Effect of nitric oxide on the expression of apelin receptor mRNA in rat caudate nucleus

Bo BAI, Yu-Hong LIU, Hai-Qing LIU

Department of Neurobiology, Taishan Medical College, Taian 271016, China

Abstract: Objective To investigate the effect of nitric oxide (NO) on the expression of apelin receptor mRNA, as well as their correlation, in the caudate nucleus of rat. **Methods** L-Arginine (L-Arg), *N*^G-nitro-L-arginine methyl ester (L-NAME) and normal saline (NS) was separately microinjected into rat caudate nucleus. Expressions of neuronal NO synthase (nNOS) mRNA and apelin receptor mRNA were detected by RT-PCR at 4, 8, 12, 24 and 48 h after microinjection, and their correlation was determined. **Results** The expressions of nNOS mRNA and apelin receptor mRNA were both significantly increased after microinjection of L-Arg, but significantly decreased after microinjection of L-NAME compared with the NS control group. The nNOS mRNA had a positive correlation with the expression of apelin receptor mRNA after microinjection of L-Arg and L-NAME. **Conclusion** The activity of NOS in the central nervous system, especially in the caudate nucleus, is one of the key factors for NO to exert many kinds of biological actions, such as modulation of central pain, as a neurotransmitter. The neurobiological action of NO in rat caudate nucleus may be associated with apelin receptors.

Keywords: nitric oxide; apelin receptor; pain; caudate nucleus; reverse transcription polymerase chain reaction

1 Introduction

Nitric oxide (NO) plays an important role in modulation of pain, cerebral ischemia, epilepsy, and morphine tolerance and dependence in central nervous system^[1,2]. The novel peptide apelin is an endogenous ligand of putative receptor protein related to the angiotensin receptor AT1 (APJ). Besides several physiological actions, it is engaged in immunoregulation^[3]. Previous studies showed that apelin could increase the NO production in the central nervous system^[4]. In other word, the physiological effect of apelin is associated with NO. Caudate nucleus is one of the high-level centers engaged in pain modulation. We have previously investigated the pain threshold of rats by means of radioimmunoassay and immunohistochemistry. Our data suggested that NO in the caudate nucleus could transmit pain messages^[5]. However, the molecular mechanisms of NO participating the pain modulation in the caudate nucleus, as well as the relationship between NO and the expression of apelin receptor mRNA, have not been reported. This

study was designed to investigate the changes of neuronal nitric oxide synthase (nNOS) mRNA and the apelin receptor mRNA by RT-PCR and tried to determine the action pathway of NO and its internal relationship to the apelin receptor.

2 Materials and methods

2.1 Animal treatment Healthy male Wistar rats ($n = 45$, 220-250 g) were supplied by Shandong University Medical Animal Center. All of the animals were given sufficient water and food for one week. After intraperipheral administration of pentobarbital sodium (40 mg/kg), the anesthetized animals were fastened to the brain locators (Jiangwan I-C, Shanghai, China). Following the Pellegrino LJ atlas^[6], a stainless steel tube (diameter 0.6 mm) was implanted at the coordinate of A 2.0, LR 2.5 and H 5.0 and fixed by 502 glue and self-curing denture acrylic. The experiment was done 3-5 d after the operation.

The animals were randomly divided into three groups: L-Arginine (L-Arg), *N*^G-nitro-L-arginine methyl ester (L-NAME) and normal saline (NS) groups. Three samples were taken at each time point. The whole RT-PCR process was repeated three times separately for each sample.

The injector was inserted into a fixed catheter with its point sticking out 1 mm. L-Arg (5 mmol/L, 0.5 μ L) or L-NAME (4 mmol/L, 0.5 μ L) was microinjected slowly.

Corresponding author: Bo BAI
Tel: 86-538-6229908
E-mail: bbai@tsmc.edu.cn
Article ID: 1673-7067(2007)03-0180-05
CLC number: Q426
Document code: A
Received date: 2007-01-29

2.2 Instruments and reagents The instruments were PCR system (No. iCycler, Thermal Bio-Rad) and running gel imaging analysis system (Gel Doc 2000, Bio-Rad Co., USA).

The main reagents were: L-Arg and L-NAME (both from Sigma), TRIzol Reagent (Invitrogen), reverse transcription kit (Promega), PCR reaction system such as Taq DNA polymerase (Takara Co., Japan) and agarose (BioAsia Co., Shanghai, China).

