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·Original Article·

Nitric oxide inhibits excitatory vagal afferent input to nucleus tractus solitarius neurons in anaesthetized rats

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Abstract: Objective Endogenous nitric oxide (NO) has been implicated in the regulation of neuronal activity which mediates cardiovascular reflexes. However, there is controversy concerning the role of NO in the nucleus tractus solitarius (NTS). The present study aims to elucidate the possible physiological role of endogenous NO in modulating the excitatory vagal afferent input to NTS neurons. **Methods** All the experiments in the rat were conducted under anaesthetic conditions. Ionophoresis method was used for the application of NO donor or nitric oxide synthase (NOS) inhibitor, and single unit recording method was employed to detect the effects of these applications on vagal afferent- or cardio-pulmonary C-fibre reflex-evoked neuronal excitation in NTS. **Results** Ionophoresis applications of *L*-arginine (*L*-Arg), a substrate of NOS, and sodium nitroprusside (SNP), a NO donor, both attenuated the vagal afferent-evoked discharge by (51.5±7.6)% (n = 17) and (68.3±7.1)% (n = 9), respectively. In contrast, application of D-Arg at the same current exerted no overall effect on this input. Also, both *L*-Arg and SNP inhibited spontaneous firing of most of the recorded neurons. In contrast, ionophoresis application of N^G-nitro-*L*-arginine methyl ester (L-NAME) enhanced vagal afferent-evoked excitation by (66.3±11.4)% (n = 7). In addition, ionophoresis application of *L*-Arg and SNP significantly attenuated cardio-pulmonary C-fibre reflex-induced excitation in the tested NTS neurons. **Conclusion** Activation of local NO pathway in the NTS could suppress vagal afferent-evoked excitation, suggesting that NO is an important neuromodulator of visceral sensory input in the NTS.

Keywords: nitric oxide; nucleus tractus solitarius (NTS); ionophoresis; vagus afferents; cardiopulmonary reflex; rat

1 Introduction

Nucleus tractus solitarius (NTS) is an important site of the visceral afferent pathway, receiving vagus nerve input. Neurons in the NTS can be selectively activated by either myelinated or non-myelinated vagal afferent fibres derived from various receptors[1,2]. Additionally, NTS is innervated and modulated by many other regions of the central nervous system (CNS), and involved in cardiovascular homeostasis by integrating information from various sensory input[3-5]. Numerous neurotransmitters have been implicated in autonomic regulations in the NTS[6].

Since nitric oxide (NO) has been well recognized as an endogenous neurotransmitter, a neuromodulator and an intercellular messenger, it has become clear that endogenous NO may be involved in the regulations of neuronal activities

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that mediate cardiovascular reflexes[7,8]. Being an unstable molecule, NO is produced by nitric oxide synthase (NOS) under normal conditions in the brain. Evidence from histological studies shows that vagal afferents to the NTS containing NOS and vagal deafferentiation decrease the NOS immunoreactivity in the NTS [9-11]. NOS immunoreactivity has also been found in many nuclei of neurons within the medulla oblongata that sends projections to the NTS[12-14], which suggests that NO generated in the NTS may modulate vagal afferent integration.

However, controversy still exists concerning the role of NO in the NTS, since some studies have suggested that NO is excitatory within the baroreflex pathway[15-17], while others have demonstrated the NO-mediated inhibitory actions or even no effect[18-22]. In this study, N^G-nitro-*L*-arginine methyl ester (L-NAME, an inhibitor for NOS), *L*-arginine (*L*-Arg, a substrate of NOS) and sodium nitriprusside (SNP, the NO donor), were administered to NTS neurons by microionophoresis. By using single unit recording method, the responses of NTS neurons, activated by stimulations of cervical vagus nerve and cardiopulmonary receptors, L-NAME, *L*-Arg and SNP, were tested. The results demonstrate that activations of NO pathways in the NTS could suppress the vagal C-fibre excitatory input to NTS neurons.

2 Materials and methods

Male Sprague-Dawley rats (weighing 280-360 g) used in this study were housed under standard conditions at (21±1) ºC, with free access to food and water under a 12-h light-dark cycle. All the experiments were approved by the Local Ethical Committee for Laboratory Animals in accordance with national animal research guidance. At the end of the experiments, animals were euthanized with overdose of urethane.

