Role of neuronal nitric oxide synthase in hypoxia-induced anapyrexia in rats

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Steiner, Alexandre A., Evelin C. Carnio, and Luiz G. S. Branco. Role of neuronal nitric oxide synthase in hypoxia-induced anapyrexia in rats. J Appl Physiol 89: 1131–1136, 2000.—Anapyrexia (a regulated decrease in body temperature) is a response to hypoxia that occurs in organisms ranging from protozoans to mammals, but very little is known about the mechanisms involved. Recently, it has been shown that the NO pathway plays a major role in hypoxia-induced anapyrexia. However, very little is known about which of the three different nitric oxide synthase isofoms (neuronal, endothelial, or inducible) is involved. The present study was designed to test the hypothesis that neuronal nitric oxide synthase (nNOS) plays a role in hypoxia-induced anapyrexia. Body core temperature (Tc) of awake, unrestrained rats was measured continuously using biotelemetry. Rats were submitted to hypoxia, 7-nitroindazole (7-NI; a selective nNOS inhibitor) injection, or both treatments together. Control animals received vehicle injections of the same volume. We observed a significant (P < 0.05) reduction in Tc of ∼2.8°C after hypoxia (7% inspired O2), whereas intraperitoneal injection of 7-NI at 25 mg/kg caused no significant change in Tc. 7-NI at 30 mg/kg elicited a reduction in Tc and was abandoned in further experiments. When the two treatments were combined (25 mg/kg of 7-NI and 7% inspired O2), we observed a significant attenuation of hypoxia-induced anapyrexia. The data indicate that nNOS plays a role in hypoxia-induced anapyrexia.

Increased survival of the tested species if the animals are allowed to become hypothermic during hypoxia exposure (20). Although this protective response is extremely widespread among taxa, little is known about the mechanisms involved. Evidence has accumulated that hypoxia-induced hypothermia is likely to be produced by a downward resetting of the thermoregulatory set point (6, 15). This regulated decrease in body temperature has been referred to as anapyrexia (9, 15, 38).

The description of endothelium-derived relaxing factor in 1980 by Furchgott and Zawadzki (14) started a revolution in the understanding of not only blood pressure control but also a number of other physiological systems (1, 3–5, 8, 11–13, 17, 19, 22, 24, 25, 27–29, 31, 32, 35, 37). Endothelium-derived relaxing factor, identified as nitric oxide (NO) (33), activates soluble guanylate cycase and increases cyclic GMP (cGMP) levels in vascular smooth muscle (34), the central nervous system (39), and several other tissues (27).

The family of NO synthase (NOS), the enzymes that produce NO in vivo, consists of two different classes, i.e., the inducible and constitutive forms (10). At least three isoforms of NOS exist: neuronal (n) and endothelial (e), as the constitutive isofoms, and inducible NOS (iNOS) (3, 10, 12). nNOS is encountered in spinal cord, brain, kidney, and sympathetic ganglia (36), and, according to Schmidt et al. (36), most NO in the brain may be synthesized by the action of nNOS. eNOS is largely found in endothelial cells and has a substantial role in blood pressure control (12, 14). iNOS is widely distributed throughout the immune cells, including macrophages and glial cells, and has been shown to be induced by several stimuli, including endotoxin (22). A number of studies have shown that L-arginine (a NOS substrate) analogs inhibit NO synthesis nonspecifically (27). More recently, the specific NOS isoform inhibitor 7-nitroindazole (7-NI) has been used to assess the role of the nNOS subtype in several processes involving NO (28, 29).

