Brain Research Bulletin 84 (2011) 258–263

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

Nitric oxide synthase expression in the medullary respiratory related nuclei and its involvement in CO-mediated central respiratory effects in neonatal rats

Jie Zhang[1], Ying He[1], Yan Ding, Hua Zhou, Yuhong Tang, Li Chen, Yu Zheng*

Department of Physiology, West China School of Preclinical and Forensic Medicine, Sichuan University, 3-17 Renmin South Road, Chengdu, Sichuan 610041, PR China

ARTICLE INFO

Article history:
Received 20 July 2010
Received in revised form 6 December 2010
Accepted 5 January 2011
Available online 19 January 2011

Keywords:
Respiratory regulation
Carbon monoxide
Nitric oxide
Neuronal nitric oxide synthase
Neonatal rat

ABSTRACT

The present study was conducted in order to observe the potential participation of the nitric oxide synthase–NO pathway in CO-mediated regulation of respiration of neonatal rats. An immunofluorescent histochemical technique was used to examine the existence of the neuronal nitric oxide synthase, a key enzyme of synthesizing NO, in medullary respiratory nuclei. The rhythmic respiratory-like discharges of hypoglossal rootlets of medullary slices were recorded to test the role of the nitric oxide synthase in CO-mediated respiratory effects. We observed neuronal nitric oxide synthase expressed in the medullary respiratory nuclei in conjunction with CO lengthened expiratory duration, decreased respiratory frequency, and increased inspiratory amplitude. These CO-mediated respiratory effects could be partially eliminated by prior treatment of the slices with Nω-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase. The results suggest that nitric oxide synthase–NO pathway might be involved in the CO-mediated central regulation of respiration at the level of medulla oblongata in neonatal rats.

1. Introduction

Carbon monoxide (CO), produced during degradation of heme under action of heme oxygenase (HO), has been verified as an endogenous biological messenger [29]. Three distinct isoforms of HO have been identified, inducible HO-1 [22] and constitutive HO-2 [21] and HO-3 [13].

CO regulates physiological functions in a wide variety of tissues as an endogenous paracrine and autocrine gaseous messenger [29]. It has been demonstrated that some biological effects induced by CO may be mediated by the nitric oxide synthase (NOS)–nitric oxide (NO) pathway. In central regulation of cardiovascular functions, the cardiovascular effects of hematin, a heme molecule cleaved by HO to yield CO, were attenuated by prior administration of the NOS inhibitors, Nω-nitro-L-arginine methyl ester (L-NAME) or N-monomethyl-L-arginine (L-NMMA), demonstrating that the HO/CO pathway might couple to the activation of NOS via liberation of NO [11]. In bipolar and amacrine cells of retina, CO stimulated increases in cyclic guanine monophosphate (cGMP), which can be completely blocked by L-NAME, suggest that an increase of cGMP in response to CO might be mediated, at least partly, by CO displacing and releasing NO from intracellular storage pools [3].

In a previous study, we have observed that inhibition of heme oxygenase activity from ZnPP-IX augmented rhythmic respiratory-like discharge of hypoglossal rootlets, suggesting an involvement of endogenous CO in regulation of rhythmic respiration at the level of medulla oblongata [30]. In the present study, we further investigated our hypothesis that the NOS–NO pathway might be involved in CO-mediated central respiratory effects in neonatal rats. The expression of neuronal NOS (nNOS) in medullary respiratory related nuclei was observed by using an immunofluorescent histochemical technique and the influence of the NOS inhibitor, L-NAME, on the CO-induced respiratory effects was recorded by measuring the rhythmic respiratory-like discharge of hypoglossal rootlets of medullary slices.

2. Materials and methods

2.1. Animals

Male or female neonatal (P0–3) Sprague–Dawley rats were used in this study. All procedures were reviewed and approved by the Sichuan University Committee for the Use of Live Animals in Research, which is in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

Abbreviations: ACSF, artificial cerebrospinal fluid; cGMP, cyclic guanine monophosphate; CO, carbon monoxide; DD, discharge duration; DF, discharge frequency; DI, discharge interval; Gi, gigantocellular reticular nucleus; HO, heme oxygenase; IA, integrated amplitude; L-NAME, Nω-nitro-L-arginine methyl ester; L-NMMA, N-monomethyl-L-arginine; L-NNA, N-omega-nitro-L-arginine; LPGi, lateral paragigantocellular nucleus; NA, nucleus ambiguous; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; NTS, nucleus tractus solitarius; PB, phosphate buffer; PBC, pre-Bötzinger complex; PBS, phosphate buffer saline; ROb, nucleus raphe obscurus; rVRG, rostral ventral respiratory group; sGC, soluble guanylyl cyclase; XII, hypoglossal nucleus.

