Isoform profile of NOS enzyme in structure of rats’ solitary-vagal complex in arterial hypertension of various origin

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

The aim of this study was to characterize the nitric oxide isoforms profile in rats’ nucleus tractus solitarii (NTS) and dorsal motor nucleus (DMN) of vagus nerve in arterial hypertension (AH) of various origin (essential (EAH) – rats of SHR line, and endocrine-salt AH).

Materials and methods. The study was performed on 30 aged male rats. Among them, 20 Wistar rats were divided into two groups – control (10 rats) and 10 rats with simulated endocrine-salt AH (ESAH) and 10 SHR rats. An immunohistochemical method was used to study the of nitric oxide synthase isoforms expression features in DMN and NTS. The following parameters were determined: the content of immunoreactive material (IRM) for the studied peptides (Uif/μm2), the relative area of the IRM (%) and the IRM concentration in 1 μm2 (Uif/μm2).

Results. It was found that in rats with both models of AH, the expression indices of all three nitric oxide synthase (NOS) isoforms in the studied structures increased. In our opinion, this is due to the activation of the studied structures in AH conditions. This must be considered as an important element of high blood pressure compensating. It is achieved through the implementation by a complex of mechanisms like reducing of the sympathetic tone and increasing of the parasympathetic tone by activating a system of secondary messengers; improving neurotrophy due to the high activity of constitutive NOS isoforms; iNOS-mediated NO overproduction, as a factor in compensating for its bioavailability in conditions of local ischemia in AH.

Conclusions. Regardless of the AH etiopathogenesis in both experimental groups, the expression of all three NOS isoforms increases in the DMN and NTS structures. In rats with EAH in the DMN and NTS structures, the expression indices of NOS isoforms have their own characteristics. So in the first structure, the largest changes in the indices of the immunoreactive material content and concentration are observed for eNOS, and the relative area for iNOS. At the same time, in the NTS structure, the largest changes in the content indices are observed for iNOS, and the concentration and relative area for eNOS. In rats with ESAH in the DMN structure, the highest changes in the indices of IRM content and concentration are observed for the endothelial isoform of NOS, and the relative area – for inducible. In the NTS structure, the IRM content changed the most for nNOS, and the concentration and relative area for iNOS.

Изоформный профиль фермента NOS у структуре солитарно-вагального комплекса щурів при артеріальній гіпертензії різного генезу

М. В. Данукало, О. В. Ганчева

Мета роботи – дати характеристику ізаформного профілю синтази оксиду азоту (NOS) у структурах ядра солітарного тракту (ЯСТ) і дорсального моторного ядра (ДМЯ) щурів при артеріальній гіпертензії (АГ) різного генезу (єсенційний – ЕАГ) – щури лінії SHR, та ендокринно-сольовій АГ.

Матеріали та методи. Дослідження здійснили на статевозрілих 30 щурів-самцях, серед них – 20 тварин лінії Wistar, яких поділили на дві групи: контроль (10 щурів) і 10 щурів зі змодельованою ендокринно-сольовою АГ (ЕСАГ), 10 щурів лінії SHR – із генетичною ЕАГ.

Результати. Протягом дослідження встановлено, що в щурів обох моделей з АГ показники експресії всіх трьох ізаформ NOS у досліджуваних структурах збільшуються. На нашу думку, це пов’язано з активізацією структур, що діють на сімпатичну й парасімпатичну нервову систему, а також з інтенсивнішим синтезом NOS у структурі северно-вагального комплексу щурів.

Ключові слова: нітріковий оксид, ядро солітарного тракту, дорсальний моторний ядро, країнаспільна нерва, гіпертензія, щури.
Arterial hypertension (AH) is characterized by a variety of etiological factors and multicomponent links of pathogenesis. Most researchers agree that its development is based on disturbances in the functioning of blood pressure (BP) regulation systems [1]. The most important BP coordinators are brainstem structures, as primary analyzers of the afferent information about the condition of vascular tone. Moreover, they provide an efferent response that supports BP homeostasis in the case of its change. These structures, first of all, are the nucleus of the solitary tract (NTS) and the dorsal motor nucleus (DMN) n. vagus. They regulate BP by activating a number of reflexes; the most important of them is the baroreflex. In this context, NTS is considered as an important afferent link, while DMN is an efferent component [2]. So, it is logical to assume that a change in the NTS and DMN regulatory systems will be displayed on the BP regulation.