2.1 Animal preparation Animal preparation was conducted as previously reported[23,24]. Briefly, rats were anaesthetized with pentobarbitone sodium (60 mg/kg, i.p.), supplemented with anaesthesia when necessary (6 mg/kg, i.v.). After that, tracheotomy was performed in the low part of the neck for ventilation. Catheters were inserted into the femoral artery for the measurement of blood pressure, or into the vein for the administration of supplemental anaesthetic agents and drugs. A silicone cannula filled with phenylbiguanide (PBG, 200 μg/mL) was laid within the right atrium by inserting it through the right jugular vein. Rectal temperature was maintained at 37 ºC with a Harvard homeothermic blanket system (Harvard Apparatus, South Natick, MA, USA). Animals were ventilated at O_2-enriched conditions and positive end expiratory pressure (1cm H_2O) was achieved using a positive pressure ventilator (Harvard rodent ventilator, model 683). End-tidal CO_2 was continuously measured using a fast response CO_2 meter (model FM1, The Analytical Development Company Ltd, Hoddesdon, Herts, UK) and maintained close to 4%. Arterial blood samples were regularly collected and the pH-blood gas analyzer (model 238, Ciba Corning Diagnostics Ltd, Halstead, UK) was used to measure the pH value and the partial pressures of the dissolved blood gases that were maintained in the following ranges: pH 7.3-7.4; PO_2, 90-130 mmHg; PCO_2, 40-50 mmHg, by slow infusion (i.v.) of sodium bicarbonate (1 mol/L) or adjustment of the respiratory pump.

The rat was fixed in a stereotaxic frame and the phrenic nerve was isolated in the low part of the neck in a dorsolateral approach. The nerve was cut at its peripheral part, and the proximal end was de-sheathed and placed on bipolar platinum recording electrodes. Phrenic nerve activity was amplified (NL 104, Neurolog; gain 2K) and filtered (NL 125, Neurolog, 0.5-5 kHz). Using the same approach, the right cervical vagus nerve was dissected from the sympathetic trunk and placed on the bipolar silver wire electrodes for electrical stimulation (50-500 μA, 1 ms, 0.3-1 Hz) with an isolated stimulator (Digitimer DS2A) triggered by a programmer (Digitimer 4030). The exposed parts of both nerves were covered by paraffin wax and fixed with dental impression material (President light body dental polyvinyl siloxane, Coltene). To expose the dorsal surface of the caudal brainstem, the nuchal muscles were removed, after which the occipital bone was removed and the dura overlying the brainstem was cut and reflected laterally. For the single cell recording, neuromuscular block was conducted using decamethonium bromide with an initial dose of 3 mg/kg (i.v.), followed by administrations of 3 mg/kg per hour, or a single dose of α-

bungarotoxin (140 μg/kg, i.v.). During neuromuscular block, the extent of anaesthesia was assessed by detecting the stability of arterial blood pressure, the heart rate and the cardiovascular responses to the paw pinch.

2.2 Protocol Extracellular recording was made from neurons in the medial region of the NTS (< 1 mm lateral to midline) within 1-2 mm caudal to obex, an area known to contain neurons receiving cardiopulmonary afferent input. 'Piggy-back' electrode constructed from a single barrel recording electrode glued to a 5- or 7-barrelled microelectrode made from borosilicate glass (Clarke Electromedical, Reading, UK) as previously reported [24,25]. The recording barrel contained NaCl (4 mol/L) while the other barrels contained pontamine sky blue dye prepared in 0.5 mol/L sodium acetate and the drug of *L*-Arg, L-NAME, SNP or DL-homocysteic acid (DLH), respectively. Neuronal recording was amplified (×1000, NL 104, Neurolog) and filtered (0.5-5 kHz; NL 125, Neurolog). NTS neurons were identified by their orthodromic responses to electrical stimulation of the cervical vagus nerve at 2× threshold for evoking activity[24]. NTS neurons receiving non-myelinated vagal input mediate a wide range of functions. In the present study, one functional subpopulation of these neurons was identified by their responses to cardiopulmonary afferent stimulation from the right atrial administration of PBG (12-24 μg/kg, 60-120 μL/kg). Drugs were applied to the vicinity of the recorded neurons by ionophoresis (Neurophore, Medical Systems, Digitimer Ltd). Between drug ejection periods, a retaining current of 10-15 nA was applied to each drug barrel. When neuronal firing rate was steady, the effects of NOS substrate, NO donor and/or NOS inhibitor given alone or in combination were tested. In all the experiments, the possible current artifacts were minimized using the automatic current balancing available on the Neurophore System. In some experiments, recording sites were marked by ionophoresis ejection of pontamine sky blue dye. At the end of the experiment, the brainstem was removed and fixed in 10% formal saline. Frozen sections (50 μm) were cut and the recording sites were localized and mapped onto the standard sections of a rat brainstem[26].

2.3 Data capture and analysis Arterial blood pressure, tracheal pressure, electrocardiogram (ECG), phrenic nerve activity and neuronal activity were recorded on a PC accessed via an A/D interface (Cambridge Electronic Design (CED) 1401plus). Off-line analysis of recorded data was made using Spike 2 software (CED). Single unit activity was discriminated using a Spike Processor (D130, Digitimer Ltd) and displayed as a rate histogram. To investigate the effects of drugs on the ongoing NTS neuronal activity, the baseline and drug-evoked neuronal firing rates (averaged over a 20-60 s period) were measured and compared. Peri-stimulus time histograms (PSTHs, 40 stimuli) were constructed to investigate the effects of the drugs on the vagal-evoked responses of NTS neurons. The total numbers of evoked spikes before and during ionophoresis application of the drugs were compared. The responses of NTS neurons to pulmonary C-fiber afferent stimulation from PBG injection into the right atrium was analyzed by counting the total number of spikes within 5 s following PBG injection, during which only the pulmonary chemoreceptor was stimulated. Drugs were classified as excitatory or inhibitory if the activity increased or decreased by more than 10%. All the data were presented as mean±SEM and analyzed with the student's paired *t*-test.