* Corresponding author. Tel.: +86 28 8550 3433; fax: +86 28 8550 3204.
E-mail address: yzheng@scu.edu.cn (Y. Zheng).

[1] These authors contributed equally to this work.

0361-9230/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.brainresbull.2011.01.004

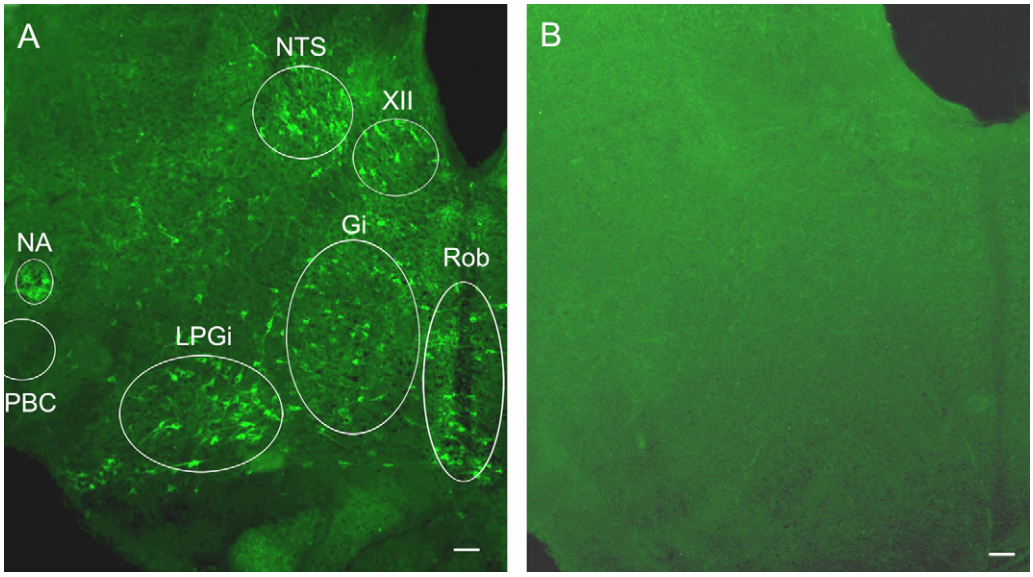

Fig. 1. Immunofluorescent staining of nNOS in medulla oblongata of neonatal rats. (A) Distribution of nNOS-positive immunoreactive neurons in medulla oblongata. Gi: gigantocellular reticular nucleus; LPGi: lateral paragigantocellular nucleus; NA: nucleus ambiguous; NTS: nucleus tractus solitarius; ROb: nucleus raphe obscurus; XII: hypoglossal nucleus. (B) Negative control. Scale bars are all 200 μm.

2.2. Immunofluorescent staining

The animals were anaesthetized using inhaled ether and then decapitated. The brainstem was dissected and immersed in 4% paraformaldehyde in a 0.1 M phosphate buffer (PB, pH 7.4) overnight at 4 °C, and then immersed in 30% sucrose in 0.1 M PB solution at 4 °C until falling to bottom in the specimen containers. The specimens were serially sectioned into 25 μm-thick slices at the level of medulla from the obex to the rostral until the inferior olive nucleus disappeared. The subsequent immunofluorescent staining was done as described in a previous study [12]. In brief, the sections were blocked in a 0.01 M phosphate buffer saline (PBS) containing 5% bovine serum albumin, 10% donkey serum and 0.3% Triton X-100 for 2 h at 37 °C, then incubated with rabbit polyclonal antibodies against nNOS (1:400, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 37 °C. The samples were overnighted at 4 °C in a medium containing 1% bovine serum albumin, 5% donkey serum and 0.3% Triton X-100 in PBS. The binding site of the primary antibody was revealed by incubating with FITC-conjugated donkey anti-rabbit IgG (1:200, Jackson, Philadelphia, USA) for 2 h at room temperature in PBS containing 0.3% Triton X-100. The sections were examined under a Leica fluorescence microscope. PBS was substituted for the primary antibody and was used as negative control.

