Effect of nitric oxide synthase inhibition on hypercapnia-induced hypothermia and hyperventilation

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Barros, Renata C. H., and Luiz G. S. Branco. Effect of nitric oxide synthase inhibition on hypercapnia-induced hypothermia and hyperventilation. J. Appl. Physiol. 85(3): 967–972. 1998.—Hypercapnia elicits hypothermia in a number of vertebrates, but the mechanisms involved are not well understood. In the present study, we assessed the participation of the nitric oxide (NO) pathway in hypercapnia-induced hypothermia and hyperventilation by means of NO synthase inhibition by using N\(^{-}\)-nitro-L-arginine (L-NNA). Measurements of ventilation, body temperature, and oxygen consumption were performed in awake unrestrained rats before and after L-NNA injection (intraperitoneally) and L-NNA injection followed by hypercapnia (5% CO\(_2\)). Control animals received saline injections. L-NNA altered the breathing pattern during the control situation but not during hypercapnia. A significant (P < 0.05) drop in body temperature was measured after both L-NNA (40 mg/kg) and 5% inspired CO\(_2\), with a drop in oxygen consumption in the first situation but not in the second. Hypercapnia had no effect on L-NNA-induced hypothermia. The ventilatory response to hypercapnia was not changed by L-NNA, even though L-NNA caused a drop in body temperature. The present data indicate that the two responses elicited by hypercapnia, i.e., hyperventilation and hypothermia, do not share NO as a common mediator. However, the L-arginine-NO pathway participates, although in an unrelated way, in respiratory function and thermoregulation.

body temperature; ventilation; carbon dioxide

A REDUCTION IN BODY TEMPERATURE (\(T_b\)) is generally observed when an animal encounters stress such as hypoxia (36) and hypercapnia (5). Although this response is extremely widespread among taxa (5), little is known about the potential mediators of hypothermia and also about the effect of \(T_b\) on the hypercapnic drive to breathe.

The endothelium-derived relaxing factor, which has been definitively identified as nitric oxide (NO) (23), has started a revolution in the understanding of several mammalian systems since its description in 1980 by Furchgott and Zawadzki (9). NO is an active regulatory molecule at the periphery (23) and also serves as a neuronal messenger in the brain (18). It is synthesized from L-arginine, and the enzyme responsible for this synthesis is NO synthase (NOS). L-arginine analogs such as \(N^{-}\)-nitro-L-arginine (L-NNA) have been used as inhibitors of NO synthesis. Compared with other analogs, L-NNA crosses the blood-brain barrier relatively easily and strongly inhibits NOS in the central nervous system (cf. Ref. 34).

It has become increasingly clear that NO may play a role in ventilatory (12) and \(T_b\) (3) control in mammals.

NOS is present in medullary and pontine respiratory regions (8), where a central respiratory neural network involving NO may exist. At the periphery, NO acts as an inhibitory transmitter in the carotid bodies (6). As to its temperature effects, NO plays a role in thermoregulation during exercise in horses (22) and acts as an endogenous antipyretic factor in endotoxin-induced fever in rabbits (11). Recently, it has been shown that NO participates in hypoxia-induced hypothermia (3).

No reports are available about the effect of NOS inhibition on hypercapnia-induced hyperventilation and hypothermia. Branco and Wood (5) demonstrated that hypercapnia causes behavioral hypothermia in the toad Bufo marinus and that this response is mediated by central chemoreceptors, i.e., at the same site where hypercapnia elicits hyperventilation not only in amphibians (4) but also in mammals (29). Because both responses to CO\(_2\) inhalation may share to some extent the same central sensor, they may also share some mediators, and NO seems to be a strong candidate. The purpose of the present study was to examine the role of the L-arginine-NO pathway in the hyperventilatory and hypothermic responses to CO\(_2\) inhalation of conscious rats.

MATERIALS AND METHODS

Animals

Experiments were performed in awake adult male Wistar rats (Rattus norvegicus; Rodentia: Muridae), weighing 230–300 g, housed under conditions of controlled temperature (25 ± 2 (SD) °C), and exposed to a daily 12:12-h light-dark cycle. The animals were allowed free access to water and food. The rats used in this study were divided into two groups, an experimental group treated with a NOS blocker (L-NNA) and a control group (n = 8 rats) injected with saline. The experimental group was divided into three subgroups (n = 8 rats/subgroup), according to the NOS-blocker dose used.