2.4 Drugs and solutions The following drugs were freshly dissolved in 0.9% saline, including PBG (200 μg/mL; Sigma), L-NAME (100 mmol/L; Sigma; pH 8.0), *L*-Arg (100 mmol/L; Sigma; pH 8.0), *D*-Arg (100 mmol/L; Sigma; pH 8.0), SNP (100 mmol/L; Sigma; pH 8.0), and DLH (100 mmol/L; BDH, Poole, Dorset; pH 8.5). The pH was adjusted using 0.1mol/L HCl or 0.1 mol/L NaOH. Pontamine sky blue dye (20 mg/mL; BDH, Poole, Dorset) was dissolved in 0.5 mol/L sodium acetate. Drugs administered by microionophoresis were ejected as anions by applying ejecting currents to the drug-containing barrels. During non-ejection period, a retaining current with an appropriate polarity was applied to the drug barrels to prevent the passive diffusion of the drug from the electrode tip.

3 Results

3.1 Characterization of the NTS neurons receiving vagal afferent input The NTS neuron in the present study was identified according to the location and the response to electrical stimulation of the cervical vagus nerve (Fig. 1). A total

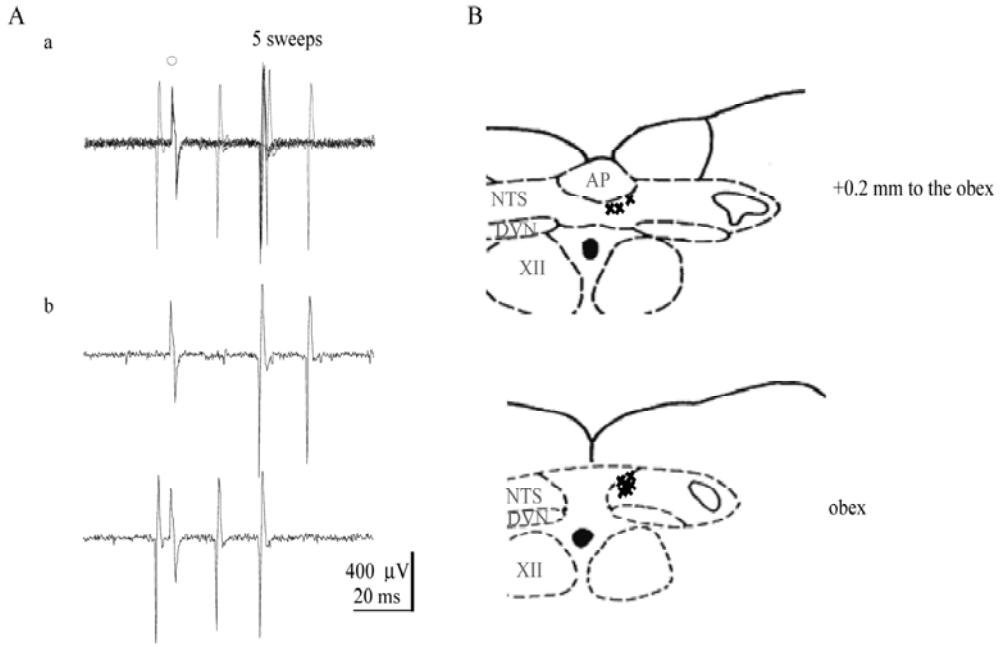

Fig. 1 Identification of neurons receiving vagus afferent input in the NTS. A: original traces showing vagus nerve stimulation (50-500 μA, 1 ms, 0.5-1 Hz)-evoked excitatory input in one of the NTS neurons. The sign "o" indicated the stimulus artifact. (a) The superimposed 5 sweeps showing evoked excitatory input with the latency in the C-fiber range; (b) traces showing evoked excitatory input (top) was not collide by the spontaneous firing spike within certain time range (bottom). B: diagrams showing the recording sites (marked with *) after histological recovery of pontamine sky blue staining of electrophysiological recording sites. AP, area postrema; NTS, nucleus tractus solitarius; DVN, dorsal vagus nucleus; XII, hypoglossal nucleus.

number of 32 NTS neurons were identified, receiving excitatory orthodromic C-fiber input following stimulation of the vagus nerve, with a mean latency of (30.4±1.5) ms (n = 32). Among them, 4 neurons also received short latency input assumed from B-fibers in the vagus nerve with a mean latency of (4.2±0.4) ms (n = 4).