2.3 Extraction of total RNA At 4, 8, 12, 24 or 48 h after the injection, the rats were decapitated and their encephala were obtained. Bilateral caudate nuclei were rapidly isolated at the hypothermia and then separately put into the homogenate containing 1 mL TRIzol reagent for incubation for 5 min at room temperature. After 0.2 mL of chloroform was added, the homogenate was violently oscillated for 15 s and stored for 2-3 min at room temperature, then centrifuged at 12 000 g for 10 min at 4 °C. The upper aqueous phase was put into another 1.5 mL centrifugal tube and mixed with 500 µL of isopropanol for 10 min at room temperature, then centrifuged at 12 000 g for 10 min again. The pellets were washed with 75% alcohol (prepared by DEPC water), and centrifuged additionally at 7 500 g for 5 min. Discard the supernatant fluid. The pellet was dried off for 5-10 min at room temperature and dissolved in 20 µL of RNase-free water. The extracted total RNA was preserved at -80 °C. Two microlitres were used for primary quantification with ultraviolet spectrophotometer.

2.4 Reverse transcription Two micrograms of total RNA and 0.5 µg of random primer, with supplement of de-ionized water to 15 µL, were heated at 70 °C for 5 min and then ice-bathed for 5 min. The 5 × reactive buffer solution [dNTPs (10 mmol/L) 1.25 µL, RNAase inhibitor fermentas (40 U/µL) 1 µL, M-MLV reverse transcription enzyme (200 U/µL) 1 µL] were added, and de-ionized water was supplemented to total volume of 25 µL. The solution was hatched at 37°C for 1 h and then heated at 70 °C for 10 min. The resultant product was preserved at -20 °C.

2.5 PCR amplification and agarose gel electrophoresis PCR primers were synthesized by Invitrogen Company. The detection primers of nNOS, apelin receptor and 18 s rRNA (as the internal control) discovered the relevant mRNA sequences^[7] (NM_052799, NM_031349 and DQ235090, respectively) from NCBI GenBank. The primers were as follows: nNOS, forward: 5'-CAGACCTGATTTCGAGGAGGTG-3', reverse: 5'-TGAAGGTGGTCTCCAGATGTG-3'; apelin receptor, forward: 5'-GCTCAGCAGCTACCTCAT

CTT-3', reverse: 5'-GGC CACCATAGAGTAGTCCAT-3'; and 18 s, forward: 5'-GTAACC CGT TGAACC CCA TT-3', reverse: 5'-CCATCC AAT CGG TAG TAG CG-3'.

PCR amplification reaction parameters of nNOS and 18 s were: pre-degeneration for 4 min at 94 °C, degeneration for 30 s at 94 °C, annealing for 30 s at 60 °C, elongation for 30 s at 72 °C, for 20-30 cycles, and finally a continual elongation for 5 min at 72 °C. PCR amplification reaction parameters of apelin receptor were: pre-degeneration for 4 min at 94 °C, degeneration for 30 s at 94 °C, annealing for 30 s at 55 °C, elongation for 40 s at 72 °C, for 34 cycles, and finally continual elongation 5 min at 72 °C. Ten microlitre of PCR product was used for electrophoresis at 1% agarose gel for observation and photography under ultraviolet lamp. The pictures were stored in the running gel imaging system, and analyzed with Quantity One software (Bio-Rad, USA). The absorbance represented the expression capacity. The relevant quantity of expression was estimated by the absorbance ratio compared with 18 s rRNA. The whole RT-PCR process was repeated at least 3 times.

2.6 Statistical analysis All variables were presented as mean ± SD. The statistical processing was carried out using SPSS10.0 with one-way ANOVA. Correlation analysis was performed with Pearson's correlation method.

3 Results

3.1 Effects of NO on the expression of nNOS mRNA in the caudate nucleus At 4 h after microinjection of L-Arg into rat caudate nucleus, the expression of nNOS mRNA began to rise, reached the peak value of 1.81 ± 0.09 ($P < 0.01$) at 12 h, and then steadily lowered down. However, there was still significant difference between L-Arg group and NS group within 24 h. The nNOS mRNA began to decrease at 8 h after microinjection of NOS inhibitor L-NAME and decreased to 0.37 ± 0.13 at 12 h ($P < 0.01$). Then the expression steadily increased, but was still significantly lower than that in the NS control group within 8-24 h ($P < 0.05$). (Fig. 1, 2)

3.2 Effects of NO on the expression of apelin receptor mRNA in the caudate nucleus At 8 h after microinjection of L-Arg into the caudate nuclei, the expression of apelin receptor mRNA began to increase, and reached the peak value of 1.88 ± 0.06 ($P < 0.01$) at 12 h. And then it steadily lowered down, but there was still significant difference between the L-Arg and the NS control groups within 24 h. At 8 h after microinjection of NOS inhibitor L-NAME, the expression of apelin receptor mRNA began to decrease, and at 12 h it

Fig. 1 Results of RT-PCR. The products of RT-PCR for 18 s, nNOS and apelin receptor are 151 bp, 203 bp, and 259 bp, respectively. A: 18 s rRNA; B: nNOS in L-NAME group; C: nNOS in L-Arg group; D: apelin receptor in L-NAME group; E: apelin receptor in L-Arg group. M, 1, 2, 3, 4, 5 and 6 are DL2000 marker, 4 h, 8 h, 12 h, 24 h, 48 h and NS, respectively.