It has been established today that gaseous transmitters act as important regulators of the neurons’ functional activity. The most studied of them is nitrogen monoxide (NO). A number of researchers have already demonstrated disturbances in the NO system in the BP regulatory centers of the brain in AH. It was found that they are associated with both quantitative and qualitative features of NO systems’ components (presence of substrate, isoform profile of the enzyme, bioavailability of NO) [3,4]. Unfortunately, the process of directly determining NO amount in tissues is expensive and time-consuming. Therefore, indirectly, the NO amount in tissues can be measured by the level of nitric oxide synthase (NOS) isoforms expression. It is proved that each isoform is involved in the implementation of a certain complex of NO effects. The nitrogen monoxide produced by the endothelial synthesis of NO leads to vasodilation, inhibition of aggregation and adhesion of platelets and leukocytes, activation of endothelial progenitor cells, stimulation of angiogenesis; neuronal isoform – regulates synaptic transmission, acts as a neurotransmitter and neuroprotector; inducible isoform – to activation of nNOS, and concentration and pla cing for iNOS.

The study was conducted on 30 adult male rats, of which 20 animals of the Wistar strain, and 10 Sprague–Dawley rats – with genetically determined AH. For the study of NOS isoform expression in the structures of the solitary-vagal complex, we used the immunohistochemical method. The parameters were determined: the content of the immunoreactive material (IRM) to the investigated substance (\( \text{EId}_{\text{A}}, \text{EId}_{\text{B}} \)), relative area of the IRM (%) and concentration of the investigated substance in 1 mm\(^2\) (EId/m\(^3\)).

It has been established today that gaseous transmitters act as important regulators of the neurons’ functional activity. The most studied of them is nitrogen monoxide (NO). A number of researchers have already demonstrated disturbances in the NO system in the BP regulatory centers of the brain in AH. It was found that they are associated with both quantitative and qualitative features of NO systems’ components (presence of substrate, isoform profile of the enzyme, bioavailability of NO) [3,4]. Unfortunately, the process of directly determining NO amount in tissues is expensive and time-consuming. Therefore, indirectly, the NO amount in tissues can be measured by the level of nitric oxide synthase (NOS) isoforms expression. It is proved that each isoform is involved in the implementation of a certain complex of NO effects. The nitrogen monoxide produced by the endothelial synthesis of NO leads to vasodilation, inhibition of aggregation and adhesion of platelets and leukocytes, activation of endothelial progenitor cells, stimulation of angiogenesis; neuronal isoform – regulates synaptic transmission, acts as a neurotransmitter and neuroprotector; inducible isoform – to the production of a large number of free radicals and their mediated cytotoxicity.
In this regard, it can be argued that in order to obtain a holistic picture of NO-dependent processes, it is important to study the entire NOS isoform profile in the structures under study. All three NOS isoforms were found in NTS and DMN [6], but data about the features of their expression in brainstem regulatory centers in AH are insufficient.

**Aim**

Therefore, the aim of this work was to characterize the NOS isoform profile in rats’ NTS and DMN structures in arterial hypertension of various origins.

**Materials and methods**

The study was performed on 20 mature male Wistar rats and 10 male rats of the SHR line (generally accepted model of essential AH (EAH) in humans [7], mean weight – 249.9 ± 12.0 g). 10 male Wistar rats were in a control group (mean weight – 187.2 ± 6.3 g), in other 10 we modeled endocrine-saline hypertension (ESAH), which is analogue of endocrine-associating AH in humans (mean weight – 263.7 ± 7.2 g).

The rats used in the study were obtained from the nursery “Biomodelservice” Kyiv. The experimental part of the study was carried out in accordance with the “General Ethical Principles of Animal Experiments” (Ukraine, 2001), which are adjusted with the statement of Europe Parliament Council 2010/63EU and Council from 22 of September 2010 on the protection of animals used for scientific purposes (Council Directive 2010/63EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes).

