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Participation of nitric oxide in the nucleus isthmi in CO_2-drive to breathing in toads

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Abstract

The nucleus isthmi (NI) is a mesencephalic structure of the amphibian brain. It has been reported that NI plays an important role in integration of CO_2 chemoreceptor information and glutamate is probably involved in this function. However, very little is known about the mechanisms involved. Recently, it has been shown that nitric oxide synthase (NOS) is expressed in the brain of the frog. Thus the gas nitric oxide (NO) may be involved in different functions in the brain of amphibians and may act as a neurotransmitter or neuromodulator. We tested the hypothesis that NO plays a role in CO_2-drive to breathing, specifically in the NI comparing pulmonary ventilation, breathing frequency and tidal volume, after microinjecting 100 nmol/0.5 µl of L-NAME (a nonselective NO synthase inhibitor) into the NI of toads (*Bufo paracnemis*) exposed to normocapnia and hypercapnia. Control animals received microinjections of vehicle of the same volume. Under normocapnia no significant changes were observed between control and L-NAME-treated toads. Hypercapnia caused a significant ($P<0.01$) increase in ventilation only after intracerebral microinjection of L-NAME. Exposure to hypercapnia caused a significant increase in breathing frequency both in control and L-NAME-treated toads ($P<0.01$ for the control group and $P<0.001$ for the L-NAME group). The tidal volume of the L-NAME group tended to be higher than in the control group under hypercapnia, but the increase was not statistically significant. The data indicate that NO in the NI has an inhibitory effect only when the respiratory drive is high (hypercapnia), probably acting on tidal volume. The observations reported in the present investigation, together with other studies on the presence of NOS in amphibians, indicate a considerable degree of phylogenetic conservation of the NO pathway amongst vertebrates.

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Key words
- Nitric oxide
- Amphibian
- Hypercapnia
- Ventilation
- Nucleus isthmi
- Toad
- CO_2
- L-NAME

Introduction

The breathing pattern of most fish, birds and mammals is continuous, whereas that of most air-breathing fish, amphibia, and reptiles is not. These animals exhibit one of two basic types of intermittent breathing in which lung ventilation occurs in single events or is grouped into episodes of many breaths separated by non-ventilatory (apneic) periods of variable duration (1). The emergence of CO_2 chemosensitivity in amphibians near meta-

morphosis may be an important factor in the production of breathing episodes (2).

In bullfrogs, the area where brainstem transections affected the breathing pattern included the nucleus isthmi (NI) (2). This nucleus is a mesencephalic structure of the amphibian brain located between the roof of the midbrain and the cerebellum (3). NI goes through substantial cellular arrangement and differentiation during amphibian metamorphosis, a period also associated with the onset of episodic lung ventilation in bullfrogs (4). The NI also plays an important role in CO_2 chemodetection or, more likely, in the integration of CO_2 chemoreceptor information (2). NI is an important site for the control of breathing and glutamate is probably involved in this function (2).

Glutamate released from one presynaptic terminal acts on both *N*-methyl-D-aspartate (NMDA) and non-NMDA classes of glutamate receptors. It has been shown that activation of NMDA receptors results in an increase in intracellular calcium which can stimulate constitutive nitric oxide synthase (NOS), an enzyme that generates nitric oxide (NO) from the amino acid L-arginine (5,6). The NO formed diffuses to the presynaptic terminal where it stimulates guanylate cyclase and elevates cyclic GMP concentration (7), leading to a further increase in the release of glutamate and a greater augmentation of synaptic transmission (6). A number of recent studies have shown that arginine analogs such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) inhibit NO synthesis (6).

More recently, it has been shown that NOS is expressed in a subpopulation of neurons throughout the brain of the frog (8). The distribution pattern reveals certain similarities to that of other species. Expression of NOS is not limited to a particular system. Therefore, NO may be involved in different functions in the brain of amphibians.

