that suggests an excessive and aggressive effect on the myocardium of this isoform, accompanied by the production of peroxynitrite instead of nitric oxide. Such an excess of iNOS expression may be associated with stimulation of the GITR gene (glucocorticoid-induced TNF receptor), which increases tumor necrosis factor content that directly stimulate the iNOS gene [34]. That is, such a large amount of iNOS is the result of direct exposure to steroids. Moreover, studies in gene-knocked out mice have shown an interesting dependence of BP on NOS isoforms. Thus, in studies by Morishita et al. (2005) with triple switching off of n/i/eNOS - / - the degree of hypertension was the same as with isolated eNOS knockout or double blocking of n/eNOS - / -. At the same time, the development of hypertension was not observed at isolated blockade of nNOS -/- (Sällström et al. 2008) and iNOS -/- (Ihrig et al. 2001) [35].

Our results of the NOS isoform profile study suggest a compensatory direction of its balance change in order to increase of nNOS and eNOS. This increase in the content of mRNA and IRM to constitutional isoforms in the myocardium indicates the need for them due to their active involvement in limiting hemodynamic stress and the damaging effects of steroids.

Disruption of NO, ROS and peroxynitrites mechanisms of production is an important etiological factor of cardiovascular disease, therefore it was logical to study the levels of end metabolites: nitrites – marker of NO, and nitrotyrosine – a marker of ROS [36]. A decrease in nitrites concentration and a significant increase in nitrotyrosine level obtained in the present study, indicates the development of nitroso-oxidative stress, which is probably the result of "switching" iNOS from NO production-producing isoform to a source of free radicals, which in turn cause free radical damage to the myocardium along with action of steroids, pressure and volume overload due to hypertension.

Conclusions

1. The development of experimental endocrine-salt hypertension in the rats leads to a stable increase in mean blood pressure by 65 % compared to control.

2. Endocrine-salt arterial hypertension in Wistar rats is characterized by an increased mRNA content of all three isoforms of nNOS, eNOS and iNOS by 2.7, 2.8 and 5.7 times, respectively, compared to the control; increased expression of immunoreactive material to isoforms in transverse fibers by 14.3 %, 16.2 % and 18.5 %, respectively; in longitudinal fibers IRM to nNOS was higher by 8.3 %, to iNOS - by 8. 5%, but to eNOS it was lower by 7.6 %. At the same time, nitrites level decreased by 11.7 % and nitrotyrosine concentration was significantly higher, exceeding the control value by 88.5 %.

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