Fig. 2 Changes of nNOS mRNA in the rat caudate nucleus at different times after injection of chemicals. * $P < 0.05$, ** $P < 0.01$ vs NS (control) group.

Fig. 3 Changes of apelin receptor mRNA in rat caudate nucleus at different times after injection of chemicals. * $P < 0.05$, ** $P < 0.01$ vs NS (control) group.

decreased to 0.41 ± 0.04 ($P < 0.01$). It increased steadily thereafter, but was still significantly lower than that in the NS group within 8-24 h ($P < 0.01$). (Fig. 1, 3)

3.3 Correlation analysis of nNOS mRNA versus apelin receptor mRNA expression

After microinjection of L-

NAME into rat caudate nuclei, Pearson's correlation coefficient of nNOS mRNA versus apelin receptor mRNA expression showed dramatic positive correlation ($r = 0.997$, $P < 0.01$). After L-Arg microinjected, Pearson's correlation coefficient of nNOS mRNA versus apelin receptor mRNA expression also showed positive correlation ($r = 0.950$, $P < 0.05$). After microinjection of normal saline there was no correlation in Pearson's correlation coefficient ($r = 0.310$, $P > 0.05$) between nNOS mRNA expression and apelin receptor mRNA expression.

4 Discussion

The effects of NO, an important message molecule in the nervous system, have been increasingly valued^[2]. It is demonstrated that NO exerts effects in normal physiological activity. Lots of data indicate that NO also exerts effects in hyperalgesia development and maintenance. Experiments suggested that inhibition of NOS activity in acute and chronic pain could decrease hyperalgesia^[8].

The production of NO is mediated by NOS, which degrades L-Arg. Now it is recognized that NOS is the key enzyme to synthesize NO, and NOS inhibitor could reduce NO production^[5]. NOS has 3 subtypes: neuronal NOS (nNOS), endothelial NOS (eNOS) and induction NOS (iNOS). Ca^{2+} /CaM-dependant nNOS is widely distributed in the central nervous system^[9]. Because of its unstable nature, easy diffusion and only 3-5 s of biological half life, NO is difficult to be directly used in experiments. Consequently, NOS, NOS substrates, specific or non-specific inhibitors, or NO donor substances are commonly used to reflect the effects of NO.

In this study, L-Arg, L-NAME and NS were separately microinjected into rat caudate nuclei and the following expressions of nNOS mRNA and apelin receptor mRNA were determined by RT-PCR technique. The results showed that the expression of nNOS mRNA was stronger in the L-Arg group but lower in the L-NAME group than the NS group, and both had statistical significance. NO rapidly diffuses among neurons and combines soluble guanylate cyclase (GC) to form NO-heme-GT complex, which catalyzes guanine triphosphate to produce cGMP. cGMP stimulates cGMP-dependent protein kinase to influence the signal conduction of cell receptor^[10,11].

Apelin, a natural ligand to APJ, is widely expressed in the central nervous system and lots of peripheral organs. Studies demonstrate that apelin can exert many kinds of physiological effects through paracrine and autocrine modes, e.g. activate phospholipase C (PLC) via APJ receptor, increase intracellular Ca^{2+} level by way of PLC-inositol triphosphate (IP_3), activate Ca^{2+} /CaM-dependent nNOS, induce NO production, and exert powerful physiological effects by NO-cGMP pathway, and these effects can be greatly inhibited by NOS inhibitor^[12-15].

Data substantiate the important roles of NO and apelin played in the central pain-regulating actions^[1,14,15]. However, the effects of NO on the apelin expression and their correlation had not been reported. This research investigated the changes of apelin receptor mRNA at a given time by microinjection of L-Arg, L-NAME and NS into the caudate nuclei. The results revealed that the expression of apelin receptor mRNA was significantly increased in the L-Arg group but obviously decreased in the L-NAME group compared with the NS control. Both had statistical significance within a given time. The expressions of nNOS mRNA and apelin receptor mRNA showed a significant correlation by using Pearson's correlation analysis.

In conclusion, L-Arg can induce more NO production in the presence of nNOS, but L-NAME can decrease the NO production by inhibiting the nNOS activity. The physiological actions of NO may be associated with the activation of apelin receptor.

Acknowledgements: This work was supported by the Natural Science Foundation of Shandong Province, China (No. Y2003D01 and No. Y2005C54) and a grant from Department of Science and Technology of Shandong Province, China (No. 2001BB1CDA1).