The recording sites for electrophysiological study were stained by pontamine sky blue dye after the recording was finished. Twelve marked sites in 9 rats were then successfully recovered histologically and all of them were located in the caudal-median position of the NTS (Fig. 1B). All the other recording sites were either close to the marked sites in the same animal or within the similar stereotaxic coordination, thus were assumed to be in the same brainstem region as shown in Fig. 1B.

3.2 Inhibitory effects of *L*-Arg and SNP on vagal-evoked excitation of NTS neurons Among the 32 recorded NTS neurons, 18 neurons were used to test the effect of ionophoresis application of *L*-Arg, a NO precursor, on vagal afferent-evoked discharge. Ionophoresis application of *L*-Arg (10-160 nA) inhibited the vagal afferent-evoked excitation in a dose-dependent way in 17 tested neurons by (51.5±7.6)%, as compared to the control level, with an inhibition range between 11%-100% (Fig. 2A). There was a slight potentiation (7%) of the evoked discharge in the remaining NTS neuron. In contrast, *D*-Arg application at the same current (80-160 nA) exerted no effect on the evoked excitation in all the 7 NTS neurons, with the excitatory response change being only (1.8±0.7)% (n = 7), as compared to the control.

Similarly, ionophoresis application of SNP (40-160 nA), a NO donor, inhibited the vagal afferent-evoked excitation in 9 out of the 10 tested neurons in a dose-dependent way, with an overall inhibition rate being (68.3±7.1)% of the control (Fig. 2B). Meanwhile, SNP treatment (160 nA) extered no effect on the remaining tested neuron.

Moreover, the ionophoresis applications of *L*-Arg and SNP (40-160 nA) to the vicinity of the NTS neurons also inhibited both spontaneous and DLH-evoked excitation in

