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Characteristics of the nitric oxide system indicators in the left ventricle myocardium in SHR

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Key words: NOS isoforms, nitric oxide synthase, ventricular remodeling, left ventricle, heart, essential hypertension, rats, SHR.

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The aim was to determine the morpho-functional parameters of the left ventricular myocardium NO system in the rats with essential hypertension (SHR line).

Material and methods. We used a combination of modern highly informative methods, namely: research of NOS isoform profile (nNOS, iNOS eNOS) in the myocardial slices along with an assessment of their synthesis and expression of the corresponding mRNA; NO derived nitrites level determination directly in the myocardium homogenates and concentration of nitrotyrosine in blood plasma of rats.

The results of the performed studies have shown that high blood pressure in the SHR was accompanied by a significant increase in the concentrations of all three NOS isoforms in the myocardium and increased expression of their mRNA. Higher concentration of nitrites by 18.8 % was detected in the SHR group compared with the control animals. The concentration of nitrotyrosine in blood plasma of rats with essential hypertension was also increased by 25 %.

Conclusions. The predominance of IRM to constitutive isoforms of NOS with low IRM content to iNOS was noted in the myocardium of the control group rats while in SHR rats higher IRM values were marked for all NOS isoforms. The formation of hypertension is accompanied by high content of NO end metabolites and the development of systemic nitroso-oxidative stress with the increase of nitrotyrosine concentration.

Ключові слова: ізоформи NOS, синтаза оксиду азоту, ремоделювання шлуночка серця, лівий шлуночок, серце, есенціальна артеріальна гіпертензія, щури, SHR.

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Характеристика показників системи оксиду азоту в міокарді лівого шлуночка в щурів лінії SHR

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Мета роботи – визначення морфофункціональних показників системи NO міокарда лівого шлуночка в щурів з есенціальною артеріальною гіпертензією (лінії SHR).

Матеріали та методи. Використали комплексний підхід із застосуванням сучасних і високоінформативних методів: дослідження у зрізах міокарда ізоформного профілю NOS (nNOS, iNOS eNOS) з оцінюванням їхнього синтезу за експресією відповідних мРНК; визначення рівня кінцевого метаболіту NO-нітритів безпосередньо в гомогенатах міокарда й концентрації нітротирозину у плазмі крові щурів.

Результати. Показано, що формування стійкого підвищення артеріального тиску в щурів лінії SHR супроводжувалося вірогідним збільшенням у зрізах міокарда концентрацій усіх ізоформ NOS і збільшенням показників експресії їх мРНК. У групі SHR встановлена вища концентрація нітритів (на 18,8 %) порівняно з показником контрольних тварин. Щодо концентрації нітротирозину у плазмі крові щурів, то у групі щурів з есенціальною артеріальною гіпертензією також встановили збільшення показника на 25 %.

Висновки. У щурів контрольної групи в міокарді виявили переважання ІРМ до конститутивних ізоформ NOS із низьким значенням вмісту ІРМ до iNOS, а в щурів SHR встановили вищий вміст ІРМ до всіх ізоформ NOS. Формування артеріальної гіпертензії супроводжується збільшеним вмістом кінцевих метаболітів NO та формуванням системного нітрозо-оксидативного стресу з підвищенням концентрації нітротирозину.

Ключевые слова: изоформы NOS, синтаза оксида азота, ремоделирование сердца желудочка, левый желудочек, сердце, эссенциальная артериальная гипертензия, крысы, SHR.

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Характеристика показателей системы оксида азота в миокарде левого желудочка у крыс линии SHR

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Цель работы – определение морфофункциональных показателей системы NO миокарда левого желудочка у крыс с эссенциальной артериальной гипертензией (линии SHR).

Материалы и методы. Применили комплексный подход с использованием современных и высокоинформативных методов: исследование в срезах миокарда изоформного профиля NOS (nNOS, iNOS eNOS) с оценкой их синтеза и экспрессии соответствующих мРНК; определение уровня конечного метаболита NO-нитритов непосредственно в гомогенатах миокарда и концентрации нитротирозина в плазме крови крыс.

Результаты. Показано, что формирование стойкого повышения артериального давления у крыс линии SHR сопровождалось достоверным увеличением в срезах миокарда концентраций всех изоформ NOS и увеличением показателей экспрессии их мРНК. В группе SHR установлена более высокая концентрация нитритов (на 18,8 %) по сравнению с показателем контрольных животных. Что касается концентрации нитротирозина в плазме крови крыс, то в группе крыс с эссенциальной артериальной гипертензией установлено увеличение показателя на 25 %.

