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FEATURES OF NITRIC OXIDE SYNTHASE ISOFORMS EXPRESSION IN RAT LOCUS COERULEUS NEURONS IN EXPERIMENTAL HYPERTENSION OF VARIOUS ORIGINS

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The aim of this work was to establish the expression of of nitric oxide synthase isoforms in the locus coeruleus in rats under experimental arterial hypertension of various origins (essential and endocrine-saline). To achieve the aim, the study was performed on 20 Wistar mature male rats and 10 male rats of the SHR line. 10 Wistar male rats made up a control group, other 10 were modeled endocrine-saline hypertension. The object of the study was the brain stem of rats. To study the pattern of expression of nitric oxide synthase isoforms serial sections of the brainstem were processed with immunofluorescence method of enzyme identification. It was found that the pattern of expression and balance between different isoforms of nitric oxide synthase in the locus coeruleus of rats with arterial hypertension differs from the pattern of normotensive animals. The features of expression of nitric oxide synthase isoforms in arterial hypertension depends on the mechanism of its development. Thus, the highest expression indices for all three isoforms of the enzyme were observed in essential hypertension (model - SHR line). There was an increase in the area of the immunoreactive material for all three isoforms in endocrine-saline hypertension, whereas their concentration increased only for endothelial isoform of nitric oxide synthase whereas the concentration of the neuronal isoform did not change, while the inducible isoform decreased.

Key words: brain stem, locus coeruleus, NOS isoforms, arterial hypertension.

Today the participation of locus coeruleus (LC) in the regulation of various functions of the body is being widely discussed in the scientific literature [1, 2]. Mechanisms of systemic blood pressure (BP) control by LC neurons have been clearly established [3, 4]. This predetermines the interest in studying the functional state of LC in hypertension. Recently, the system of nitric oxide (NO) and the enzyme necessary for the formation of NO –nitric oxide synthase (NOS), which is represented by three isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS), have been considered to be modulators of the central nervous system neurons functional state [5,6]. All three isoforms of NOS were detected in the LC [7, 8, 9, 10].

In our opinion, the imbalance in the NOS isoforms expression in the LC structure can change its functional state, by modulating the interneuronal relationships, vascularization and neurotransmission.

The aim of the work was to detect and describe the features of expression of NOS isoforms in the LC structure of rats under experimental models of arterial hypertension - essential and endocrine-saline.

Materials and methods

The study was performed on 20 mature male Wistar rats and 10 male rats of the SHR line 250-270 grams weight. 10 male Wistar rats made up a control group, other 10 were modeled endocrine-saline hypertension (ESH). ESH was modeled by intraperitoneal injection of prednisolone (30 days, 2 times a day, 7:00 a.m. – 2 mg/kg, 8:00 p.m. - 4 mg/kg, with forced intake of 5 ml of a 2,3% solution of NaCl). The mean arterial pressure in the control was 83,8 mmHg; in ESH was 137,8 mmHg; SHR - 125,8 mmHg. 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 Parliment 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).

The material of the study was the brain stem of experimental animals. Decapitation was performed under thiopental anesthesia (40 mg/kg intraperitoneally). Topographic identification of the structure was carried out with stereotaxic rat brain atlas [11].

Expression of NOS isoforms was studied by an immunohistochemical method. Serial 7 μm brain stem sections after preliminary histochemical processing were incubated with rabbit IgG to nNOS (1:200 dilution of Santa Cruz Biotechnology, USA), with rabbit IgG to eNOS (Santa Cruz Biotechnology, USA) (dilution 1:200), with mouse IgG to iNOS conjugated to FITC (Santa Cruz Biotechnology, USA) (1:200). Then, secondary murine anti-rabbit IgG antibodies conjugated with FITC (Santa Cruz Biotechnology, USA) (1:200) were applied to glasses coated with primary IgG to nNOS and eNOS (after 3 times washing in phosphate buffer solution).

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 AXIOSKOP microscope through a sensitive camera COHU 4922 (COHU Inc., USA). Images obtained in this way were processed interactively, with a determination of the zone corresponding to a LC with statistically significant fluorescence. In the selected zone of interest the relative area of the immunoreactive material (IRM) (%) and the concentration of the studied NOS isoform in 1 μm^2 (Uif / μm^2) were determined. The microphotographs of the LC were processed using the image analysis program - Image J. To determine the reliability of the differences in the sam-

ples studied the Student's test and, if necessary, the Whitney-Mann criterion, with the Bonferoni and Dann amendment, were used, respectively. Differences were considered significant for p <0,05.