ESAH was modeled by intraperitoneal injection of medication prednisolone (30 days, 2 times a day, 7.00 am – 2 mg/kg, 8.00 pm – 4 mg/kg, with forced intake of 5 ml of a 2.3 % solution of NaCl) [8]. To measure BP, the animal is housed in special immobilization boxes located on a platform, which is heated and constantly maintains a constant temperature of 37–39 °C. The peculiarity of the boxes is that they are light-tight. This minimizes the additional irritation and shaking of the animal. The blood pressure measurement procedure is carried out in absolute silence. The average time to register BP is 3–7 minutes. During this time it is possible to take 3–5 preliminary measurements and 3–5 control measurements. The unit automatically calculates systolic, diastolic pressures and heart rates [9]. BP indices in the control were 110/75 ± 5 mmHg; in EAH – 165/100 ± 5 mmHg; ESAH was 155/90 ± 5 mmHg.

The object of the study was the brain stem of experimental animals. Decapitation was performed under thiopental anesthesia (40 mg/kg intraperitoneally). Topographic identification of the NTS and DMN structures was carried out with the help of stereotoxic rat brain atlas [10].

Histochemical processing for immunohistochemical examination of NTS and DMN structures was conducted in a next way. After decapitation immediately, within 2–3 minutes, the brain was removed and it was placed for 20 hours in a Buen solution which was prepared ex tempore from a saturated aqueous solution of picric acid (1.2 %), concentrated formaldehyde (35–40 %) and glacial acetic acid in a ratio of 15 : 5 : 1, respectively. After 20 hours of fixation, the brain was subjected to 2 hours of washing under cold running water to wash out picric acid. The procedure was followed by dehydration of the organ in ascending concentrations of ethanol, namely: 50 %, 60 %, 70 %, 80 %, 90 %, 96 %, 100 % -1, 100 % -2, then in solutions: ethanol 100 % + chloroform 2 : 1, ethanol 100 % + chloroform 1 : 1, chloroform + paraffin (McKormick, USA) 1 : 3 (T = +37 °C), placed in liquid for 1 hour paraffin (McKormick, USA) (T = +56 °C) and then placed in paraplast blocks.

The expression of NOS isoforms was studied by an immunohistochemical method. All antibodies used were diluted 1 : 200 (Santa Cruz Biotechnology, USA). Serial 7 μm brain stem sections after above described histochemical processing were incubated with rabbit IgG to nNOS, with rabbit IgG to eNOS, with mouse IgG to iNOS conjugated to FITC. Then, secondary murine anti-rabbit IgG antibodies conjugated with FITC were applied to glasses coated with primary IgG to nNOS and eNOS (after 3 times washing in phosphate buffer solution) and placed in a mixture of glycerol / phosphate buffer (9 : 1). Specificity control was carried out by applying blocking peptides corresponding to primary antibodies. Further stages of immunohistochemical staining were carried out similarly to the method described above [11].

Immunofluorescence studies of brainstem sections prepared by the above-described method were performed in the ultraviolet spectrum using a 38 HE filter (Zeiss, Germany) on AxioImager-M2 microscope (Zeiss, Germany) through a sensitive camera AxioCam-5HRm (Zeiss, Germany). Images obtained in this way were processed interactively, with a determination of the zone corresponding to an NTS and DMN with statistically significant fluorescence. In the selected zone of interest, the content (Un), relative area of the immunoreactive material (IRM) (%) and the concentration of the studied NOS isoform in 1 μm² (Uif/μm²) were determined. The microphotographs of the NTS and DMN were processed using the image analysis program – Image J [12]. To determine the reliability of the differences in the samples studied, the Student’s test and, if necessary, the Whitney-Mann criterion were used, respectively. Differences were considered significant for P < 0.05 [13].

**Results**

A comparative analysis of NOS isoforms expression indices in the DMN structure showed significant differences between the control and experimental groups. Thus, nNOS expression values showed that in EAH rats, the content, concentration and relative area of the IRM were higher than the control values by 34.4 %, 30.5 %, and 18.0 %, respectively. At the same time, in ESAH rats only the content and concentration of IRM to nNOS were significantly higher by 70.9 % and 13.0 %, respectively (Table 1).

