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The pattern of the NOS isoforms expression in arcuate nucleus of hypothalamus in experimental hypertension

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In is known, that one of the main regulators of the blood pressure (BP) is hypothalamus. The efficiency of the BP regulation in a changeable environment is dependable on its coherence and coordination. This intrahypothalamic coordinator is an arcuate nucleus. One of the main conditions of the proper neuronal function is combination of their functional stability and plasticity, which is strictly dependable on adequate blood supply, metabolic and trophic processes. One of the key regulators of the above mentioned features is nitric oxide (NO).

The purpose was to establish the pathogenetic link of NO synthases isoforms imbalance in Arc of hypothalamus with formation of hypertension in etiologically different models of essential hypertension (SHR) and endocrine-saline model (ESM).

Materials and methods. Study was conducted on 48 mature male rats with weight of 250–270 gram and age of 13–14 months, which were allocated into 3 experimental groups of 16 animals: the 1st group was control with Wistar rats; the 2nd group comprised of Wistar rats with ESM and the 3rd was made of SHR.

Results. Formation of the hypertension in ESM and SHR rats led to similar changes of the pattern of NOS isoforms expression. We found the significant increase of the IRM content to nNOS and iNOS, but decrease to eNOS. In the same time, the constitutive isoforms concentration (nNOS and eNOS) was significantly lower compared with control group, and iNOS concentration in both groups with hypertension was higher than in control group (6 % and 9 %, $p < 0.05$, respectively).

Conclusions: In rats with normal BP in Arc, the most expressed NOS isoform was the endothelial one. We found the typical changes in the pattern of the NOS isoforms allocation. We also found the increase of the content of all isoforms with the decrease of the concentration of constitutional ones (nNOS and eNOS), but the increase of the iNOS concentration.

Ключові слова: гіпоталамус, експресія, оксид азоту, артеріальна гіпертензія, щури.

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Патерн експресії ізоформ NOS в аркуатному ядрі гіпоталамуса при експериментальній артеріальній гіпертензії

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Відомо, що основним центральним регулятором артеріального тиску (АТ) є гіпоталамус та його ядра. Від злагодженості та скоординованості їхніх дій залежатиме ефективність регуляції артеріального тиску в умовах, що змінюються. Таким внутрішньогіпоталамічним координатором є аркуатне ядро. Важливою умовою ефективності роботи нейронів у структурі є поєднання функціональної стабільності з пластичністю нейрональних процесів, що забезпечується адекватним кровопостачанням, обмінними та трофічними процесами. Одним із ключових регуляторів описаних вище процесів у ЦНС є оксид азоту (NO).

Мета роботи – встановити патогенетичний зв'язок дисбалансу ізоформ NOS в АрЯ гіпоталамуса з формуванням артеріальної гіпертензії при етіологічно різних її моделях – есенціальній (щури лінії SHR) та ендокринно-сольовій (ЕСГ).

Матеріали та методи. Дослідження здійснили на 48 статевозрілих щурах-самцях масою 250–270 г, віком 13–14 місяців, які були поділені на три експериментальні групи по 16 тварин у кожній: 1 контрольна – щури лінії Wistar, 2 – щури лінії Wistar з ендокринно-сольовою моделлю гіпертензії; 3 – щури лінії SHR зі спонтанною гіпертензією.

Результати. Сформована АГ у щурів лінії SHR і ЕСГ призводила до однакових змін у патерні експресії ізоформ NOS. Спостерігалося вірогідне збільшення вмісту IPM до nNOS та iNOS, але зниження його до eNOS. При цьому концентрації конститутивних ізоформ ферменту nNOS та eNOS ставали вірогідно нижчими, ніж у контролі, а концентрація iNOS в обох групах з АГ перевищила значення щурів із нормальним артеріальним тиском на 9 % ($p < 0,05$) у SHR і в щурів з ЕСГ – на 6 % ($p < 0,05$).

Висновки. У щурів із нормальним артеріальним тиском в АрЯ гіпоталамуса найбільш представленою з ізоформ є ендотеліальна форма ферменту. При різних за етіологічним фактором АГ (есенціальна або ендокринно-сольова) в АрЯ гіпоталамуса спостерігаються однотипні зміни в патерні розподілу ізоформ NOS. Характерно збільшення вмісту у структурі ядра всіх трьох форм ферменту зі зниженням концентрації IPM до конститутивних ізоформ nNOS та eNOS, але збільшенням її до iNOS.