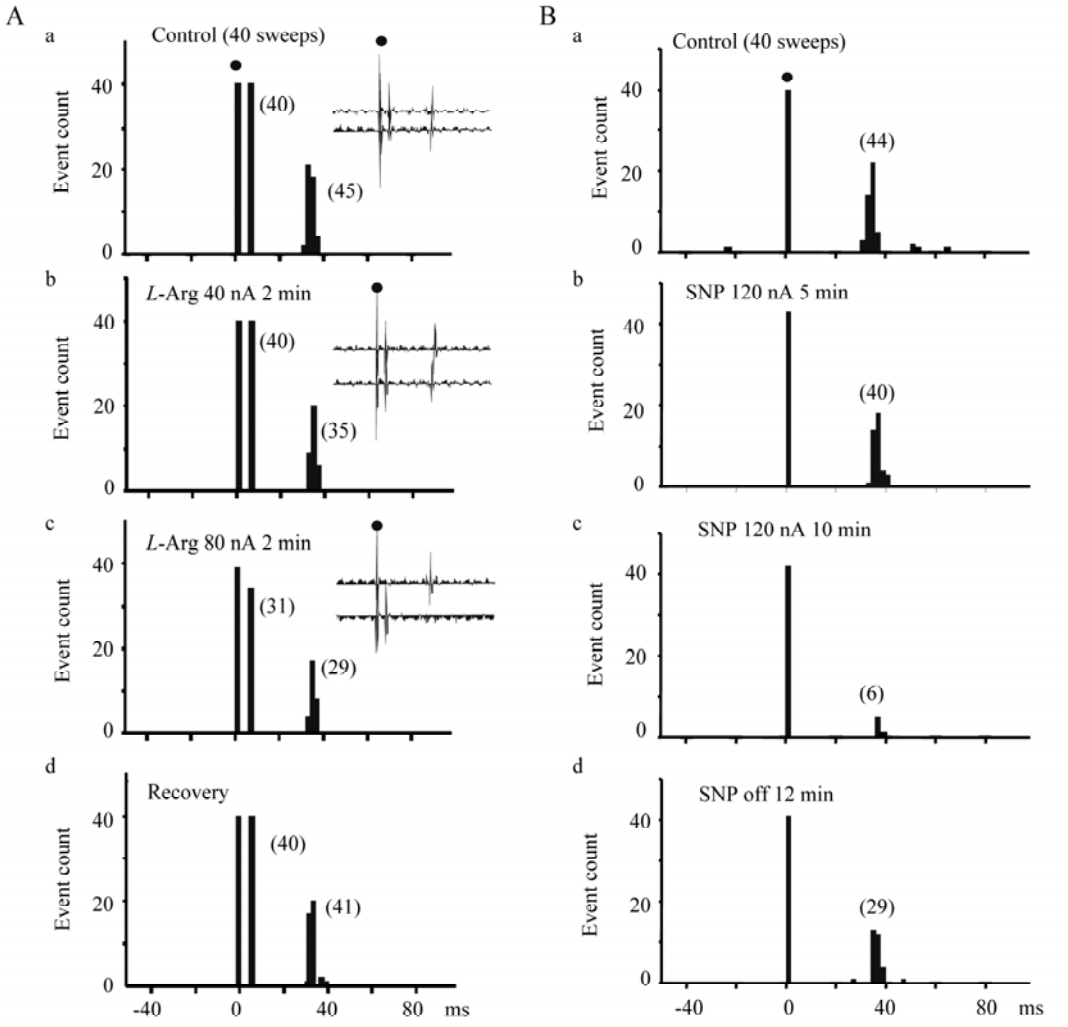

Fig. 2 The inhibitory effects of *L*-Arg and SNP in NTS neurons. Peri-stimulus time histograms (PSTH, 40 sweeps) of vagal afferent-evoked activities of NTS neurons showed the responses (a) under control conditions, (b and c) during ionophoresis drug application, and (d) recovery after drug concession. Vagal stimulation-evoked excitation of NTS neurons could be inhibited by both *L*-Arg and SNP in a dose-dependent manner. The numbers in the bracket indicated the spike count. The inserts in A were the original traces of vagal afferent stimulation-evoked discharge. The round spot indicated the stimulus artifact.

most of the tested neurons (Fig. 3). As a control, ionophoresis administration of NaCl at the same current (40-160 nA) showed no obvious effect on the neuronal firing of the tested NTS neurons.

3.3 The excitatory effect of NOS inhibitor L-NAME on NTS neurons Ionophoresis application of L-NAME (40-120 nA) increased the vagus nerve stimulation-evoked NTS neuronal firing rate in all the 7 tested NTS neurons by (66.3±11.4)%, with a range between 20%-112%, in a dose dependent way (Fig. 4). Moreover, this effect could be almost completely reversed by co-application with *L*-Arg in all the 5 tested NTS neurons (Fig. 4A).

In addition, ionophoresis application of L-NAME also induced excitation of all the tested NTS neurons by increasing their spontaneous firing (Fig. 4B-a). This effect was mainly due to the depolarization of the membrane potential together with the enhancement of presynaptic neurotransmitter release, which was evidenced with increased synaptic activities during L-NAME application, as revealed by successful *in vivo* intracellular recordings in 2 NTS neurons (Fig. 4B-b).

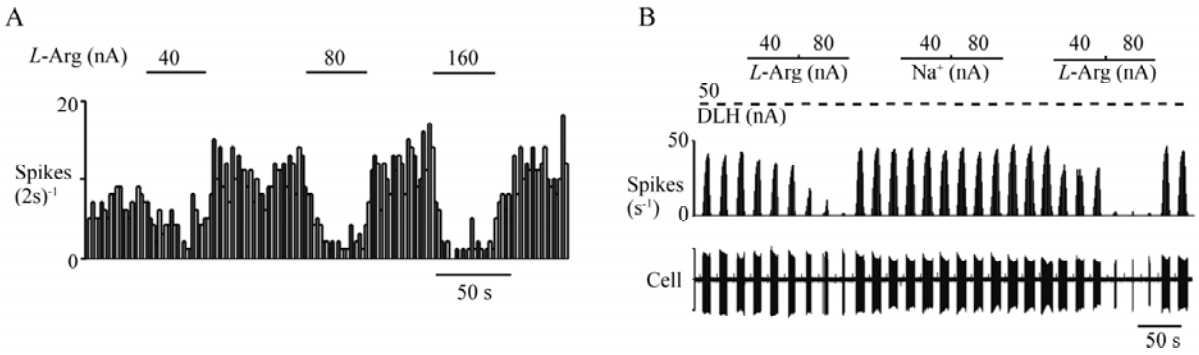

Fig. 3 The inhibitory effects of *L*-Arg on spontaneous and DLH-evoked NTS neuronal activities. A: a continuous rate histogram showing the inhibitory effect of ionophoresis administration of *L*-Arg on one NTS neuron, at 40 nA, 80 nA and 160 nA currents. B: a continuous rate histogram (top) and the original cell recording traces showed that DLH (50 nA)-evoked NTS neuronal activity was inhibited by co-application of *L*-Arg at 40 nA and 80 nA currents, respectively, but not by Na^+.