It has been recently shown that the NI plays an important role in respiratory control and in CO_2 chemodetection in bullfrogs (2). It is possible that the NO pathway is involved in this control. The aim of the present study was to assess the role of the NO pathway in CO_2-drive to breathing specifically in the NI by microinjecting a nonselective NOS blocker (L-NAME) into the NI of toads (*Bufo paracnemis*) under conditions of normocapnia and hypercapnia.

Material and Methods

Bufo paracnemis toads (mass 126.8 ± 2.4 g, N = 16) were collected in the vicinity of Ribeirão Preto, SP, Brazil, during the rainy summer months. The toads were maintained in containers with free access to water and basking area. Food was withheld for two weeks before the experiment.

Surgical methods

Animals were anesthetized by submergence in 0.3% MS-222 and a silastic tube segment, 1.5 mm in outer diameter, was introduced into the right femoral artery. The animal's head was then fixed to a stereotaxic apparatus and the skin covering the skull was removed with the aid of a bone scraper. An opening was made in the skull above the midbrain region using a small drill. A guide cannula prepared from a hypodermic needle segment 14 mm in length and 0.6 mm in outer diameter was attached to the tower of the stereotaxic apparatus and placed in contact with the exposed surface of the midbrain inside the NI region. The orifice around the cannula was filled with a paste consisting of a mixture of equal parts of paraffin and glycerine. The cannula was fixed to the skull with acrylic cement. The experiments were initiated 48 h after brain surgery.

Measurements of blood pressure, heart rate and ventilation

Arterial blood pressure (BP) was measured by connecting the arterial catheter to a

pressure transducer (Narco, P-1000B) and the signals from the transducer were recorded on paper (Narcotrace 80). Heart rate (HR) was determined by counting pressure pulses.

Pulmonary ventilation (V_I) was measured directly using a pneumotachographic method (9) based on the Poiseuille principle that the laminar flow of a gas is proportional to the pressure gradient across a tube. A lightweight transparent face mask provided an air-tight connection between the nostrils and a Fleisch tube. Inspiratory and expiratory gas flows were monitored by means of a differential pressure transducer (Validyne 451871) connected to the same physiograph.

Experimental protocols

Two days after surgery, the animals were housed individually in a plastic chamber kept at the experimental temperature of 25°C. The animal chamber was continuously flushed with humidified room air. In one group (N = 8), experimental animals received one microinjection of 100 nmol/0.5 µl L-NAME (Sigma Chemical Co., St. Louis, MO, USA) dissolved in mock cerebrospinal fluid (mCSF). The basic composition of the mCSF solutions in mEq/l was: 83.6 Na^+, 2.7 K^+, 0.9 Ca^{2+}, 0.45 Mg^{2+} and 0.45 SO_4^{2-}. Bicarbonate and chloride concentrations were adjusted in order to attain the desired pH values for the individual perfusate solutions. Control animals (N = 8) were treated with intracerebral microinjections of vehicle in the same volume. Intracerebral injections were performed with a thin dental needle introduced until its tip was 2 mm below the cannula end. A volume of 0.5 µl was injected over a period of 30 s using a Hamilton microsyringe. The movement of an air bubble inside the PE 10 polyethylene tubing connecting the microsyringe to a dental needle confirmed drug flow. Doses and methods of administration were chosen on the basis of a previous study (10).

Once conditions were stable in the normocapnic condition, BP and V_I were recorded. A hypercapnic gas mixture of 3% CO_2 (AGA, Sertãozinho, SP, Brazil) was then applied for 30 min.

Histology

At the end of experiments the animals were sacrificed with ether and perfused through the heart with Ringer followed by 10% formalin. A dental needle was inserted through the guide cannula and a 0.5 µl microinjection of Evan's blue was performed. The heads of the animals were removed and fixed in 10% formalin. The brains were removed from the skull, immersed in paraffin, sectioned on a microtome, and stained with hematoxylin-eosin for light microscopy determination of the region reached by the microinjection needle.