Results and discussion

In the course of the study it was found that the studied parameters of NOS isoforms expression in the LC in intact animals and animals with different models of arterial hypertension had significant differences.

Thus, the concentration indices of all NOS isoforms in SHR rats were significantly higher (nNOS by 43,8%, iNOS by 35,8%, eNOS by 54,9%) in comparison with the control group. In animals with ESH significant differences were established only for iNOS and eNOS isoforms: iNOS concentration significantly decreased by 5,5%, and eNOS significantly increased by 15%. It should be mentioned that the inter-model differences of the concentration of the immunoreactive material to NOS isoforms in ESH rats in comparison to SHR rats were also reliable. In the group of SHR rats the concentrations of all studied enzymes were higher: nNOS by 44,9%, iNOS by 43,7%, eNOS by 34,6% than in rats with ESH (Table 1).

The relative area of the immunoreactive material for NOS isoforms in the LC in hypertensive rats was significantly increased: in SHR: nNOS by 41%, iNOS by 49,8%, eNOS by 35,2%; ESH: nNOS by 19%, iNOS by 22,7%, eNOS by 17,4%. In the ESH rats, the relative area of the IRM to the isoforms was significantly lower than in the SHR rats: nNOS by 18,4%, iNOS by 22,1% and eNOS by 15,1% (Table 2).

Table 1

Concentrations (Uif/μm^2) of NOS isoforms in the LC in rats of experimental groups, (M ± m)

Animal lines / Isoforms of NOS	Wistar, n=10	SHR, n=10	ESH, n=10
nNOS	6,48±0,12	9,32±0,18[1]	6,43±0,15[2]
iNOS	7,04±0,1	9,56±0,19[1]	6,65±0,15[1, 2]
eNOS	5,97±0,13	9,25±0,14[1]	6,87±0,16[1, 2]

Notes: 1). (1) - significant differences in the indices (p <0,05) in the rats of the experimental groups toward to control.
2). (2) significant differences in the indices (p <0,05) in rats with ESH are significant toward to the SHR rats.

Table 2

The relative area of the immunoreative material (%) to the NOS isoforms in the LC of rats in experimental groups (M ± m)

Animal lines / Isoformes of NOS	Wistar, n=10	SHR, n=10	ESH, n=10
nNOS	42,30±0,81	59,65±0,56[1, 2]	50,37±0,72[1, 2]
iNOS	40,80±0,71	61,13±0,71[1, 2]	50,06±0,68[1, 2]
eNOS	42,42±0,88	57,35±0,98[1, 2]	49,80±0,58[1, 2]

Notes: 1). (1) - significant differences in the indices (p <0.05) in the rats of the experimental groups toward to control.
2). (2) significant differences in the indices (p <0.05) in rats with ESH are significant toward to the SHR rats.

It has been proved that NO has a stimulating effect on the LC neurons [12]. This fact was shown in studies by Zhi-Qing Xu et al. and confirmed by the electrophysiological method [13]. Taking into account these facts, we can suppose that an increase of the nNOS expression in SHR rats in the LC indicates a high degree of excitation/activity of the neurons of this structure. At the same time in rats with ESH model this fact is not observed.

The increase of the expression of eNOS which was observed in the LC of rats with experimental hypertension may indicate an increase in vascularization within the LC, which may also be due to an increase in the activity of neurons in the structure under study. This fact was noted in both models of arterial hypertension.

The fact of iNOS expression increase in the LC of hypertensive rats may result from several causes. Previously, it was believed that iNOS expression in neurons occurs only during an immune response. Increased iNOS expression was associated with neurones death due to activation of phagocytic NADP oxidase in microglia [14]. But to this date it has been shown that iNOS can be produced constitutionally in neurons, microglia and astrocytes [15]. At the moment, its role in these cells is actively discussed.

Conclusions

Based on the results of our study, we can draw the following conclusions:

1. The expression and the balance of NOS isoforms in the LC neurons of the brain stem in hypertensive animals have differences in comparison with normotensive animals and depend on the etiological factor of hypertension.