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HYPOXIA ELICITS A NUMBER OF COMPENSATORY RESPONSES IN ORGANISMS, RANGING FROM PROTOZOANS TO MAMMALS, INCLUDING A REDUCTION IN BODY TEMPERATURE (8, 16, 18, 20, 38, 40, 41). ACTIVE RESPONSES TO HYPOXIA, SUCH AS INCREASED PULMONARY VENTILATION AND CARDIAC OUTPUT, ARE, HOWEVER, OXYGEN CONSUMING, WHICH SETS A LIMIT TO THEIR USEFULNESS. THEREFORE, A DECREASE IN BODY TEMPERATURE MAY BE BENEFICIAL DUE TO A REDUCTION IN OXYGEN CONSUMPTION, A LEFTWARD SHIFT OF THE OXYHEMOGLOBIN DISSOCIATION CURVE WITH A RESULTING IMPROVEMENT OF OXYGEN LOADING IN THE LUNGS, AND A DECREASE IN VENTILATION WITH A RESULTING BLUNTING IN THE ENERGETICALLY COSTLY RESPONSES TO HYPOXIA (CF. REF. 40). THE IMPORTANCE OF THIS RESPONSE IS EMPHASIZED BY REPORTS THAT SHOW AN INCREASED SURVIVAL OF THE TESTED SPECIES IF THE ANIMALS ARE ALLOWED TO BECOME HYPOТЕРИСM DURING HYPOXIA EXPOSURE (20). ALTHOUGH THIS PROTECTIVE RESPONSE IS EXTREMELY WIDESPREAD AMONG TAXA, LITTLE IS KNOWN ABOUT THE MECHANISMS INVOLVED. EVIDENCE HAS ACCUMULATED THAT HYPOXIA-INDUCED HYPOTHERMIA IS LIKELY TO BE PRODUCED BY A DOWNWARD RESETTING OF THE THERMOREGULATORY SET POINT (6, 15). THIS REGULATED DECREASE IN BODY TEMPERATURE HAS BEEN REFERRED TO AS ANAPYREXIA (9, 15, 38).

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paires the febrile response in rats (35), whereas NO seems to act as an endogenous antipyretic factor in the central nervous system of rabbits and rats (1, 19). NO plays a permissive role in active vasodilatation in the rabbit ear (13) and in systemic vasopressin-induced hypothermia in rats (37). Moreover, our laboratory recently demonstrated that the NO pathway plays a major role in hypoxia-induced anapyrexia (8). However, the NOS isoform involved has not been identified.

Therefore, the aim of the present study was to test the hypothesis that the nNOS isoform plays a role in hypoxia-induced anapyrexia, using the selective nNOS inhibitor 7-NI.

MATERIALS AND METHODS

Animals. Experiments were performed on adult male Wistar rats weighing 200–250 g, housed at controlled temperature (25.3 ± 0.6°C) and exposed to a daily 12:12 h light-dark cycle. The animals were allowed free access to water and food.

In all experimental protocols, body temperature ranged from 36.9 to 38.0°C during the control period and no group’s baseline value differed significantly from the vehicle group. During the experiments, the mean chamber temperature was 26.2 ± 0.5°C, and room temperature was 25.8 ± 0.5°C.

Surgery. Animals were anesthetized with 2,2,2-tribromoethanol (Aldrich, Milwaukee, WI) and a paramedian laparotomy was performed for the insertion of a biotelemetry probe capsule (model VM-PH; Mini-Mitter, Sunriver, OR) into the peritoneal cavity. The wound was then closed with skin sutures and the implanted capsule was used for measurements of body core temperature (Tc). The surgical procedures were performed over a period of 10 min. After surgery, animals were treated with 100,000 units of benzylpenicillin and allowed to recover for 4 days.

Rats used for arterial blood pressure measurements were anesthetized with 2,2,2-tribromoethanol and implanted with a polyethylene catheter in the femoral artery for direct blood pressure recording. The arterial catheter was composed of a segment of PE-10 tubing (4.5 cm) heat bonded to a 15-cm-long PE-50 catheter. The catheter was filled with 0.3% heparin in sterile saline (150 mM NaCl). The PE-10 segment was introduced into the femoral artery until the tip reached the abdominal aorta. The catheter was secured in position with thread, and the PE-50 segment was passed under the skin to be finally extruded on the dorsum of the animals. After surgery, animals were also treated with 100,000 units of benzylpenicillin and allowed to recover for 2 days before experimentation. During this period, the catheters were flushed daily with heparinized saline.