Ключевые слова: гипоталамус, экспрессия, оксид азота, артериальная гипертензия, крысы.

Паттерн экспрессии изоформ NOS в аркуатном ядре гипоталамуса при экспериментальной артериальной гипертензии

Ю. М. Колесник, О. В. Ганчева, С. В. Тищенко

Известно, что основным центральным регулятором артериального давления (АД) является гипоталамус и его ядра. От согласованности и скоординированности их действия будет зависеть эффективность регуляции АД в изменяющихся

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условиях. Таким внутригипоталамическим координатором является аркуатное ядро. Важным условием эффективности работы нейронов в структуре является сочетание функциональной стабильности с пластичностью нейрональных процессов, что обеспечивается адекватным кровоснабжением, обменными и трофическими процессами. Одним из ключевых регуляторов вышеописанных процессов в ЦНС является оксид азота (NO).

Цель работы – установить патогенетическую связь дисбаланса изоформ NOS в АрЯ гипоталамуса с формированием артериальной гипертензии при этиологически разных её моделях – эссенциальной (крысы линии SHR) и эндокринно-солевой (ЭСГ).

Материалы и методы. Исследование было проведено на 48 половозрелых крысах-самцах массой 250–270 г, возраст – 13–14 месяцев, которые были разделены на три экспериментальные группы по 16 животных в каждой: 1 контрольная – крысы линии Wistar, 2 – крысы линии Wistar с эндокринно-солевой моделью гипертензии; 3 – крысы линии SHR со спонтанной гипертензией.

Результаты. Сформированная АГ у крыс линии SHR и с ЭСГ приводила к однотипным изменениям в паттерне экспрессии изоформ NOS. Наблюдалось достоверное увеличение содержания ИРМ к nNOS и iNOS, но снижение его к eNOS. При этом концентрации конститутивных изоформ фермента nNOS и eNOS становились достоверно ниже, чем контроль, а концентрация iNOS в обеих группах с АГ превысила значения крыс с нормальным АД на 9 % ($p<0,05$) у SHR и у крыс с ЭСГ – на 6 % ($p<0,05$).

Выводы. У крыс с нормальным АД в АрЯ гипоталамуса наиболее представленной из изоформ является эндотелиальная форма фермента. При разных по этиологическому фактору АГ (эссенциальная или эндокринно-солевая) в АрЯ гипоталамуса наблюдаются однотипные изменения в паттерне распределения изоформ NOS. Характерно увеличение содержания в структуре ядра всех трёх форм фермента со снижением концентрации ИРМ к конститутивным изоформам nNOS и eNOS, но увеличением её к iNOS.

It is known, that hypothalamus is one of the main central regulators of the blood pressure (BP) [1]. A great number of its nuclei are involved in this function: ventromedial, paraventricular, supraoptic, periventricular, and arcuate (Arc) nucleus [2]. The BP regulation in changing conditions is dependable from coordination and consistency of these nuclei. The intrahypothalamic coordinator is arcuate nucleus [3]. Due to the topographical features and the abundance of the projections to other nuclei, this structure is considered as integrative and commutative center, which maintains interaction of both intra- and extrahypothalamic structures, which regulate autonomic function. Peruzzo called Arc the "window to hypothalamus" [4]. The important precondition of the effective neuronal activity is the combination of functional stability and plasticity provided by the adequate blood supply, metabolic and trophic processes [5].

It is proved for now, that one of the key regulators of the processes stated above in central nervous system (CNS) is nitric oxide (NO) [2,5,6]. There are data about the NO involvement into the sympathetic regulation, neurotransmission, vasodilation and apoptosis induction [2,6,7]. The implementation and direction of the NO effects depends not only on the amount and place of its synthesis, but also on the enzyme isoform mediated this synthesis. There are three isoforms of NO synthases: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). Thus, nNOS modulates a lot of physiological processes including learning, memory formation, and neurotransmission. Additionally, there are data about the involvement of nNOS into central BP regulation [8–10]. eNOS is a powerful vasodilator, also well-known inhibitor of platelets adhesion and aggregation, thus playing an important role in adequate blood supply [11]. In CNS, iNOS produced by activated macrophages and glial cells provides the toxic effect on microorganisms and tumor cells, but in the case of hyperactivity it also induces apoptosis and necrosis [12,13]. Thus, it was proved the NO produced with the iNOS activity causes peroxynitrite-mediated neuronal apoptosis [12–14].