Calculations and statistical analysis

All values are reported as means ± SEM. BP, HR and V_I were calculated on the basis of 10-min recording periods. BF was quantified by analyzing the number of respiratory events (lung breaths) per minute. Tidal volume (V_T) was obtained from the integrated area of the inspired flow signal. V_I ($V_I = V_T$ x BF) was expressed as ml BTPS (body temperature and pressure, saturated with water vapor) kg^{-1} min^{-1}. The effects of hypercapnia were evaluated by analysis of variance (ANOVA) and the difference between means was assessed by Tukey's test. A P value of less than 0.05 was considered significant.

Results

Effects of L-NAME microinjection into the nucleus isthmi on ventilatory response to hypercapnia

Figure 1A shows the effect of L-NAME microinjection on V_I. Under normocapnia

no significant changes in V_I were observed between control and L-NAME-treated toads. Hypercapnia caused a significant ($P<0.01$) increase in V_I only after L-NAME treatment.

Figure 1B shows the effects of L-NAME microinjection on BF during normocapnia and hypercapnia. Under normocapnia, no difference in BF was observed between control and L-NAME-treated animals, whereas exposure to hypercapnia caused a significant increase in BF ($P<0.01$ for the control group and $P<0.001$ for the L-NAME group). Figure 1C shows that during hypercapnia, the V_T of L-NAME group tended to be higher than in the control group, but the increase was not statistically significant.

Figure 2 shows the pulmonary ventilation recordings obtained after mCSF or L-NAME microinjections during normocapnia and hypercapnia.

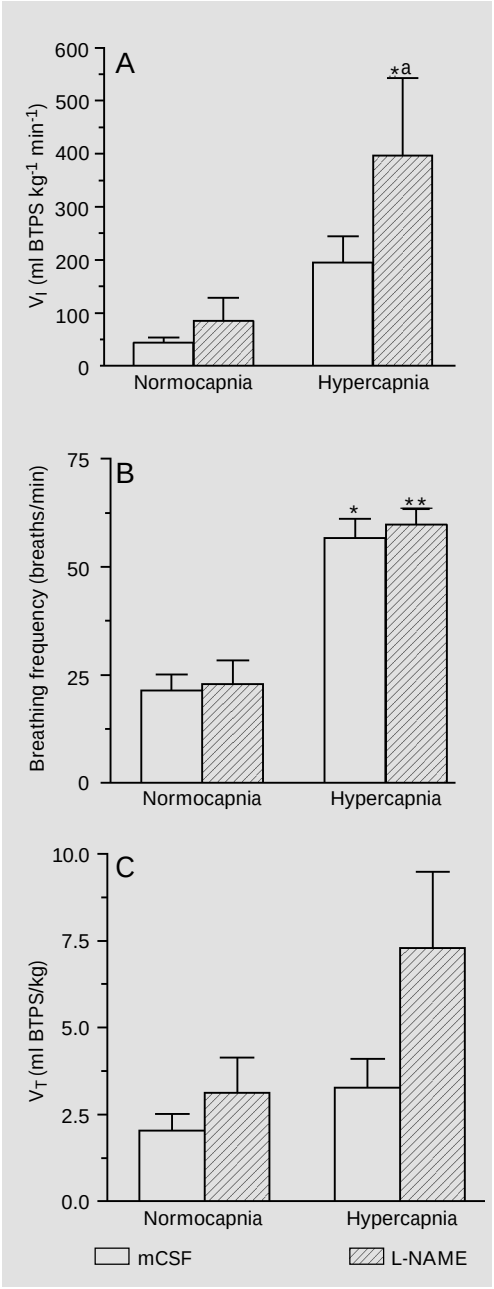

Figure 1 - Effect of microinjection of L-NAME (100 nmol/0.5 µl) or mCSF (0.5 µl) into the nucleus isthmi on V_I (A), BF (B) and V_T (C), in animals exposed to normocapnia and hypercapnia (3% CO_2). *P<0.01, **P<0.001 indicate a significant difference in mean values before and after 30 min of 3% CO_2. [a]P<0.01 indicates a significant difference between control and the L-NAME group (Tukey's test).