A comparison of nNOS expression values in animals of experimental groups with AH showed their different directions. Thus, in ESAH rats, compared to EAH rats, a significantly higher IRM content was noted by 27.1 %, but significantly
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In AH groups, the indices of iNOS expression significantly increased in comparison with normotensive control. So, in EAH animals, the IRM content to iNOS was higher by 40.9 %, the IRM concentration – by 29.0 %, and the relative area – by 21.4 %. The iNOS expression indices in ESAH group in comparison to the control were significantly higher: content by 67.8 %; concentration – 12.4 %, relative area – by 8.9 %. A comparative analysis of data between AH groups showed a similar change with nNOS values. At the same time, in the ESAH group in comparison with EAH, with significant predominance of the IRM content to iNOS by 19 %, lower concentrations were observed by 12.8 % and the relative area of the IRM by 10.6 % (Table 1).

The eNOS expression data in DMN structure of experimental animals showed that in EAH rats all studied parameters were significantly lower than the values of normotensive control. The content of IRM to eNOS was higher by 92.2 %, the concentration of IRM –by 31.7 %, the relative area of the IRM – by 11.9 %. In ESAH group, the content of IRM to eNOS in the DMN structure of was by 74.6 % higher, the concentration – by 16.7 % higher, and the relative area did not significantly differ from the control values. When comparing the parameters of the experimental groups with AH, it was found that absolutely all studied parameters of enzyme isoforms expression in animals with ESAH were significantly lower than the values of rats with EAH. The IRM content was

| Experimental groups | Content of IRM (Unit.) | Concentration of IRM (U/m²) | Relative area of the IRM (%) |
|---------------------|------------------------|-----------------------------|-----------------------------|
| nNOS                |                        |                             |                             |
| Control             | 244.24 ± 10.65         | 59.88 ± 1.38                | 45.74 ± 1.30                |
| EAH                 | 328.41 ± 17.36*        | 78.19 ± 2.34*               | 54.00 ± 0.63*               |
| ESAH                | 417.56 ± 14.03***      | 67.67 ± 1.06***             | 44.94 ± 0.56*               |
| iNOS                |                        |                             |                             |
| Control             | 266.43 ± 11.78         | 57.24 ± 1.21                | 44.64 ± 1.11                |
| EAH                 | 375.57 ± 12.69*        | 73.89 ± 1.08*               | 54.20 ± 0.75*               |
| ESAH                | 447.28 ± 13.86***      | 64.38 ± 0.62***             | 48.42 ± 0.56*               |
| eNOS                |                        |                             |                             |
| Control             | 269.38 ± 10.92         | 57.45 ± 1.33                | 45.27 ± 1.02                |
| EAH                 | 517.99 ± 14.45*        | 75.66 ± 0.96*               | 54.99 ± 0.61*               |
| ESAH                | 470.36 ± 14.09***      | 67.09 ± 0.99***             | 44.43 ± 0.73*               |

*: significant difference in parameters (P < 0.05) of rats of the experimental groups in relation to the control; #: significant difference in parameters (P < 0.05) of rats between groups with experimental arterial hypertension.

Table 1. Parameters of NOS isoforms expression in the DMN structure of experimental animals (M ± m)

| Experimental groups | Content of IRM (Unit.) | Concentration of IRM (U/m²) | Relative area of the IRM (%) |
|---------------------|------------------------|-----------------------------|-----------------------------|
| nNOS                |                        |                             |                             |
| Control             | 282.30 ± 10.47         | 64.17 ± 1.31                | 44.52 ± 1.08                |
| EAH                 | 399.76 ± 13.82*        | 77.55 ± 1.80*               | 53.47 ± 0.56*               |
| ESAH                | 480.31 ± 13.74***      | 67.60 ± 0.60***             | 45.52 ± 0.47*               |
| iNOS                |                        |                             |                             |
| Control             | 308.95 ± 12.84         | 58.11 ± 1.23                | 45.03 ± 1.10                |
| EAH                 | 473.81 ± 11.25*        | 73.60 ± 0.90*               | 52.48 ± 0.58*               |
| ESAH                | 467.31 ± 12.06*        | 63.97 ± 0.75***             | 50.25 ± 0.67*               |
| eNOS                |                        |                             |                             |
| Control             | 398.32 ± 10.75         | 61.82 ± 1.04                | 42.21 ± 1.07                |
| EAH                 | 486.00 ± 12.26*        | 79.13 ± 0.95*               | 51.93 ± 0.60*               |
| ESAH                | 537.98 ± 14.27***      | 64.86 ± 0.85***             | 45.62 ± 0.61***             |