Ventilation (\(V_e\)) Measurements

Measurements of \(V_e\) were performed by the body plethysmograph method (2). The animal was placed in a Plexiglas chamber (5 liters) and allowed to move about freely while the chamber was flushed with humidified air or a normoxic hypercapnic gas mixture of 5% CO\(_2\). Each time \(V_e\) was measured, the flow was interrupted and the chamber was sealed for short periods of time (no more than 3 min), and the oscillations in air temperature caused by breathing could be measured as pressure oscillations. This procedure was performed once per experimental condition. Calibration for volume was obtained during each measurement by injecting the chamber with a known amount of air (0.2 ml) by using a graduated syringe. Signals from a differential air transducer displayed on a paper recorder (Hewlett-Packard) allowed the
calculation of respiratory frequency (f) and tidal volume (VT) by appropriate correction factors (20).

Tb Measurements

Tb was determined by inserting a thermocouple probe into the colon of the animals every time a Tb measurement was made. Before the experiments, the rats were habituated to temperature measurements, which were performed quickly to avoid any stress-induced elevations in Tb.

O2 Consumption (VO2) Measurements

VO2 was measured by using an O2 analyzer (Saylor Servomex, type OA. 272) in a closed-flow system. Animals were placed in the same 5-liter Plexiglas chamber ventilated with humidified air or a normoxic hypercapnic gas mixture of 5% CO2. At the end of the control or experimental period, the flow was interrupted to perform the measurements. The chamber was then totally sealed, and six samples of chamber air (30 ml) were taken at 3-min intervals and passed through the O2 analyzer. Therefore, the chamber remained sealed for 15 min in each condition. This period was needed to collect sufficient data to ensure good accuracy of the slopes. A curve of %O2 evolution was constructed, and its slope was given the value of VO2. Because we obtained resting VO2 values agreeing with data in the literature, we concluded that this method was acceptable.

Experimental Protocols

Experiment 1: Measurements of VE and Tb. Each animal was placed in the chamber flushed with room air. After the animals remained calm (at least 30 min), control Tb and then VE were measured. Subsequently, experimental rats were treated with l-NNA (Sigma Chemical) by intraperitoneal bolus injection of 10, 20, or 40 mg/kg body weight, and control rats were treated with the same volume of saline (150 mM NaCl). Two hours after l-NNA or saline administration, measurements of Tb and VE were obtained. Subsequently, a normoxic hypercapnic gas mixture of 5% CO2 (AGA) was flushed through the chamber for 30 min. At the end of the experiment, VE and Tb were measured again. Saline was the vehicle in which l-NNA was dissolved. The volume of each injection was 0.5 ml.

Experiment 2: Measurements of VO2. Animals were placed in the chamber ventilated with room air for at least 30 min until they remained quiet, and the basal VO2 was measured. The three subgroups of rats received an intraperitoneal bolus injection of 10, 20, or 40 mg/kg body weight of l-NNA, and the control group received an intraperitoneal injection of saline, and VO2 was measured 2 h later. Subsequently, a normoxic hypercapnic gas mixture of 5% CO2 was flushed through the chamber for 30 min, and VO2 was measured again.

Statistical Analysis

Values are reported as means ± SE (unless otherwise stated). The effect of saline or l-NNA injection on VE, VT, f, T0, and VO2 was evaluated by using one-way ANOVA. The Tukey-Kramer multiple-comparisons test (VE, VT, f, and T0) and the Kruskal-Wallis test (VO2) were applied as post hoc tests. Point-by-point comparisons of mean values before and after hypercapnia were performed by Student’s t-test. Values of P < 0.05 were considered to be significant.

RESULTS

During the two sets of experiments, the mean chamber temperature was 25.81 ± 0.69 (SD) °C, and room temperature was 24.62 ± 0.76°C. Individual values of VE, T0, and VO2 during air breathing and before any injection ranged from 601.6 to 965.86 ml BTPS·min−1·kg−1, from 36.10 to 36.70°C, and from 14.84 to 24.12 ml STPD·min−1·kg−1, respectively, and no group differed significantly from the saline group in baseline values.