We assume the change of the balance of NOS isoforms in the Arc may lead to violation of central hypothalamic domain of BP regulation and, probably, to the formation and development of hypertension (HT).

Considering stated above we believe that deep and detailed study of the NOS isoforms balance in Arc in HT caused by different factors will contribute to understanding the pathophysiological mechanisms of HT formation and to the search of new molecular targets of antihypertensive therapy.

The purpose of our study was to find out the pathogenetic link between the NOS isoforms imbalance and the formation of hypertension in etiologically different models: the essential in spontaneously hypertensive rats (SHR) and endocrine-saline model (ESM).

Materials and methods

Study was performed on 48 mature male rats with weight of 250–270 gram in age of 13–14 month, which were allocated to 3 experimental groups of 16 animals: the 1st control group comprised of Wistar rats; the 2nd consisted of Wistar rats with endocrine-saline model of hypertension; the 3rd group – of SHR. Experiment was performed according to the national "General ethical principles of the animal experiments" (Ukraine, 2001), concerted with Council Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

For modelling of ESM the Wistar rats were treated with prednisolone intramuscularly at 7 o'clock in dose of 2 mg/kg and at 20 o'clock in dose of 4 mg/kg during 30 days combined with the force watering with 5 ml of saline (2.3 %). From the beginning of the experiment and each 7 days we performed the BP assessment using the non-invasive system BP-2000 (Visitech Systems, USA). Rats of the 1st group showed the systolic pressure of 110 ± 5 mm Hg. The 2nd group showed 110 ± 5 mm Hg at the beginning of experiment, 145 ± 5 mm Hg on the 7th day and 165 ± 5 since

21 days of the modelling. The systolic pressure in the 3rd group was 165±5 mm Hg.

The object of the study was hypothalamus of experimental animals. We performed the immunofluorescence assessment of the NOS isoforms expression in serial paraffin-embedded slices, which were incubated with rabbit IgG (Santa Cruz Biotechnology, USA) to nNOS, iNOS and eNOS, respectively in dilution of 1:200. Then slices were incubated in plastic chambers during 24 hours (T=+4 °C), then we applied mouse anti-rabbit IgG conjugated with FITC (Santa Cruz Biotechnology, USA) in dilution of 1:200 and incubated them 2 tweis (T=+37 °C) during 45 minutes, and then covered them in glycerol/FBS (9:1). The specificity control was performed as described, but with previous application of blocking peptide to the corresponding antibodies in dilution of 1:50.

The assessment of immunofluorescence was performed in ultraviolet spectrum of emission 390 nm using 38HE light filter with high emission (Carl Zeiss) in Axioscope microscope (Carl Zeiss). The images obtained using 8-bit camera AxioCam-ERc5s (Carl Zeiss) were analyzed with ImageJ in interactive way. We marked the regions of interest in the field of view of 15 000 μm^2, where we calculated the contain of the immunoreactive material (IRM) in conditional units of immunofluorescence (U_{if}) as well as IRM concentration in 1 μm^2 ($U_{if}/_{\mu m}{}^2$). We assessed no less than 200 areas of view in each series. The obtained data was analyzed in EXCEL-7.0 (Microsoft Corp). We counted the mean and its standard error. With the aim to assess the differences in the study results we used Student's t-criterion with evaluation of significant differences according to Student's tables. Statistically significant difference was considered at $p<0.05$.

Results and discussion

During the immunofluorescence analysis of hypothalamus of rats with topographic identification of anterior hypothalamic field [15] we found that IRM to NOS isoforms were allocated predominantly in dorsolateral of Arc diffusely in neuronal cytoplasm and axons. High intensity of fluorescence was found in granules of IRM in neurons of control group and ESM on the periphery of cytoplasm and in axons (*Fig. 1–3*), whereas in SHR we found no granules to nNOS (*Fig. 1B*).