Выводы. У крыс контрольной группы в миокарде отмечено преобладание ИРМ к конститутивным изоформам NOS с низким значением содержания ИРМ к iNOS, а у крыс SHR установлено более высокое содержание ИРМ ко всем изоформам NOS. Формирование АГ сопровождается увеличенным содержанием конечных метаболитов NO и формированием системного нитрозо-оксидативного стресса с повышением концентрации нитротирозина.

Today it is well known that the development of arterial hypertension (AH) is accompanied by endothelial dysfunction [1]. Increased blood pressure in arterial hypertension begins due to hyperepinephrinemia which leads to angiospasm. The result is an increase in total peripheral vascular resistance, an increase in heart rate, stroke volume and cardiac output. In the case of prolonged maintenance of such angiospasm, irreversible morpho-functional changes in the vessel wall (its thickening, decrease of elasticity, dilation and increase in rigidity index) are formed. The nitric oxide system (NO) plays an essential role in this event. Endothelial dysfunction is formed both through lack of nitric oxide and reduction of its bioavailability as well as by its hyperproduction, as a result of oxidative stress [2]. The main source of NO is the nitric oxide synthase enzyme (NOS), represented by three isoforms: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). Depending on the type of activated isoform, its amount and activity the effect of NO – either vasodilation or vasoconstriction and cell damage will be realized [3]. Thus, the NOS isoforms can be considered as a key factor in NO effects regulation, acting as a protective or aggressive agent, which disturbs the function and damages the cardiovascular system [4].

The target organ, which is one of the first to response to hemodynamic overload during arterial hypertension, is the heart. At the same time, remodeling of the left ventricle develops at the preclinical stage, initially as a component of high blood pressure (BP) compensation and later after the exhaustion of physiological adaptive mechanisms, as an element of pathological myocardial remodeling (PMR) [5]. It has been established that the development of hypertrophy with the interstitial fibrosis and initiation of apoptosis in cardiomyocytes make the basis of PMR pathogenesis. Other components of PMR are those processes similar to endothelial dysfunction, which are termed as myocardial dysfunction [6]. Moreover, these processes mutually reinforce each other. Violations of cardiac muscle contraction, energy supply and innervation of cardiomyocytes occur as a result of the myocardial dysfunction development. These particular processes are influenced by the universal regulatory gasotransmitter NO, formation of which is controlled by NOS isoforms. However, the insufficient clarity of the nitric oxide system role in hypertension, the uncertainty in peculiarities of the NOS isoforms profile in the myocardium in different etiological forms of arterial hypertension, require an in-depth research and conduction an experimental study.

We encountered a large number of contradictory and conflicting data while planning the experiment and analyzing the data of other scientists. In our opinion, this is due to the fact that most of scientists focus their study on the one method of research (more often in vitro) or determine a limited number of parameters.

The aim

That is why, in order to achieve the intended goal to determine the morpho-functional parameters of the left ventricular myocardium NO system in the rats with essential hypertension (SHR line), an integrated approach was chosen. We used modern highly informative methods, namely: analysis of NOS isoform profile (nNOS, iNOS eNOS) in myocardial slices with their synthesis estimation by expression of the corresponding mRNA; determination of NO derived nitrites level directly in the myocardium homogenates. We also studied nitrotyrosine concentration in blood plasma of rats in order to prove the presence of oxidative stress.

Materials and methods

The experiment was conducted in 20 male rats 220–290 g weight, 6–10 months old which were divided into 2 experimental groups: the control group (10 intact normotensive male Wistar rats) and the experimental group – 10 male SHR (a model of essential hypertension).

The experimental part of the study was carried out exactly according to the National “Common Ethical Principles of Animal Experiments” (Ukraine, 2001), which are in accordance 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. 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 registration No. 039/14 dated June 25, 2014, valid until June 24, 2019). All devices used for study are certificated and undergo annual metrological control (Laboratory of Experimental Pathophysiology, License 2CK2 YMK2 T6PB SG5N SJLS4).

Systolic and diastolic BP levels 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 blood pressure 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 = (2(diastolic pressure) + systolic pressure)/3. Mean BP was 83.8 ± 0.96 mm Hg in the control group rats and 125.8 ± 1.12 mm Hg in SHR.

Animals were euthanized via rapid decapitation after thiopental anesthesia (45 mg/kg body weight, intraperitoneally).