Experiment 1

Figure 1 shows the pulmonary VE recordings obtained after saline or l-NNA injections during air or 5% CO2 breathing. Animals treated with the NOS blocker had a ventilatory response during air breathing that consisted of episodes of many breaths separated by episodes of few breaths. This response was dose dependent but not evenly distributed: 25, 50, and 62.5% of the animals treated with 10, 20, and 40 mg/kg, respectively, showed this changed ventilatory pattern, whereas no rat injected with saline was affected. No difference in animal behavior was observed between control and treated groups. The breathing pattern was recovered during CO2 inhalation, when both groups, experimental and control, showed similar recordings.

Figure 2 illustrates the effect of the NOS blocker on Tb during air or 5% CO2 breathing. When saline was injected intraperitoneally, no change in Tb was observed. Conversely, l-NNA injected at the 40 mg/kg dose elicited a significant reduction in Tb (P < 0.05; 1-way ANOVA) of 0.74 ± 0.05°C, whereas 10 and 20 mg/kg l-NNA caused no significant change. The changes
in $T_b$ caused by NOS blockade were not altered by hypercapnia. When inspired CO$_2$ was increased from 0 to 5%, a significant ($P < 0.05$; paired $t$-test) decrease in $T_b$ was observed in the saline group.

### Experiment 2

The individual curves of $\%O_2$ evolution showed accurate slopes, i.e., maximum changes observed were

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**Table 1. Effect of peripheral injection of L-NNA and saline on $\dot{V}e$, $V_t$, and $f$ in rats during hypercapnia**

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>10 mg/kg</th>
<th>20 mg/kg</th>
<th>40 mg/kg</th>
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<tbody>
<tr>
<td></td>
<td>Pre-L-NNA</td>
<td></td>
<td></td>
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<tr>
<td>$V_t$, ml/kg</td>
<td>7.48 ± 0.16</td>
<td>7.41 ± 0.33</td>
<td>7.26 ± 0.52</td>
<td>7.40 ± 0.35</td>
</tr>
<tr>
<td>$f$, breaths/min</td>
<td>106.00 ± 4.25</td>
<td>105.00 ± 4.91</td>
<td>112.08 ± 6.69</td>
<td>107.50 ± 5.90</td>
</tr>
<tr>
<td>$V_e$, ml·min$^{-1}$·kg$^{-1}$</td>
<td>790.46 ± 31.64</td>
<td>772.30 ± 28.49</td>
<td>796.34 ± 43.55</td>
<td>795.50 ± 30.40</td>
</tr>
<tr>
<td>L-NNA</td>
<td></td>
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<tr>
<td>$V_t$, ml/kg</td>
<td>7.17 ± 0.47</td>
<td>8.45 ± 0.95</td>
<td>8.41 ± 0.56</td>
<td>9.35 ± 0.41</td>
</tr>
<tr>
<td>$f$, breaths/min</td>
<td>108.00 ± 6.09</td>
<td>101.77 ± 7.94</td>
<td>104.78 ± 8.87</td>
<td>98.56 ± 8.35</td>
</tr>
<tr>
<td>$V_e$, ml·min$^{-1}$·kg$^{-1}$</td>
<td>771.22 ± 58.56</td>
<td>843.77 ± 105.23</td>
<td>870.26 ± 70.77</td>
<td>902.76 ± 52.55</td>
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<tr>
<td>L-NNA and 5% CO$_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_t$, ml/kg</td>
<td>11.30 ± 0.75*</td>
<td>11.56 ± 0.98*</td>
<td>10.64 ± 0.46*</td>
<td>12.98 ± 1.03*</td>
</tr>
<tr>
<td>$f$, breaths/min</td>
<td>147.00 ± 10.76*</td>
<td>127.03 ± 4.10*</td>
<td>132.86 ± 7.08*</td>
<td>136.41 ± 6.94*</td>
</tr>
<tr>
<td>$V_e$, ml·min$^{-1}$·kg$^{-1}$</td>
<td>1,636.66 ± 85.87*</td>
<td>1,472.95 ± 140.46*</td>
<td>1,418.95 ± 107.90*</td>
<td>1,798.69 ± 206.94*</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 8$ group. L-NNA, N$\rightarrow$-nitro-L-arginine; $V_e$, ventilation; $V_t$, tidal volume; $f$, respiratory frequency. *Significant difference in mean values before and after 30 min of 5% CO$_2$ exposure ($P < 0.05$; paired $t$-test).
Effects of L-NNA and Hypercapnia on V̇E