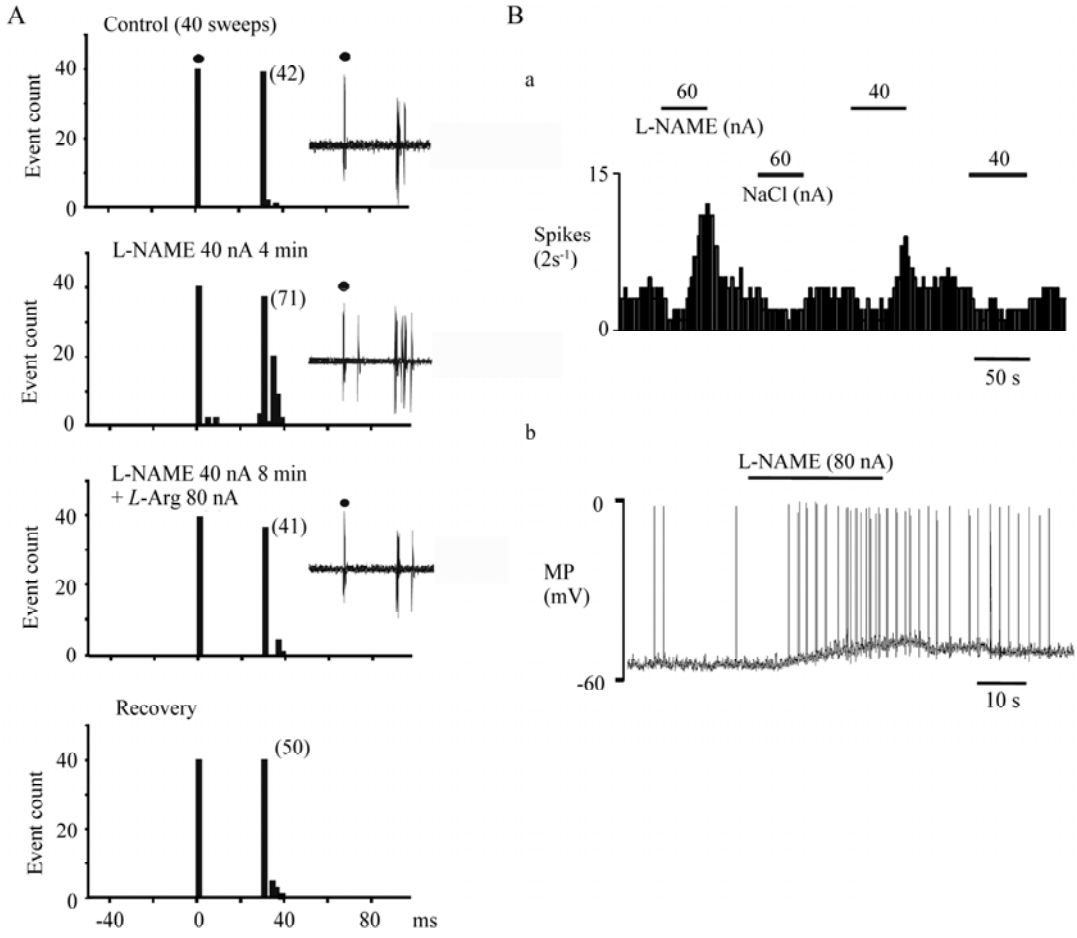

Fig. 4 Effect of ionophoresis application of L-NAME on NTS neurons. A: peri-stimulus time histograms (40 sweeps) showed vagal afferent-evoked discharge in one NTS neuron (a) under normal conditions, (b) during ionophoresis administration of L-NAME, (c) co-application of L-NAME and *L*-Arg, (d) recovery after cessation. The inserts were the 5 sweeps original traces superimposed. B: (a) continuous rate histogram showed the excitatory response of one NTS neuron to the ionophoresis administration of L-NAME, but not to the ejection of Na^+ at the same current, (b) an intracellular recording trace in one NTS neuron showed that the ionophoresis application of L-NAME depolarized the membrane potential and increased the synaptic events.

3.4 Inhibitory effects of L-Arg and SNP on pulmonary C-fiber reflex-induced NTS neuron excitation Among those identified neurons receiving C-fiber vagal excitatory input, 6 neurons were also tested with pulmonary C-fiber reflex input induced by PBG injection into the right atrium. The pulmonary C-fiber reflex could be induced by PBG (8-30 μg/kg) injection into the right atrium for chemoreceptor stimulation. And the effect of NO on pulmonary C-fiber reflex-evoked NTS neuronal activity was investigated by ionophoresis applications of *L*-Arg and SNP into the vicinity of the recorded NTS neurons. As shown in Fig. 5A, all the 6 tested NTS neurons were excited by PBG injection accompanied with hypotension and apnea similar as the previous report[24]. Pulmonary C-fiber reflex induced a great increase of neuronal firing, from a baseline firing rate of (1.06±0.14) Hz to the peak of (8.40±1.30) Hz within 5 s after PBG injection[25]. However, among these 6 neurons, *L*-Arg significantly ($P < 0.01$) inhibited pulmonary C-fiber stimulation-induced NTS neuronal excitation in 4 tested NTS neurons (Fig. 5B). The neuronal activity increased to (618.2±47.2)% ($n = 4$) of the baseline firing rate during PBG reflex, from baseline firing of (1.08±0.18) Hz to the peak of (6.50±0.96) Hz during reflex. However,