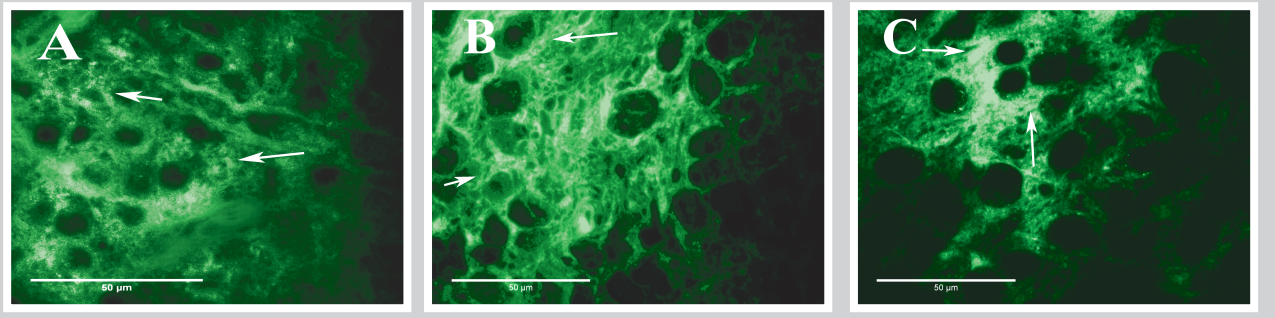

Fig. 1. Pattern of the nNOS expression in neurons of Arc in Wistar (A), SHR (B) and ESM (C). Indirect immunofluorescence, 400×. Arrows show IRM to nNOS.

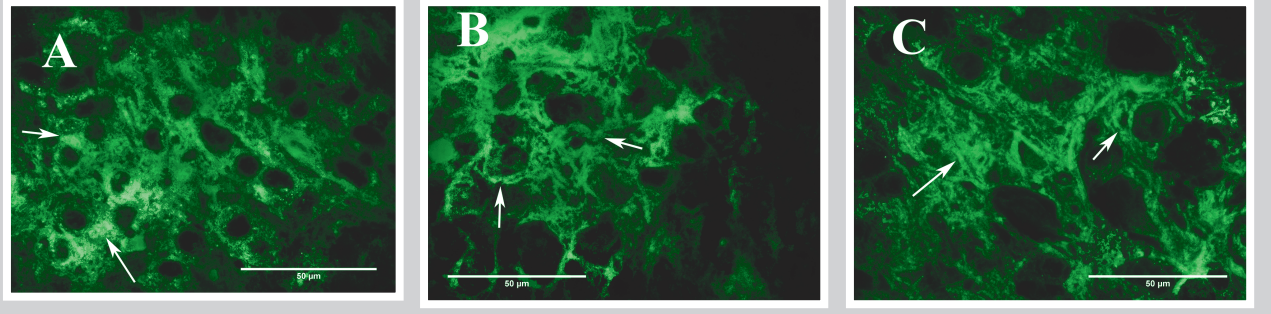

Fig. 2. Pattern of the eNOS expression in neurons of Arc in Wistar (A), SHR (B) and ESM (C). Indirect immunofluorescence, 400×. Arrows show IRM to nNOS.

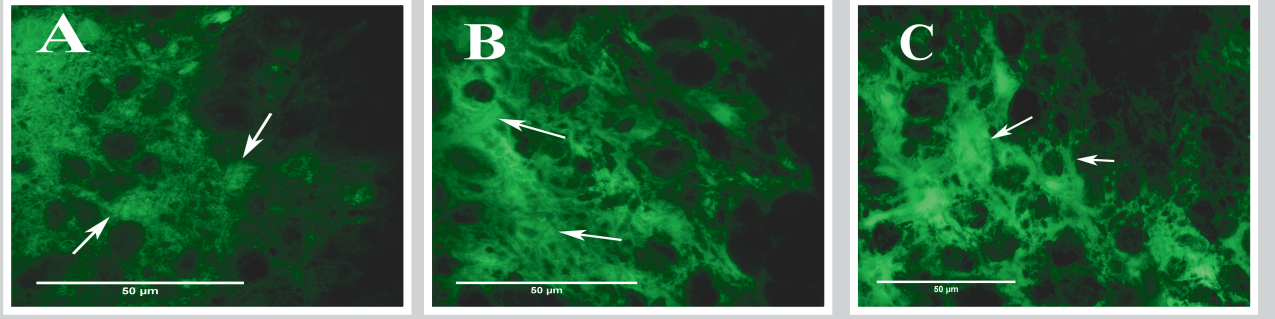

Fig. 3. Pattern of the iNOS expression in neurons of Arc in Wistar (A), SHR (B) and ESM (C). Indirect immunofluorescence, 400×. Arrows show IRM to nNOS.