The study objects in the experimental animals were blood plasma, in which the nitrotyrosine level was determined, and the left ventricle fragment, which was divided into two parts, one of which was homogenized using a

Silent Crusher S homogenizer (Heidolph, Germany), the second fragment of the heart after standard histological preparation was fixed in paraplast blocks and then serially sectioned into 5 μm-thick slices using a rotary microtome Microm-325 (MicromCorp, Germany).

Concentration of immunoreactive material to NOS isoforms was determined with immunofluorescence method in accordance with the protocol of immunohistochemical study and manufacturer's instructions [7].

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 against 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 analyzing 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. [8]. Total RNA was isolated from the myocardium tissue using Trizol RNA Prep 100 (Isogen, RF), according to the manufacturer's protocol. The RNA was resuspended in water, quantified and tested with the RT-PCR assay using the RT-PCR kit; RT (Synthol, RF). RT-PCR was performed in a final volume of 25 μl containing 10 μl of the 2,5X reaction mixture, 11 μl dd H2O, 1 μl of primers (*Table 1*) of Random-6, 1 μl of reverse transcriptase and 2 μg of RNA. The reverse transcription was performed at 45 °C for 45 minutes and then it was heated for 5 minutes at 92 °C. For RT-PCR in real time with gene specific primers CFX96 ™ Real-Time PCR Detection Systems (Bio-Rad Laboratories Inc., USA) were used in accordance with the manufacturer's recommendations and Maxima SYBR Green/ROX qPCR Master Mix (2X) reagent kit (Thermo Fisher Scientific, Inc.). Master Mix included Maxima Hot Start Taq DNA polymerase and dNTPs in optimized PCR buffer. The samples were amplified in a volume of 25 μl of the reaction mixture at a concentration of 0,3 μM of forward and reverse primers, 12.5 μl Maxima SYBR Green/ROX qPCR Master Mix (2X), template DNA ≤500 ng / reaction, nuclease-free water up to 25 μl. All primers were designed using the Primer-BLAST software (NIH, USA) and were synthesized by Metabion (Germany). Amplification was performed with the following settings: 10 min at 95 °C for initiating denaturation followed by 50 cycles of denaturation at 95 °C for 15 sec., primer annealing for 30 sec. at 58–63 °C and extension at 72 °C for 30 sec.

Table 1. Primers design

Gene	Primer	Tm, °C	Product size (bp)	Position
nNOS	F=GACGCAGATGAGGTTTTCAGC R=GGGGGCAGGAGGATCCAG	59.87 61.17	45	4477/4478
iNOS	F=GTTCCTCAGGCTTGGGTCTT R=CCGTGGGGCTTGTAGTTGAC	59.6 60.95	49	143/144
eNOS	F=CCCAGGAGAGATCCACCTCA R=CAGCACATCCTGGGTTCTGT	60.03 59.96	58	2899/2900
actin, beta (Actb)	F=ACAACCTTCTTGCAGCTCCTC R=TCGTCATCCATGGCGAACTGG	60.54 60.76	64	72/73

F: forward primer; **R**: reverse primer; **Tm**: melting temperature.

Registration of the fluorescence intensity occurred automatically at the end of each cycle extension step in the SYBRGreen channel.

As the reference gene, the actin beta gene (Actb) gene was used to determine the relative value of changes in expression level of studied genes. A comparative Ct method (ΔΔCt method) was used to express the relative level of gene expression. Statistical analysis of the PCR data was performed using the CFX Manager™ software (Bio-Rad, USA). Negative controls were included in the experiment: without the cDNA matrix adding to the PCR reaction, without the mRNA matrix adding to the cDNA synthesis, without the enzyme adding in the cDNA synthesis. All amplification reactions were performed on individual samples in three replicates.

The level of nitrites in homogenates of the left ventricular myocardium was determined by the biochemical Griess nitrite test on the Libra S 32 PC spectrophotometer [9]. The blood plasma nitrotyrosine concentration in rats was determined by immunoassay according to the instructions for the reagent set (Hycultbiotech, HK501 – Nitrotyrosine).

All statistical computations were performed in 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 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 P_{St}<0.05 were considered statistically reliable [10].

Results

The results of conducted studies reveal that the persistent increase of BP in SHR was accompanied not only by a significant increase in the concentrations of all three NOS isoforms (nNOS, iNOS, eNOS) in the myocardial slices (*Fig. 1-A*), but also by an increase in their mRNA expression (*Fig. 1-B*).