Effects of L-NAME microinjection into the nucleus isthmi on blood pressure and heart rate response to hypercapnia

Table 1 shows the effects of treatments on BP and HR. None of the experimental conditions had any significant effect on BP and HR.

Discussion

The present study provides evidence that the gas NO plays a role in NI neurotransmission involved in CO_2-drive to breathing since intracerebral microinjection of a nonselective NOS blocker (L-NAME) increased the ventilatory CO_2 response.

Despite recent advances, the mechanisms of neurorespiratory control in amphibians are not fully understood. Most early studies on pulmonary ventilation in amphibians focused on the mechanics of pulmonary ventilation, the mechanism behind the positive pressure inflation of the lungs (11,12). Additionally, the periodic pattern of ventilation was described as breath-holding alternating with bursts of pulmonary ventilation, which in some anuran amphibians are initiated by stepwise pulmonary deflation followed by lung reinflation (13,14). In anurans, studies have evaluated the chemical drive to breathe and the receptors involved. The ventilatory responses to hypoxia were assessed in *Bufo paracnemis* (15), and the other studies characterized the arterial O_2 receptors (16,17), as

well as the central acid-base receptors of *Bufo paracnemis* (18,19). The respiratory control of ectotherms resembles the mammalian control system; the rhythmogenic and pattern-forming elements in each are adapted to meet the demands determined by the environment, behavior, metabolic needs, and breathing mechanisms. However, studies about neurorespiratory control in ectotherm vertebrates are scarce (20,21). Recently, Kinkead et al. (2) found that NI plays an important role in respiratory control by maintaining eucapnic motor output and allowing full expression of the CO_2 response, since bilateral lesions of NI in bullfrogs by microinjection of kainic acid caused a reduction of eucapnic ventilation and of CO_2 chemosensivity. However, it has been previously shown that kainic acid causes damage at the site of injection together with seizures and loss of cells some distance from the injection (22). Thus, it is often difficult to attribute the changes observed in this experimental preparation to damage to the brain area of interest. Additionally, the experiments were performed approximately 1.5 h after the kainic acid injections, despite the fact that this neurotoxin has a strong and long-lasting excitatory effect (23).

The experimental approach used in the present study differs considerably from that followed by Kinkead et al. (2). They performed this study in decerebrate, paralyzed and unidirectionally ventilated bullfrogs. These differences preclude a detailed comparison between their study and the present data although both investigations provide support for a participation of the NI in integration of CO_2-drive to breathing.

Glutamate is widely distributed in the central nervous system and is supposed to act as a neurotransmitter in the NI for the control of breathing (2). NO is linked to glutamatergic neurotransmission in the central nervous system (24,25) and activation of glutamate receptors in the nucleus tractus solitarius (NTS) and paragigantocellular nucleus is necessary to maintain normal levels of blood pressure and ventilation and also for a normal ventilatory CO_2 response to occur (26). It has been demonstrated that NO enhances the excitability and spontaneous discharge rates of neurons in the NTS (27) and may act as a retrograde messenger in an L-glutamate-releasing positive feedback system, also in the NTS, involved in the increase of ventilation during hypoxia (24). NO has an excitatory effect on the discharge rates of neurons in the pontine respiratory group, whereas L-NNA produces a disruption of the pneumotaxic mechanism (28).