Body temperature measurement. Tc was measured by biotelemetry. The cages of animals previously implanted with the temperature probe were placed on an RLA 3000 telemetry receiver. The output of the receiver was fed to a Vital View data acquisition system, and data were displayed graphically on a video monitor.

Determination of the effect of hypoxia on body temperature. Rats were housed in a plastic chamber (5 liters) ventilated with humidified room air for at least 2 h before control Tc was determined. In all experimental protocols, control Tc was determined as the mean of four measurements made at 10-min intervals. Ten minutes later (time zero), a humidified hypoxic gas mixture of 7% inspired O2 (AGA) was flushed through the chamber for 120 min. Subsequently, the chamber was ventilated again with humidified room air, under the same conditions as before, for an additional 80-min period. During the experiments, Tc was recorded every 10 min. The severity of hypoxia used (7% oxygen) was chosen on the basis of previous studies from our laboratory (8, 38).

Determination of the effect of 7-NI on body temperature. The same animal chamber (now continuously flushed with humidified room air only) was used for all experiments. After the animals habituated to the experimental conditions (~2 h), control Tc was determined and experimental rats were then treated with 7-NI (Calbiochem-Novabiochem, La Jolla, CA) by intraperitoneal injection of 25 or 30 mg/kg body wt. Tc was then recorded every 10 min, for a total of 200 min. 7-NI was dissolved in vehicle consisting of dimethyl sulfoxide-sesame oil (1:9). The volume of each injection was 0.5 ml. Doses, method of administration, and period of time after injection when Tc was determined were chosen on the basis of previous studies (23, 26, 28, 29). Control animals were treated with an intraperitoneal injection of the same volume of vehicle.

Determination of the combined effects of hypoxia and 7-NI on body temperature. After an initial 2-h period, 7-NI (25 mg/kg) or its vehicle was injected intraperitoneally while the chamber was kept ventilated with room air. Thirty min after injection, a hypoxic gas mixture (7% inspired O2) was applied for an additional 120-min period. Subsequently, the chamber was ventilated again with humidified room air for 80 min. Tc was recorded every 10 min throughout the experiment.

Determination of the effect of 7-NI on mean arterial pressure and heart rate. The effect of 7-NI on mean arterial blood pressure (MAP) and heart rate (HR) was determined in unanesthetized, freely moving rats using a Narco polygraph (NarcoTrace 80) connected to a pressure transducer (Narco model P-10000B). A paper recording speed of 0.5 mm/s was used to minimize blood pressure fluctuation artifacts. HR was measured by actual pulse counting at a paper recording speed of 5 mm/s. A 30-min period was initially used to measure stabilized control values of MAP and HR. Subsequently, animals were injected intraperitoneally with 7-NI at the higher dose used in the present study (30 mg/kg body wt) or its vehicle, and MAP was recorded continuously for 1 h. The volume of each injection was 0.5 ml.

Statistical analysis. All values in this study are given as means ± SD. Changes in Tc were evaluated by repeated measures ANOVA. The difference between means was assessed by the Tukey-Kramer multiple-comparisons test. When necessary, two way ANOVA was used, followed by a point-by-point unpaired t-test to assess differences between groups. Values of P < 0.05 were considered significant.

RESULTS

Effect of hypoxia on Tc. Figure 1 shows the effect of hypoxia on Tc. Hypoxia caused a significant reduction in Tc, whereas room air caused no change. When inspired O2 was reduced from 21% (room air) to 7%, Tc dropped quickly during the first 50 min and continued to drop more slowly up to 90 min, after which a plateau value was observed. Immediately after room air was applied again, Tc started to return to baseline control or normoxic values.