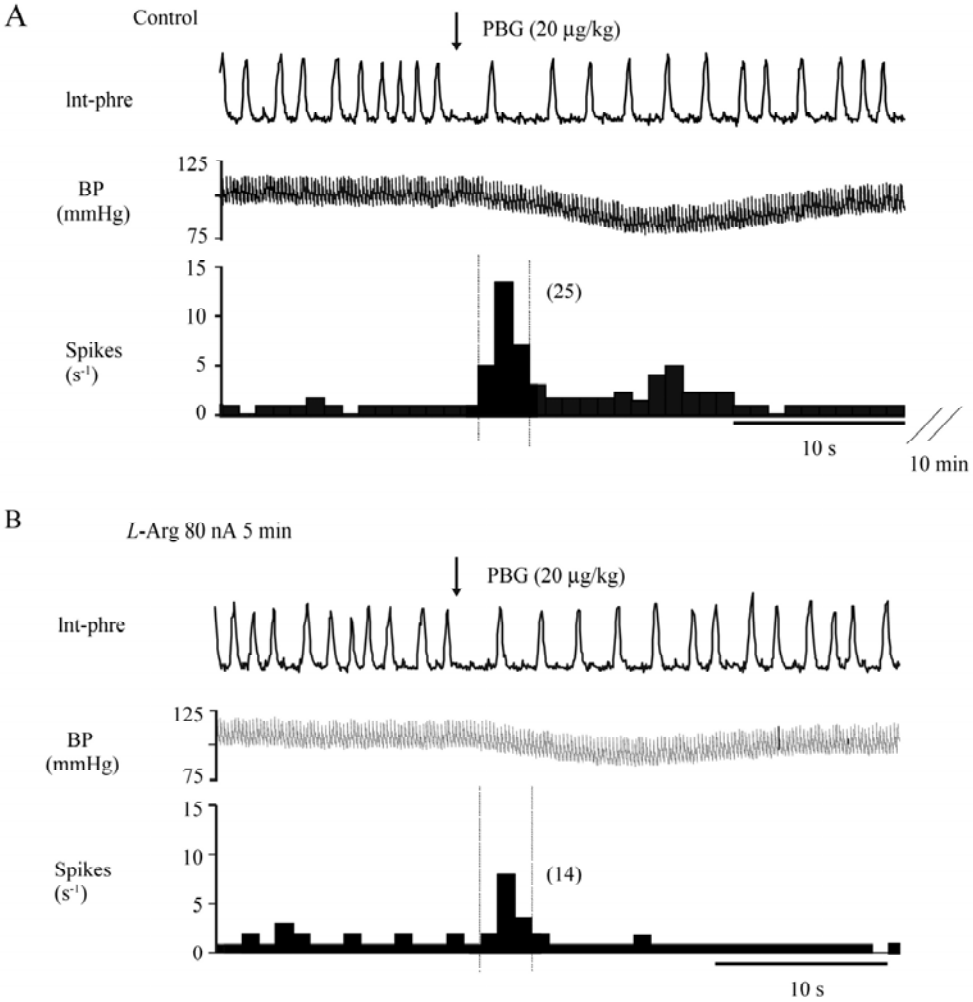

Fig. 5 The inhibitory effect of *L*-Arg on pulmonary C-fiber reflex-induced NTS neuronal activity. A: the right atrium PBG application-induced reflex evoked an increase of activity in one NTS neuron; B: the ionophoresis pre-administration of *L*-Arg (80 nA) for 5 min attenuated the excitatory effect of PBG reflex on this NTS neuron. Results in A and B were from the same neuron, with a 10-min interval. Int-phre: integrated phrenic nerve activity. BP: arterial blood pressure. Spikes: rate histogram of the NTS neuronal activity. The vertical line indicated the 3-s window after PBG injection and the number indicated the evoked spikes within this window.

with ionophoresis application of *L*-Arg (10-100 nA), the neuronal activity only increased to (263.2±74.1)% (n=4) of the baseline firing rate during PBG reflex, from baseline firing of (1.05±0.41) Hz to the peak of (2.00±0.41) Hz during reflex. Similarly, SNP application (40-80 nA) also significantly ($P < 0.05$) inhibited pulmonary C-fiber reflex-induced NTS neuron excitation in 3 tested neurons. The NTS neuronal activity during control PBG reflex increased to (857.6±207.3)% (n = 3) of the baseline firing rate, from (1.07±0.03) Hz to the peak of (9.00±2.08) Hz during reflex, while with SNP application to the vicinity of the recorded neuron, the activity only increased to (355.0±73.9)% (n = 3) of the baseline firing rate, from (1.80±1.02) Hz to the peak of (5.00±1.43) Hz during reflex.

4 Discussion

In the present study, the effects of NOS inhibitor, NO precusor and NO donor on NTS neuronal activation induced by the stimulation of cervical vagus nerve were investigated. Our results demonstrate that activation of NO pathway could suppress the vagal afferent stimulation-induced excitation in a subgroup of NTS neurons, while inhibition of neuronal nitric oxide synthase (nNOS; NOS-I) could enhance it. Besides, this group of NTS neurons receives non-myelinated C-fiber vagal afferent-transmitted signal as previously reported[23,24]. In addition, results show that the cardiopulmonary reflex-induced excitation of NTS neurons is also inhibited by local activation of NO pathway.