Higher concentration of IRM to nNOS by 22.8 %, and 1.6 times increase of its mRNA were found in SHR in comparison with the control group. Concentration of IRM to eNOS in SHR was reliably increased by 6.8 % and in 2.3 times increase its mRNA in comparison with the intact rats. Reliability of the increased concentration

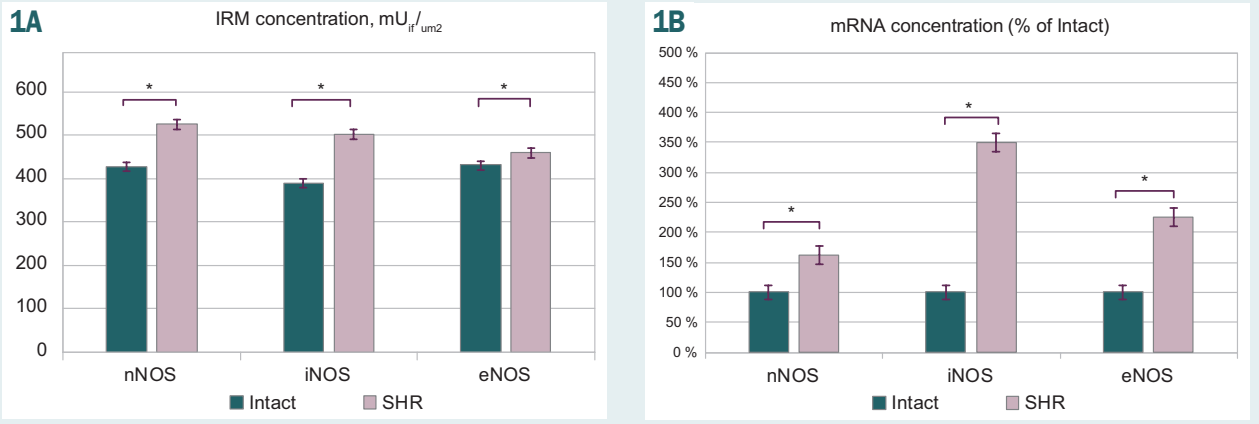

Fig. 1. Concentration of IRM (A) and mRNA expression (B) of NOS isoforms (nNOS, iNOS and eNOS) in the left ventricular myocardium of experimental rats. The data are presented as M ± m.

*: reliability of the differences P < 0.05.

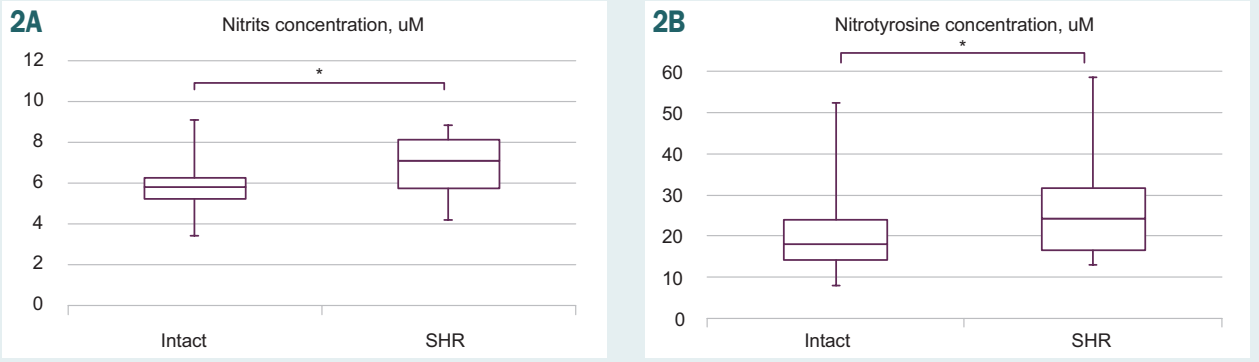

Fig. 2. Concentration of nitrites (A) in left ventricular myocardium homogenates and nitrotyrosine (B) in blood plasma of the experimental groups rats. The data are presented as median, the 1st and the 3rd quartiles, min and max.

*: reliability of the differences P < 0.05.

of IRM to iNOS by 29.7 % was detected. iNOS mRNA concentration was 3.5 times more than in control animals.

Studies of NO end metabolites level were also done in order for assessing the state of the nitric oxide system. In the SHR group 18.8 % higher concentration of nitrites was detected in comparison with the control animals. Nitrotyrosine concentration in blood plasma in the SHR was also increased by 25 % (*Fig. 2A-B*).