Effect of 7-NI on Tc. When vehicle or 7-NI, at the dose of 25 mg/kg body wt, was injected intraperitoneally, no significant change in Tc was observed; however, when 30 mg/kg of 7-NI were injected by the same route, a significant reduction in Tc was observed. These data are plotted in Fig. 2.
Effect of 7-NI on hypoxia-induced anapyrexia. Figure 3 shows the effect of hypoxia on Tc of rats pretreated with vehicle or 7-NI at the dose that causes no change in Tc (25 mg/kg). Similar to hypoxia only, the Tc of control animals dropped quickly during the first 20 min and continued to drop more slowly until 100 min under hypoxic conditions. Immediately after room air was applied again, Tc started to return to baseline control values. Moreover, we observed that 25 mg/kg of 7-NI significantly attenuated hypoxia-induced anapyrexia compared with the group treated with the vehicle. It is important to point out that, after hypoxia exposure, Tc in the group pretreated with 7-NI dropped significantly 20 min after the drop seen in the group pretreated with the vehicle.

Effect of intraperitoneal injection of 7-NI on MAP and HR. Table 1 shows the effect of intraperitoneal injection of 7-NI (30 mg/kg) or its vehicle on MAP and HR. Treatments caused no change in MAP or HR.

**DISCUSSION**

The present study provides evidence that nNOS plays a role in hypoxia-induced anapyrexia, because injection of the nNOS inhibitor 7-NI attenuated the reduction in Tc caused by hypoxia. Recently, it has been shown that NOS inhibition with the nonspecific NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) abolishes hypoxia-induced anapyrexia (8), suggesting that NO plays a major role in hypoxia-induced anapyrexia. We now report that the NO arising from the nNOS isoform is necessary to produce hypoxia-induced anapyrexia.

Previous studies have demonstrated that hypoxia per se causes anapyrexia (8, 16, 18, 38, 20, 40, 41), a response that was confirmed to occur under the experimental conditions used in the present study (Fig. 1). Additionally, a recent study (41) has shown that the Tc of rats falls to 30–32°C with 7% inspired O2 if they are kept at 17°C. However, the mechanisms responsible for hypoxia-induced anapyrexia have only recently been suggested. Some lines of evidence indicate arginine vasopressin (AVP) as one putative mediator (40), but a recent study showed that the blockade of AVP receptors, peripherally as well as centrally, does not alter

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<th>Table 1. Effect of 7-NI on mean arterial pressure and heart rate</th>
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Values are means ± SD, n = 4 rats. Control values were obtained before any treatment. Treatments caused no change in mean arterial pressure (MAP) or heart rate (HR). Experiments were performed at 25.4 ± 0.7°C. 7-NI, 7-nitroindazole.
the magnitude of hypoxia-induced anapyrexia (38). Besides AVP, many other mediators, such as lactate, adenosine, and histamine (for review, see Ref. 40), have also been suggested, but none of the possible candidates can trigger a full blown hypothermic response. A route mediating the reduction in $T_c$ may be the impairment of central oxidative phosphorylation, because intracerebroventricular injection of inhibitors of oxidative phosphorylation, such as azide or cyanide, reduces the preferred body temperature of toads (9). Moreover, exclusion of glucose from central sites plays a major role in hypoglycemia-induced hypothermia (7, 11). Taken together, these data indicate that the role of the central nervous system in body temperature control is subject to numerous modifiers, including NO. Although recent studies have indicated that central NO may be a common mediator for hypothermic stimuli, such as hypoxia (8), hypercapnia (5), systemic vasopressin (37), and 2-deoxy-D-glucose (11), the NO synthase isoform involved remains unclear.

The importance of NO can be demonstrated by inhibition of the effects of NO, using L-arginine analogs such as L-NAME (27). More recently, from the group of the imidazoles and indazoles, 7-NI has been used as a relatively selective inhibitor of nNOS (26, 28, 29). In the present study, we used 7-NI to verify the participation of nNOS in hypoxia-induced anapyrexia in rats.