NO in the NTS comes from various places within the medulla oblongata or from outside projection. Immunohistochemical studies have shown that NOS-positive neurons in many medulla oblongata nuclei project to the NTS[12-14]. Vagal afferents to the NTS also contain NOS, and vagal deafferentiation decreases NOS immunoreactivity in the NTS[9-11]. Our present study, by using ionophoresis drug application method, indicates that *L*-Arg and L-NAME are likely to act on the local NO pathways within the vicinity of the recording site, which was further confirmed by the effect of SNP on vagal afferent-evoked excitation. Moreover, the minority of the NTS neurons did not respond to either *L*-Arg (n = 1) or SNP (n = 1) in the present study, indicating the diverse effects of NO in regulating the sub-groups of NTS neurons.

In the NTS, endogenous NO is produced from Arg, predominantly catalyzed by 2 enzymes: nNOS and endothelial nitric oxide synthase (eNOS; NOS-III). Reports have shown that nNOS and eNOS are constitutively active within NTS in rat and play major roles in determining the set point of arterial pressure in the spontaneously hypertensive rats[27, 28]. Since L-NAME is a non-selective NOS inhibitor, the excitatory effect observed during L-NAME ionophoresis in the present study could not be attributed to one specific NOS. The interactions between NO and other neurotransmitters or modulators, such as glutamate, Ach, AngII, and Orexin, are also critical in the regulation of cardiovascular activity in the NTS[29-34]. The revealed inhibitory effect of NO on vagal afferent input is likely due to NO-induced modulations of certain neurotransmitters within NTS, such as glutamate and/or GABA system. However, further detailed investigations are required to find out the exact role of NO in modulating vagal input in the NTS.

The role of NO in the NTS is still unclear since previous studies have produced controversial results. Some studies suggest that NO is excitatory, such as within the baroreflex pathway[15-17], whereas others demonstrate NO-mediated inhibitory or even no effects[18, 20-22]. Dias and colleagues[18] have shown that L-NAME-induced inhibition in NO production can enhance AMPA-evoked excitation, while it exerts no effect on vagus nerve stimulation-evoked excitation in the NTS. However, our study shows that NO actually has an inhibitory effect on vagal afferent-mediated excitation in the NTS. One possible explanation for this divergence may be the difference in the cell groups: the NTS neurons in their study receive myelinated B-fiber vagal afferent input with short latency at 2.5 ms, while the recorded neurons in the present study mainly receive non-myelinated vagal C-fiber afferent input with mean latency over 30 ms. The reason why NO pathways in the NTS can modulate the C- and B-fiber vagal afferent input to NTS neurons in different ways is unknown.

In conclusion, the current study indicates that NO exerts an inhibitory effect on the excitatory responses of NTS neurons to C-fiber vagal afferent and cardiopulmonary re-

ceptor input. However, the interactions between NO and other neurotransmitters still needs further investigations. We hypothesize that the functional significance of NO in the NTS is the integration of the peripheral visceral signals within NTS during different physiological and pathophysiological conditions.

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一氧化氮抑制迷走神经介导的孤束核神经元兴奋性传入

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摘要 目的 一氧化氮在延髓孤束核内调控心血管反射功能的确切机制尚未得到阐明。本实验将微电泳与细胞外神经元记录技术结合，研究一氧化氮对孤束核神经元接受迷走神经介导的兴奋性传入的调控作用，揭示一氧化氮在延髓孤束核内调控心血管反射的重要作用。 **方法** 将大鼠进行整体麻醉，通过单细胞记录孤束核神经元的电活动。微电泳给予一氧化氮供体或一氧化氮合成酶抑制剂，观察其对迷走神经电刺激或肺化学感受器刺激下诱发的孤束核神经元兴奋性反应的调控作用。 **结果** 微电泳给予*L*-arginine (*L*-Arg) 或硝普纳均能抑制迷走神经刺激诱发的孤束核神经元兴奋反应，分别降低了(51.5±7.6)% (n = 17) 和 (68.3±7.1)% (n = 9)。而在相同条件下给予*D*-Arg并未出现任何影响。并且在大部分所测神经元中，*L*-Arg和硝普纳均能抑制神经元的自放电行为。相反，微电泳给予L-NAME则会加强迷走神经刺激诱发的孤束核神经元兴奋反应，增强了(66.3±11.4)% (n = 7)。另外，微电泳给予*L*-Arg或硝普纳都能抑制肺化学感受器刺激下诱发的孤束核神经元兴奋性反应。 **结论** 在延髓孤束核内一氧化氮对迷走神经介导的心血管反射具有负调控作用。

关键词: 一氧化氮; 延髓孤束核; 微电泳; 迷走传入纤维; 心肺反射; 大鼠