*: significant difference in parameters (P < 0.05) of rats of the experimental groups in relation to the control; #: significant difference in parameters (P < 0.05) of rats between groups with experimental arterial hypertension.

| Experimental groups | Content of IRM (Unit.) | Concentration of IRM (U/m²) | Relative area of the IRM (%) |
|---------------------|------------------------|-----------------------------|-----------------------------|
| nNOS                |                        |                             |                             |
| Control             | 269.38 ± 10.92         | 57.45 ± 1.33                | 45.27 ± 1.02                |
| EAH                 | 517.99 ± 14.45*        | 75.66 ± 0.96*               | 54.99 ± 0.61*               |
| ESAH                | 470.36 ± 14.09***      | 67.09 ± 0.99***             | 44.43 ± 0.73*               |

*: significant difference in parameters (P < 0.05) of rats of the experimental groups in relation to the control; #: significant difference in parameters (P < 0.05) of rats between groups with experimental arterial hypertension.

Table 2. Parameters of NOS isoforms expression in the NTS structure of experimental animals (M ± m)

lower concentration by 13.45 % and the relative area of the IRM by 16.7 %, respectively (Table 1).
9.2 % lower, the concentration – 11.3 %, and the relative area – 19.2 % (Table 1).

The next stage was the study of the above-described parameters of the NOS isoforms expression in NTS structure. They are shown in Table 2.

Studied indices of nNOS expression in the EAH group of rats were significantly higher than the values of normotensive animals. Thus, the IRM content to nNOS exceeded by 41.6 %, the concentration – by 20.9 %, and the relative area of the IRM to isoform – by 20.1 %. At the same time, in EAH rats, only the IRM content and concentration to nNOS were significantly higher by 70.1 % and 5.3 %, respectively. Differences between AH groups in nNOS expression indices in NTS structure were found to be interesting. EAH animals in comparison with EAH showed significantly lower values of the IRM concentration and relative area to the studied enzyme by 12.8 % and 15.1 %, respectively. Moreover, the nNOS content in the NTS structure of EAH rats was significantly higher by 20.1 % compared with EAH rats (Table 2).

In the NTS structure in both experimental AH groups in relation to the control group, the iNOS expression indices were significantly higher. So, in EAH rats, the content, concentration and relative area of the IRM to iNOS were higher by 53.3 %, 26.6 %, and 16.5 %, respectively. In EAH animals, the prevalence of expression values relative to the control was 51.2 %, 10.1 %, 11.6 %, respectively. Comparison between AH groups showed, that in EAH animals in relation to the EAH group, only the IRM concentration and area to iNOS were significantly lower by 13.1 % and 4.2 %, respectively (Table 2).

Indices of eNOS expression in EAH rats showed significantly higher digital values compared to the control. So, the content of eNOS in the NTS structure was 22 % higher, the concentration – 28 %, the relative area – 23 %. Similar parameters in EAH rats showed differences from the control values by 35 % higher content, concentration – 4.9 %, and relative area – 8 %. Intergroup differences were also found during the analysis of eNOS expression data. It was found that the concentration and relative area of the IRM to eNOS in EAH animals compared with EAH animals was significantly lower by 18 % and 12 %, respectively, and the content was 10.7 % higher (Table 2).