Discussion

The fact, ascertained in the work, that the higher values concentration of IRM to nNOS and increase of its mRNA in the myocardium in the formed AH in SHR were found to be the expected result (*Fig. 1A-B*). This is due to the fact obtained by many researchers, nNOS in the cardiac muscle acts as the main endogenous source of myocardial NO [11], which provides a rapid, situational change in the level of NO in response to extracellular signals of mediators, hormones and biologically active substances [12], implements the control over parasympathetic and sympathetic regulation of cardiac rhythm, influences the myocardial contractility and relaxation [13]. Therefore, it is logical to assume that a higher concentration of nNOS in SHR is necessary for activation of both urgent and long-term adaptation mechanisms of the heart to hemodynamic overloads. This mechanism of compensation is especially important at the initial stages of blood pressure increase.

Concerning the increased concentration of IRM to eNOS and increase of its mRNA in SHR myocardium compared to control, it should be noted that this isoform is associated with the local endothelial cytoprotective mechanisms realization and maintenance of vascular homeostasis. Persistent increase in BP is accompanied by the long-term compensatory mechanisms activation, namely left ventricular hypertrophy [14] with increased blood supply and new capillaries and nerves growth. In our opinion, a significant increase in eNOS mRNA expression in SHR is due to induction of gene expression through the transcription pathway [15] as one of the components of adaptation implementation. This gene is activated under conditions of moderate hypoxia in AH and is accompanied with an increase in the level of intracellular Ca^{2+} ions, an increase in the Ca^{2+}-dependent eNOS synthesis and activity. This results in a large amount of endothelial NO formation and leads to the improvement of blood supply. This particular mechanism serves as one of the essential components of the compensatory myocardium remodeling [16].

In contrast to constitutive NOS isoforms, the high concentration of iNOS, which is considered as a mediator of nitrosative and oxidative stress, worsens endothelial and myocardial dysfunction in AH due to a large amount of peroxynitrite formation [17]. In our study, there was a reliable increase in the concentration of IRM to iNOS (*Fig.1-A*), which confirms its involvement in the formation of PMR and cell injury, the fact of which in SHR has been found in many studies [18]. An analysis of the RT-PCR results in the SHR group demonstrated a reliable increase in iNOS mRNA expression (*Fig. 1-B*). According to other researchers, high level of mRNA in the myocardium of rats with AH is associated not only with the activation of mRNA transcription in response to stimulation by pathological factors (active forms of oxygen, peroxynitrite, H_2O_2, cytokines, etc.), but also with the decrease of its breakdown that was also demonstrated [19]. Thus, it contributes to a vicious cycle – the higher is the level of unstable metabolites, the higher is the level of iNOS, which will produce further greater NO excess and results in the development of oxidative and nitrosative stress with myocardial dysfunction.

Studies of NO end metabolites level were also done. Nitrites are stable NO metabolites and are determined by the researchers as an equivalent marker of its formation. In the SHR group a higher concentration of nitrites was detected in comparison with the control animals. The obtained result can be considered as an evidence of the NOS increased activity in the myocardium of rats with essential hypertension. Nitrotyrosine concentration in blood plasma (NO-dependent marker of oxidative stress) in the group of rats with essential hypertension was also increased (*Fig. 2A-B*). This fact allows us to assert that oxidative and nitrosative stress occurs not locally in the myocardium, but has a systemic character [20].

Therefore, the conducted study allows to state that essential hypertension causes significant changes in the morpho-functional state of nitric oxide system. The important features of these changes are the peculiarities of NOS isoforms profile, the prevalence of iNOS and the presence of systemic nitroso-oxidative stress signs.

Conclusions

1. The predominance of IRM to constitutive NOS isoforms with low IRM content to iNOS has been noted in myocardium of the control group rats with normal BP.

2. The higher IRM content to all three NOS isoforms in SHR (the model of essential AH), compared to the control, is associated with the stimulation of synthetic processes due to their mRNA increased expression.

3. The higher indices of nNOS and iNOS concentration along with reduced IRM content to eNOS have been revealed in SHR myocardium.

4. Formation of hypertension is accompanied by an increased content of NO end metabolites (nitrites) and systemic nitroso-oxidative stress development with high nitrotyrosine concentration.

Conflicts of interest: authors have no conflict of interest to declare.
Конфлікт інтересів: відсутній.

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