2. Peculiarities of NOS expression in various models of hypertension depend on the leading mechanism of its development. Thus, in the case of essential hypertension (SHR rats) the highest expression indices of all three isoforms are observed. In rats with ESH an increase in the area of the IRM for all three NOS isoforms was observed probably due to bigger amount of activated neurons or a due to more diffuse enzyme distribution in the cells. At the same time their concentration which is indicative of the synthetic processes activity in neurons, increased only for NOS, whereas - the concentration of nNOS did not change, and iNOS concentration- decreased.

Prospects for further research: In order to assess more accurately the functional activity of the LC neurons, it is necessary to perform morphodensitometric examination of LC neurons.

References

1. Douglas L. F. Causes, consequences and cures for neuroinflammation mediated via the locus coeruleus: noradrenergic signaling system / F.L. Douglas, S. Kalinin, D. Braun // Journal of Neurochemistry. – 2016. – Vol. 139. – P. 154-178.
2. Aston-Jones G. Locus coeruleus: from global projection system to adaptive regulation of behavior / G. Aston-Jones, B. Waterhouse // Brain research – 2016. – Vol. 1645. – P. 75-78.
3. Wood C.S. Individual differences in the locus coeruleus-norepinephrine system: relevance to stress-induced cardiovascular vulnerability / C.S. Wood, R.J. Valentino, S.K.Wood // Physiology & behavior – 2017. – Vol. 172. – P. 40-48.
4. 4. Wang X. Optogenetic stimulation of locus coeruleus neurons augments inhibitory transmission to parasympathetic cardiac vagal neurons via activation of brainstem б1 and в1 receptors / X. Wang, R.A. Pinol, P. Byrne [et al.] // J Neurosci. – 2014. – V.34(18). – P. 6182-6189.
5. Lorenc-Koci E. Role of nitric oxide in the regulation of motor function. An overview of behavioral, biochemical and histological studies in animal models / E. Lorenc-Koci, A. Czarnecka // Pharmacological reports – 2013. – Vol. 65. – P. 1043-1055.
6. Belefontaine N. Nitric oxide as key mediator of neuron-to-neuron and endothelia-to-glia communication involved in the neuroendocrine control of reproduction / N. Belefontaine, N.K. Hanchate, J. Parkash [et al.] // Neuroendocrinology – 2011. - Vol. 92. – P. 74-89.
7. 7 Sanchez–Padilla J. Mitochondrial oxidant stress in locus coeruleus is regulated by activity and nitric oxide synthase / J. Sanchez–Padilla, J.N. Guzman, E. Ilijic [et al.] // Nat. Neurosci. – 2014. − № 17. – P. 832–840.
8. Pablos P. Contribution of nitric oxide dependent guanylate cyclase and reactive oxygen species signaling pathways to desensitization of m-opioid receptors in the rat locus coeruleus / P. Pablos, A. Mendiguren, J. Pineda // Neuropharmacology. – 2015. – Vol. 99. – P. 422-431.
9. Santamarta M.T. Involvement of neuronal nitric oxide synthase in desensitization of μ-opioid receptors in the rat locus coeruleus / M.T. Santamarta, J. Liorente, A. Mendiguren [et al.] // Journal of psychopharmacology. – 2014. – Vol. 28. – P. 903-914.
10. Shelkar G.P. Interactions of nitric oxide with б2-adrenoreceptors within the locus coeruleus underlie the facilitation of inhibitory avoidance memory by agmatine / G.P. Shelkar, S.G. Gakare, S. Chakraborty [et al.] // Br. J. Pharmacol. – 2016. – Vol. 173. – P. 2589-2599.
11. Paxinos G. The rat brain in stereotaxic coordinates / G. Paxinos, Ch. Watson. − Academic Press, 1998. – 474 c.
12. Athineos P. Nitric oxide: a universal modulator of brain function / P. Athineos // Current medical chemistry. – 2016. – Vol. 23 – P. 2643-2652.
13. Xu Z-Q. A functional role for nitric oxide in locus coeruleus: immunohistochemical and electrophysiological studies. / Z-Q.Xu V. A. Pieribone, X.Zhang [et al.] // Experimental brain research. – 1994. – Vol. 98. – P. 75-83.
14. 14.Villanueva C. Subcellular and cellular locations of nitric oxide synthase isoforms as determinants of health and disease / C. Villanueva, C. Giulivi // Free radical biology and medicine. – 2010. – Vol. 49. – P. 307-316.
15. Amitai Y. Physiologic role for «inducible» nitric oxide synthase: a new form of astrocytic-neuronal interface. / Y. Amitai // Glia. – 2010. – Vol. 58. – P. 1775-1781.