2.3. Preparation of medullary slices and recording of discharge of hypoglossal rootlets

Medullary slices for electrophysiological recording were prepared as described previously [7,20,30]. In brief, the animals were decapitated following ether anaesthesia. The brainstem was isolated in cooled artificial cerebrospinal fluid (ACSF) containing (in mM): 129 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 21 $NaHCO_3$, 1 KH_2PO_4 and 30 D-glucose, equilibrated with carbogen (95% O_2 and 5% CO_2), pH 7.4. The brainstem was secured in a vibratome with the caudal end up. A 700–1000 μm thick slice, corresponding to the level of medulla from about 100–300 μm caudal to 600–700 μm rostral to obex, was obtained and then continuously perfused with ACSF at a rate of 4–5 ml/min at 27–28 °C, pH 7.4. To obtain and maintain consistent respiratory rhythmic activity, the KCl concentration of the perfusing ACSF was raised from 3 to 7 mM, meanwhile the concentration of NaCl was reduced from 129 to 125 mM to balance the osmotic pressure. The slices were incubated for 30 min before starting experiments. Glass suction electrodes filled with ACSF were used to record the rhythmic respiratory-like discharge from the cut ends of the hypoglossal rootlets. The signals were amplified, filtered (τ = 0.001 s, F = 1 kHz) and integrated with a 50 ms time constant using a BL-420F biological signal processing system (Taimeng Biotech. Co., Chengdu, China). The rhythmic discharge duration (DD), discharge interval (DI), discharge frequency (DF, number of discharge in 1 min), and integrated amplitude (IA) of hypoglossal rootlets of the slices were analyzed. The slices were divided into four groups (n = 7 for each), control, CO, L-NAME and L-NAME + CO. In the control group, the slices were perfused with ACSF during the whole process. In the CO group, ACSF was bubbled with both pure CO gas and carbogen in an approximately 100 ml volume container at the same flow rate for 15 min immediately preceding perfusion of the slices. The estimated CO concentration in the resulting CO-ACSF was about 500 μM. Slices were then perfused with CO-ACSF for 8 min. In the L-NAME group, slices were perfused with 200 μM L-NAME-ACSF for 13 min. In the L-NAME + CO group, sample slices were perfused with 200 μM L-NAME-ACSF for 5 min and then with L-NAME-CO-ACSF for 8 min. The discharge of the hypoglossal rootlets before chemical application was recorded for 5 min as an activity baseline for each group. Following chemical sample preparations, the slices were continuously perfused with ACSF for washout.

2.4. Statistical analysis

All electrophysiological data was compared with the baseline before applying chemicals or with the control group. Normalized DD, DI, DF and IA of hypoglossal rootlets were reported as mean ± S.E.M. and statistically analyzed with repeated measure ANOVA. Statistical analysis was preformed with SPSS 13.0 for Windows. P values < 0.05 were considered statistically significant.

3. Results

3.1. Expression of nNOS in the medullary respiratory related nuclei

Immunofluorescent staining showed that nNOS-positive immunoreactive neurons could be observed in the respiratory related nuclei of the medulla oblongata, including the nucleus tractus solitarius (NTS), gigantocellular reticular nucleus (Gi), lateral paragigantocellular nucleus (LPGi), nucleus ambiguous (NA), nucleus raphe obscurus (ROb) and hypoglossal nucleus (XII), as shown in Fig. 1. The immunoreactive products were distributed in cell plasma.

3.2. Effects of L-NAME on CO-mediated central regulation of respiration

The rhythmic respiratory-like discharge of hypoglossal rootlets of the medullary slices was recorded, as shown in Fig. 2, to investigate whether NOS–NO pathway is involved in CO-mediated central regulation of respiration. Stable rhythmic activity of the rootlets could be maintained for more than 1 h in most cases and up to 8 h in some cases.

CO was bath-applied continuously for 8 min to observe its effects on the rhythmic discharge of hypoglossal rootlets of the medullary slices. The changes of discharge in the hypoglossal rootlets were prominent from the 3rd to 12th min. Compared with the control group, DI was prolonged, DF decreased, and IA increased (P < 0.05), while DD did not change significantly (P > 0.05). In the