The sites of action of L-NNA that may affect respiratory function are distributed throughout the body. At the periphery, NO acts as an inhibitory neurotransmitter in the carotid body (6), and alters arterial blood pressure, which can change V̇E probably by a baroreceptor-mediated effect (cf. Ref. 34). NO is linked to glutamatergic neurotransmission in the central nervous system (27, 34), and activation of glutamate receptors in the nucleus tractus solitarii and paragigantocellular nucleus is necessary to maintain normal levels of V̇E and also for a normal ventilatory CO₂ response to occur (26). It has been demonstrated that NO enhances the excitability and spontaneous discharge rates of neurons in the nucleus tractus solitarii (19) and that L-NNA produces a disruption of the pneumotaxic mechanism (18).

In the present study we found an unusual ventilatory pattern during air breathing 2 h after injection of L-NNA. Because this response was totally unexpected and was not the purpose of the study, some information cannot be provided with the present protocol and remains to be investigated, such as the duration of the abnormal ventilatory pattern, the time course of the dose-dependent response, and the situation during normoxia after CO₂ breathing. Although no difference in animal behavior was observed between control and treated groups, we should not exclude the possibility of a toxic effect of the drug. This seems to be unlikely because the L-NNA doses in this study and even higher ones have been regularly used without problems regarding toxic effects (34, 35). Thus we continue to think that the changes observed in the present study may be caused by a specific effect of the drug on NO. Because we used systemic administration of a NOS inhibitor, the atypical breathing pattern probably resulted from a balance between the peripheral and mainly the central systems where NO synthesis occurs. This affirmation is based on the facts that L-NNA crosses the blood-brain barrier (34) and that the altered ventilatory pattern, with episodes of many breaths separated by episodes of few breaths that we may call short nonventilatory periods, occurred in a dose-dependent way, but not after saline administration (Fig. 1). Episodic breathing is a pattern in many ectothermic vertebrates that can occur in some species of mammals in states normally associated with metabolic depression, such as hibernation and sleep (15). The lack of NO produced a fictive breathing pattern that resembled the episodic one and may support the argument that respiratory control is a system highly conserved in vertebrates. Furthermore, the present study indicates that NO plays a major role in normal respiratory function in rats.

Hypercapnia induced similar V̇E elevations in control and treated groups, which resulted from the same strategy, i.e., they were due to both VT and f increases. It seems that an additional factor (CO₂) that stimulates the respiratory center can counterbalance the effect of L-NNA. Gozal et al. (12) also found that NOS inhibition, by using N′-nitro-L-argininemethyl ester and 3,4-dimethyl-L-thiocitrulline injected intravenously, did not alter the overall ventilatory response to hypercapnia. However, changes in the ventilatory strategy consisting of VT decreases with parallel f increases were reported. These contrasting data suggest that different responses are likely to occur when different NOS blockers and different pathways of drug administration are compared. Although the mechanisms underlying these findings are unclear, NO does not seem to be important for chemosensitivity per se but may play a role in neurons underlying normal functions of respiratory timing and amplitude (19, 18).

Effects of L-NNA and Hypercapnia on T₉₀

The NO pathway participates in many systems that can interfere with T₉₀ control, including brown adipose tissue (25), vascular smooth muscle (33), and some areas of the central nervous system, especially the hypothalamus (7). An interesting event is that treatment with L-NNA caused a reduction in T₉₀ (Fig. 2) despite the fact that L-NNA should decrease cutaneous heat loss because it causes vasoconstriction in both large and small arteries (33). Most likely, L-NNA elicits hypothermia by reducing brown fat blood flow (25), which may result in a drop in the firing rate of sympathetic nerves innervating interscapular brown adipose tissue (7). Thus the available data indicate that L-NNA, at least when injected systemically, may induce
Some investigators have observed an increase in both metabolism in rats. Lai et al. (17) showed that even when caused by hypoxia and seems to be more complex than hypothermia, which has been less studied than the hypoxia-induced hypothermia simultaneously. NOS inhibition did not change the ventilatory drive to hypercapnia, even though it caused a drop in $T_b$. However, the L-arginine-NO pathway participates in normal respiratory function and thermoregulation. Several studies have been conducted to determine whether changes in $T_b$ alter the ventilatory response to hypoxia. The present study confirms that hypercapnia-induced hypothermia may involve a central control. Indeed, Kühnen et al. (16) reported a shift in the set point of $T_b$ to a lower level during hypercapnia in the golden hamster, Mesocricetus auratus. In addition, the activity of neurons in the preoptic area, which affect $T_b$, is higher during hypercapnia than during hypoxia in Wistar rats (32).