After the statistical analysis of the expression of NOS isoforms in control group we found that the highest figures of IRM contain and concentration were for eNOS. The lowest IRM contain was for nNOS compared with eNOS and iNOS (45.8 % and 24.7 % respectively, $p<0.05$). The lowest IRM concentration was found for iNOS (*Table 1*).

Hypertension in rats of SHR and ESM groups leads to the same type of changes in the expression pattern of NOS isoforms. We observed significant increase of IRM concentration and contain both to nNOS and iNOS, but decrease to eNOS (*Table 1*). The concentration of constitutive isoforms of nNOS and eNOS were significantly lower than in the control group, and iNOS concentration in both hypertensive groups were higher than in Wistar (9 % for SHR and 6 % for ESM, $p<0.05$, *Table 1*).

Comparison of the NOS isoforms expression in SHR and ESM groups showed the absence of significant of IRM contain of all isoforms, whereas the IRM concentration to nNOS was significantly lower (4 %, $p<0.05$) in ESM compared with SHR (*Table 1*).

The predominance of the eNOS expression in Arc seems to be logic and expected. It is well known, that the main role of its isoform is local production of vasodilative NOS [5], which is necessary for the regulation of cerebral blood supply and neuroptrophic processes [5,6].

The fact of the same type changes of the expression of all isoforms in etiologically different hypertension became discussible. Both in essential and endocrine-saline models we found increase of both neuronal and inducible isoforms. However, in majority of clinical and bench studies the another consistent pattern was found: the forms of hypertension were accompanied with the decrease of activity and contain of constitutive isoforms and with the increase of iNOS [16,17]. We believe in could be explained with short term and absence of affection of target organs in our experiment. Additionally, the prevalence of iNOS isoform was found also in our data, and the concentration was only higher in iNOS, and constitutive ones were decreased (*Table 1*). Finally, due to features of functioning, vascularization and innervation of hypothalamus [2–4] we assume that higher contain of all the isoforms in hypertensive rats, and first of all, the constitutive ones, are compensatory with aim to provide adequate blood supply and interneuronal interactions and plasticity [5], whereas the iNOS hyperexpression is pathologic and provides the induction of nitrosative and oxidative stress, exhaustion of antioxidant defense, leads to violation of DNA reparation mechanisms and to the neurodegeneration progression [18–20].

Conclusions

1. In normotensive rats, the most predominant form of NOS in Arc is endothelial.

2. In etiologically different hypertensions, there are the same type changes in the expression pattern of NOS isoforms to be observed in Arc. The increase of contain of all the isoforms with the decreased concentration to constitutive isoforms but the increase of iNOS is typical.

Perspectives. We plan to study the features of expression of pressor and depressor neuropeptides in Arc and find out the pathogenetical link of their state with hypertension formation.

Table 1. Indexes of the expression of NOS isoforms in Arc in rats of experimental groups (M±m)

Expression figures	Groups		
	Wistar, n=16	SHR, n=16	ESM, n=16
nNOS			
IRM contain, U_{if}	967.7±21.3	1304.5±16.7[1]	1272.6±18.4[1]
IRM concentration, $U_{if}/_{\mu m}{}^2$	78.6±1.8	73.3±1.2[1]	70.2±0.9[1,2]
iNOS			
IRM contain, U_{if}	1207.5±25.1	1371.3±26.1[1]	1395.4±15.3[1]
IRM concentration, $U_{if}/_{\mu m}{}^2$	74.5±1.7	81.6±1.7	78.8±0.9[1]
eNOS			
IRM contain, U_{if}	1410.1±46.4	1289.77±18.1[1]	1296.7±20.5[1]
IRM concentration, $U_{if}/_{\mu m}{}^2$	80.9±2.6	73.3±1.1[1]	72.9±1.2[1]

[1]: significant differences ($p<0.05$) compared with control group;
[2]: significant differences ($p<0.05$) compared with SHR group.

Authors contribution. Kolesnyk Yu. M.; supervision, made critical revision of the manuscript for key intellectual content, final approval of the version to be published. Gancheva O. V.: conceived and designed the research, made critical revision of the manuscript for key intellectual content. Tischenko S. V.: conceived and designed the research; acquired and processed the data; performed statistical analysis; drafted the manuscript; made critical revision of the manuscript for key intellectual content.

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