A number of recent studies have shown that NO accounts for a large part of the biological actions of endothelin-derived re-

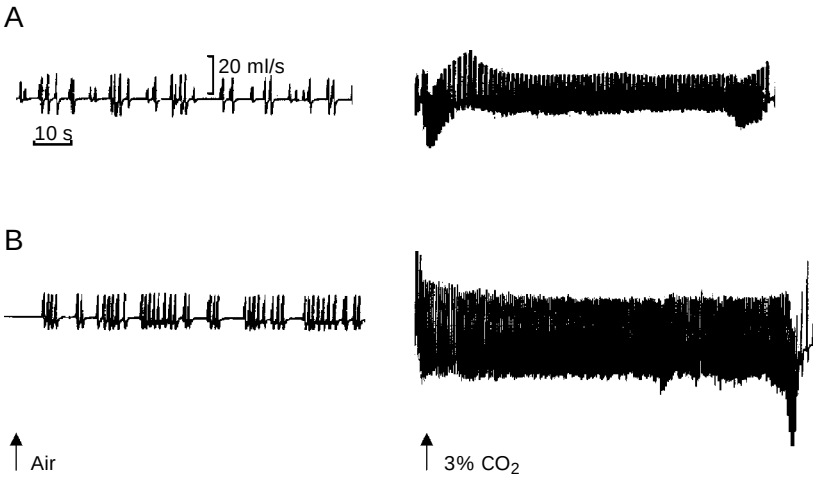

Figure 2 - Pulmonary ventilation recordings for toads illustrating the effect of intracerebral microinjection of mCSF (A) or L-NAME (B) (100 nmol/0.5 µl), during air and 3% CO_2 inhalation (arrows).

Table 1 - Effects of L-NAME microinjection into the nucleus isthmi on the blood pressure and heart rate response of *Bufo paracnemis* to hypercapnia.

Values are reported as means ± SEM. mCSF, Mock cerebrospinal fluid.

Treatment	Inspired CO_2 (%)	Blood pressure (mmHg)	Heart rate (min^{-1})
mCSF (0.5 µl)	0	36.7 ± 3.8	41.2 ± 4
(N = 8)	3	34.9 ± 2.3	46.0 ± 7.2
L-NAME (100 nmol/0.5 µl)	0	30.3 ± 4.1	47.6 ± 5
(N = 8)	3	27.7 ± 1.6	48.6 ± 6.7

laxing factor (29). The importance of NO can be demonstrated by inhibition of the effects of NO (30) using L-arginine analogs such as L-NAME. In the present study, we have chosen L-NAME because it is a nonselective inhibitor of NOS and acts on both the constitutive and inducible isoforms of the enzymes.

The microinjection of L-NAME into the NI altered the CO_2-induced hyperventilation (Figure 1). When the animals were exposed to hypercapnia after receiving L-NAME, a significant increase in ventilatory CO_2 response was observed. Probably, this might be due to the fact that NO in the NI may have an inhibitory influence on the integration of the CO_2-drive to breathing. Conversely, a number of studies indicate NO as an excitatory neurotransmitter (7,6). In agreement with our study, a previous study, Iadecola et al. (31) reported that intravenous administration of L-NAME to rats leads to a partial inhibition, i.e., ~50% inhibition of brain NOS catalytic activity. NO, besides increasing cGMP, can also inhibit NMDA-induced intracellular calcium entry and NOS activity, exercising a local negative feedback that may be an important intracellular mechanism in the regulation of NO synthesis because NO has been shown to be potentially cytotoxic (32). Such a role for NO may complicate the interpretation of data obtained by application of L-NAME. It seems that the role of NO is more complex than previously thought, and may vary according to the site under study.

NO acts as a physiological messenger molecule that may serve as a neurotransmitter in the central nervous system (29). In amphibians, NO has been suggested to be a neurotransmitter in the gut (33). We have demonstrated that NO in the NI has an inhibitory influence only when the respiratory drive is high (hypercapnia), acting on V_T. Conversely, the present observations, together with other studies on the presence of NO synthase in amphibians (8,33,34), indicate a considerable degree of phylogenetic conservation of the NO pathway amongst vertebrates.

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