The present study confirms that hypercapnia-induced hypothermia may involve other mechanisms in addition to hyperventilation or vasodilatation because the drop caused by the NOS blockade was not changed by hypercapnia. The effect of CO$_2$ inhalation on $V_E$ was maintained after treatment with L-NNA, but no additional decrease in $T_b$ was observed, suggesting that hypercapnia-induced hypothermia has a central component that may involve the NO pathway.

Effects of L-NNA and Hypercapnia on $V_O_2$

It has been shown that NOS inhibitors produce hypothermia (3). Such a reduction in $T_b$ may be accompanied by a decrease in $V_O_2$, mainly if the thermogenic response is blunted (36). In the present study, a drop in $V_O_2$ was observed 2 h after injection of the higher NO-blocker dose.

A review of the literature reveals contradictory effects of hypercapnia on $V_O_2$. Numerous factors such as intrinsic species differences may contribute to the variability in the metabolic effects of CO$_2$ inhalation. Some investigators have observed an increase in both VO$_2$ and $V_E$, the former being commonly attributed to the energetic cost of hyperventilation, for example in ponies (14). In the present study hypercapnia had no effect on VO$_2$ in rats, as also observed by Saiki and Mortola (31), who detected only a small but not significant trend for VO$_2$ to increase with 2 and 5% CO$_2$ inhalation. Finally, cats show a large drop in VO$_2$ during 4% CO$_2$ breathing at room temperature (30). Different energetic costs of hyperventilation may complicate the interpretation of such results. With this fact taken into consideration, hypercapnia might have reduced the metabolic rate, but this could not be measured because of the hyperventilation response. Thus CO$_2$ exposure had no effect on VO$_2$ in rats after saline or L-NNA, at the two lower doses. However, the drop in VO$_2$ in the subgroup injected with the higher dose of L-NNA was recovered during CO$_2$ exposure, possibly because of the hyperventilation response.

Effects of L-NNA on the Interaction Between Hypothermia and Hyperventilation During Hypercapnia

The present study indicates that the responses elicited by hypercapnia, hyperventilation, and hypothermia do not share NO as a common mediator because no interaction exists between the effects of L-NNA on hypercapnia-induced hyperventilation and hypothermia simultaneously. NOS inhibition did not change the ventilatory drive to hypercapnia, even though it caused a drop in $T_b$. However, the L-arginine-NO pathway participates in normal respiratory function and thermoregulation. Several studies have been conducted to determine whether changes in $T_b$ alter the ventilatory response to changes in inspired CO$_2$ levels, but in homeotherms the methodology always seems to be a problem, yielding divergent results. Oliever et al. (28), using a $T_b$ range of 34–40°C in anesthetized cats, concluded that $T_b$ had no important modifying effects on the response to CO$_2$. However, anesthetics impair the homeostatic mechanisms regulating $T_b$ (21), and exposure to cold affects heat production and heat loss mechanisms but does not establish a lower set-point value of $T_b$. Maskrey (21) reported that the effect of cooling on the response to breathing CO$_2$-enriched air was to raise $V_E$ in awake rats. He used an abdominal heat exchanger to cause hypothermia, which again does not affect the set point for $T_b$. Ectothermic animals seem to be a better model for studying the effect of $T_b$ on the hypercapnic drive to breathe, but data in the literature also show divergent results. In the toad Bufo paracnemis, decreases in $T_b$ elicit a reduced hypercapnic drive to breathe (4). In contrast, Jackson et al. (13) demonstrated that $V_E$ in Pseudemys turtles displays a similar sensitivity to increases in inspired CO$_2$ at 10, 20, and 30°C. The present study, conducted in conscious rats and at ambient temperature, shows that a modest but significant drop in $T_b$, caused by NOS blockade, which seems to interfere with the set point for $T_b$, had no effect on the hypercapnic drive to breathe.

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