Discussion

The data obtained in the study indicate that in the DMN and NTS structures in AH an increase in the expression of all 3 NOS isoforms is observed. And it has no dependence on the etiopathogenetic mechanisms of AH occurrence. At the same time, the increased content of NOS isoforms reflects the state of the nitrogen monoxide system as a whole and indicates its high activity in AH. The revealed high values of their indices in DMN and NTS in animals of both experimental AH models most likely indirectly indicate an increase in functional activity. It can be realized due to increased vascularization and neurotransmission. The result of the increased activity of DMN neurons will be an increase in the vagal effects. At the same time an increase of NTS functional activity leads to the implementation of a baroreflex response to a systemic BP increase, which contributes to its decrease. Moreover, the increased expression of three NOS isoforms, which will result in a significant NO content increase, becomes one of the key factors in progressive neurodegeneration in AH [14].

The NO-dependent mechanism of a change in the functional activity of neurons has been described quite well in the studies. It was shown in them that under NO influence in sympathetic neurons, cGMP inhibits cAMP, increasing its hydrolysis by PDE2A phosphodiesterase. This causes a decrease in cAMP-dependent phosphorylation of calcium channels and inhibition of neurotransmission. Whereas in parasympathetic neurons, NO, indirectly through cGMP, inhibits PDE3 phosphodiesterase, as a result, cAMP hydrolysis, on the contrary, decreases. This leads to increased cAMP-dependent phosphorylation of calcium channels and promotes the release of acetylcholine [5,15]. The result of increased DMN neurons activity will be an increase in the vagal effect, while NTS is the implementation of a baroreflex response to a systemic BP increase, which contributes to its decrease.

Evidence of the dependence of the NO effect from structure functional activity was obtained by Ogawa et al. It was shown that in NTS NO potentiates the release of glutamate by neurons [16], thus enhancing the baroreflex response to increased BP.

No less interesting was the research of the relationship between the NOS expression and the affinity of the imidazoline NTS receptors. Thus, it was experimentally found that inhibition of nNOS violates the central hypotensive effect of clonidine and relinimid. As a result of a decrease in the local formation of NO, the affinity of the imidazoline receptors decreases, resulting in an increase in the activity of the sympathetic nervous system and increased secretion of catecholamines [17]. While an increase in the NO content as a result of high expression of the enzymes that form it, it is necessary to consider it as one of the hypotensive mechanisms of the central regulation of blood pressure.

Another possible reason for the increased activity of the NO system may be the low bioavailability of nitrogen oxide, and together with its high values, it becomes one of the components of progressive neurodegeneration in AH. So, in a number of studies, it was shown that in AH, the bioavailability of NO decreases due to increased generation of superoxide radicals. Last ones’ formation can be enhanced by an increased amount of angiotensin II, which contributes to the activation of NADPH-oxidase, and excessive iNOS activity with the formation of peroxynitrite [18,19]. This may explain the increased expression of iNOS established in the work in the DMN and NTS structures. iNOS, as a calcium-independent isoform of the NOS in ischemic condition, becomes the main source of NO for the full functioning of neurons in AH, but due to its low bioavailability and large amount, the implementation of its physiological effects is impaired, and its metabolites become damage factors.

Summarizing, we can say that the high activity of the NO system in DMN and NTS in AH should be considered as an important element of compensating for increased BP, which is realized through a set of mechanisms: reducing of the sympathetic tone and increasing parasympathetic tone...
due to activating neuroregulatory programs for correcting vascular tone through a system of secondary messengers; improvement of neurotrophy due to the high activity of constitutive NOS isoforms; iNOS-mediated overproduction of NO, as a factor in compensating for its bioavailability in conditions of local ischemia in AH, but also the pathogenic factor of progressive neurodegeneration in hypertension.

Conclusions
Based on the study, the following conclusions can be made:
1. Regardless of the etiopathogenesis of arterial hypertension in both experimental groups the expression of all three NOS isoforms increases in the DMN and NTS structures.
2. In EAH rats in the DMN and NTS structure, the expression indices of NOS isoforms have their own characteristics. So in the first structure, the largest changes in the indices of the IRM content and concentration are observed for eNOS, and the relative area for iNOS. At the same time, in the NTS structure, the largest changes in the IRM content are observed for iNOS, and the concentration and relative area for eNOS.
3. In ESAH rats in the DMN structure, the highest changes in the indices of IRM content and concentration are observed for the endothelial isoform of NOS, and the relative area for inducible. In the NTS structure, the IRM content changed the most for nNOS, and the concentration and relative area for iNOS.

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