References:

- [1] Ciani E, Guidi S, Bartesaghi R, Contestabile A. Nitric oxide regulates cGMP-dependent cAMP-responsive element binding protein phosphorylation and Bcl-2 expression in cerebellar neurons: implication for a survival role of nitric oxide. *J Neurochem* 2002, 82: 1282-1289.
- [2] Haley JE. Gases as neurotransmitters. *Essays Biochem* 1998, 33: 79-91.
- [3] Masri B, Knibiehler B, Audigier Y. Apelin signalling: a promising pathway from cloning to pharmacology. *Cell Signal* 2005, 17: 415-426.
- [4] Jaszberenyi M, Bujdoso E, Telegdy G. Behavioral, neuroendocrine and thermoregulatory actions of apelin-13. *Neuroscience* 2004, 129: 811-816.
- [5] Liu YH, Liu WY, Liu HQ, Bai B. The involvement of nitric oxide in pain modulation in rat caudate nucleus. *Chin J Pain Med* 2006, 12: 163-166. (Chinese, English abstract)
- [6] Pellegrino LJ. A stereotaxic atlas of rat brain. 2nd edition. New York: Plenum Press, 1979, 18-21.
- [7] Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J Biochem Biophys Methods* 2000, 46: 69-81.
- [8] Meller ST, Pechman PS, Gebhart GF, Maves TJ. Nitric oxide mediates the thermal hyperalgesia produced in a model of neuropathic pain in the rat. *Neuroscience* 1992, 50: 7-10.
- [9] Lowenstein CJ, Glatt CS, Bredt DS, Snyder SH. Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc Natl Acad Sci USA* 1992, 89: 6711-6715.
- [10] Xu JY, Hill KP, Bidlack JM. The nitric oxide/cyclic cGMP system at the supraspinal site is involved in the development of acute morphine antinociceptive tolerance. *Pharmacol Exp Ther* 1998, 284: 196-201.
- [11] Chan MH, Chien TH, Lee PY, Chen HH. Involvement of NO/cGMP pathway in toluene-induced locomotor hyperactivity in female rats. *Psychopharmacology (Berl)* 2004, 176: 435-439.
- [12] Losano G, Penna C, Cappello S, Pagliaro P. Activity of apelin and APJ receptors on myocardial contractility and vasomotor tone. *Ital Heart J Suppl* 2005, 6: 272-278.
- [13] Tatemoto K, Takayama K, Zou MX, Kumaki I, Zhang W, Kumano K, *et al*. The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. *Regul Pept* 2001, 99: 87-92.
- [14] Szokodi I, Tavi P, Foldes G, Voutilainen-Myllyla S, Ilves M, Tokola H, *et al*. Apelin, the novel endogenous ligand of the orphan receptor APJ, regulates cardiac contractility. *Circ Res* 2002, 91: 434-440.
- [15] Charles CJ, Rademaker MT, Richards AM. Apelin-13 induces a biphasic haemodynamic response and hormonal activation in normal conscious sheep. *J Endocrinol* 2006, 189: 701-710.

大鼠尾核内一氧化氮对 apelin 受体 mRNA 表达的影响

白波, 刘玉红, 刘海青

泰山医学院神经生物学教研室, 泰安 271016

摘要: 目的 研究大鼠尾核内一氧化氮(nitric oxide, NO)的作用机理及其与 apelin 受体 mRNA 表达的相关性。

方法 大鼠尾核内微量注射 NO 前体 L-精氨酸(L-Arg)、*N*^G-硝基-L-精氨酸甲酯(*N*^G-nitro-L-arginine methyl ester, L-NAME)和生理盐水(NS), 应用逆转录-聚合酶链反应(RT-PCR)检测大鼠尾核给药 4、8、12、24 及 48 h 后一氧化氮合酶(neuronal nitric oxide synthase, nNOS) mRNA 和 apelin 受体 mRNA 表达的变化及二者的相关性。

结果 注射 L-Arg 后大鼠尾核 nNOS 和 apelin 受体的 mRNA 表达明显升高, 注射 L-NAME 后大鼠尾核 nNOS 和 apelin 受体 mRNA 表达明显降低, 且均有统计学意义。注射 L-Arg 和 L-NAME 后, nNOS mRNA 和 apelin 受体 mRNA 的表达呈正相关。**结论** 在中枢神经系统, 尤其是在尾核中, NOS 的活性是 NO 作为神经递质或调质参与包括中枢痛觉调制作用在内的多种生物学作用的关键因素之一, 尾核内 NO 的神经生物学作用可能与 apelin 受体作用相关。

关键词: 一氧化氮; apelin 受体; 痛觉; 尾核; 逆转录-聚合酶链反应