Реферат

ОСОБЕННОСТИ ЭКСПРЕССИИ ИЗОФОРМ СИНТАЗЫ ОКСИДА АЗОТА В НЕЙРОНАХ LOCUS COERULEUS КРЫС ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ АРТЕРИАЛЬНОЙ ГИПЕРТЕНЗИИ РАЗЛИЧНОГО ГЕНЕЗА

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Ключевые слова: ствол мозга, locus coeruleus, изоформы NOS. артериальная гипертензия.

Целью нашей работы было установить особенности экспрессии изоформ синтазы оксида азота в структуре голубого пятна у крыс при экспериментальной артериальной гипертензии различного генеза (эссенциальной и эндокринно-солевой). Для реализации вышеуказанной цели было проведено исследование на 20 крысах-самцах линии Wistar и 10 крысах-самцах линии SHR. 10 самцов Wistar служили контролем, у 10 – смоделировали эндокринно-солевую гипертензию. Объектом исследования был ствол мозга. Для изучения паттерна экспрессии изоформ синтазы оксида азота использовали иммунофлюоресцентный метод идентификации фермента в серийных срезах ствола мозга. В ходе проведенного исследования было установлено, что паттерн экспрессии и баланс различных изоформ синтазы оксида азота в структуре голубого пятна крыс при артериальной гипертензии имеет отличия от нормотензивных животных. Особенности экспрессии изоформ синтазы оксида азота при артериальной гипертензии зависят от механизма её формирования. Так, наиболее высокие цифры экспрессии всех трех изоформ изучаемого энзима наблюдаются при эссенциальной гипертензии, моделью которой являлись крысы линии SHR. При эндокринно-солевой гипертензии отмечено увеличение площади иммунореактивного материала для всех трех изоформ, тогда как их концентрация увеличивалась только эндотелиальной изоформы синтазы оксида азота, тогда как – концентрация нейрональной не изменялась, а индуцибельной - снижалась.

Реферат

ОСОБЛИВОСТІ ЕКСПРЕСІЇ ІЗОФОРМ СИНТАЗИ ОКСИДУ АЗОТУ В НЕЙРОНАХ LOCUS COERULEUS ЩУРІВ ПРИ АРТЕРІАЛЬНІЙ ГІПЕРТЕНЗІЇ РІЗНОГО ҐЕНЕЗУ

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Ключові слова: стовбур мозку, locus coeruleus, ізоформи NOS. артеріальна гіпертензія.

Метою нашої роботи було встановити особливості експресії ізоформ синтази оксиду азоту в структурі блакитної плями у щурів при експериментальній артеріальній гіпертензії різного ґенезу (есенціальній та ендокринно-сольовій). Для реалізації вищезазначеної мети було проведене дослідження на 20 щурах-самцях лінії Wistar та 10 щурах-самцях лінії SHR. 10 самців Wistar слугували контролем, у 10 – змоделювали ендокринно-сольову гіпертензію. Об’єктом дослідження був стовбур мозку. Для вивчення паттерну експресії ізоформ синтази оксиду азоту використовували імунофлюоресцентний метод ідентифікації ферменту в серійних зрізах стовбура мозку. В ході проведеного дослідження було встановлено, що паттерн експресії та баланс різних ізоформ синтази оксиду азоту в структурі блакитної плями при артеріальній гіпертензії має відмінності від нормотензивних тварин. Особливості експресії ізоформ синтази оксиду азоту при артеріальній гіпертензії залежать від механізму її формування. Так, найбільш високі цифри експресії всіх трьох ізоформ ферменту що вивчається спостерігаються при есенціальній гіпертензії, моделлю котрої були щури лінії SHR. При ендокринно-сольовій гіпертензії відмічено збільшення площі імунореактивного матеріалу всіх трьох ізоформ, в той час як їх концентрація збільшилась тільки ендотеліальної ізоформи синтази оксиду азоту, тоді як концентрація нейрональної ізоформи не змінювалась, а індуцибельної – знижувалась.