Because an unspecificity of 7-NI would lead us to a misinterpretation of our results, a few technical aspects of these experiments deserve comment. It has been reported that the effects of 7-NI (30 mg/kg) on nNOS are maximal within 30 min and that NO synthesis is 60% inhibited over 3 h and returns to baseline between 4 and 24 h (26). Several studies have investigated the specificity of 7-NI in inhibiting the nNOS subtype and most evidence indicates that 7-NI inhibits nNOS without affecting eNOS or iNOS (26, 28, 29). Accordingly, in the present study, no change in MAP was observed (Table 1) after 7-NI injection at the higher dose (30 mg/kg). Because the increase in MAP after the administration of NOS inhibitors has been attributed to a reduction in the activity of eNOS (27), our results showing that 7-NI does not alter MAP represent evidence that 7-NI did not block eNOS under the same experimental conditions used to determine the thermoregulatory effects of 7-NI.

Figure 2 shows that 7-NI at 30 mg/kg caused a marked decrease in $T_c$, whereas, at 25 mg/kg, no significant change in $T_c$ was observed. Because we observed a gradually increasing hypothermia as we used doses between 25 and 30 mg/kg in pilot studies, we believe that the hypothermic effect of 7-NI actually occurs within a narrow dose range and is not an all-or-none effect. The mechanism by which 7-NI at 30 mg/kg evokes a drop in $T_c$ is unknown, but some speculations can be proposed. nNOS is encountered in spinal cord, brain, sympathetic ganglia (36), and skeletal muscle (24, 25). Therefore, NOS inhibition in the sympathetic ganglia that innervate brown adipose tissue and in skeletal muscle could impair nonshivering and shivering thermogenesis, respectively, leading to a drop in $T_c$. Actually, NO has been shown to play an important role in nonshivering thermogenesis by acting on brown adipose tissue (32) and may be important for shivering thermogenesis as it may increase contractile function (24, 30, 31). On the other hand, NOS inhibition in the central nervous system could also alter $T_c$, but this is unlikely to contribute to the hypothermic effect of 7-NI at 30 mg/kg because NO in the central nervous system seems to be an antipyretic molecule (1, 19) and seems to mediate anapyrexia (5, 8, 11, 37). However, it is important to point out that NO may also be pyrexic in some brain regions (21). Clearly, further investigation is needed to determine the mechanism by which 7-NI evokes a drop in $T_c$.

Because 7-NI at 30 mg/kg per se reduces $T_c$, this effect would confound the interpretation of the results obtained with 7-NI and hypoxia combined. Thus we chose to study the effect of 7-NI on hypoxia-induced anapyrexia at the dose of 25 mg/kg because, according to pilot experiments, this is the highest dose that does not affect $T_c$.

Our observation that 7-NI attenuates hypoxia-induced anapyrexia (Fig. 3) indicates that the nNOS isoform plays an important role mediating hypoxia-induced anapyrexia. A previous study from our laboratory has already provided evidence that NO plays an important role in the reduction of $T_c$ after hypoxia exposure (8), but now we add that NO arising from nNOS contributes to the generation of anapyrexia. In our laboratory’s first study, it was observed that systemic injection of the nonselective NOS inhibitor L-NAME at 30 mg/kg prevented hypoxia-induced anapyrexia. Moreover, intracerebroventricular L-NAME at a smaller dose had a similar effect, suggesting that NO, probably generated in the central nervous system, plays an important role in hypoxia-induced anapyrexia. In contrast to this notion, a study published while this paper was in preparation observed that 7-NI does not affect hypoxia-induced anapyrexia (17). However, in that study, a considerably less severe hypoxia was applied (i.e., 11% inspired $O_2$). The occurrence of different mechanisms that depend on the degree of hypoxia could explain these differences.

It is also interesting to note that there was a 20-min delay in the drop of $T_c$ after hypoxia exposure in 7-NI-treated rats (Fig. 3), whereas the overall shape of the curve was not changed at all. Therefore, it is tempting to speculate that NO arising from nNOS exerts its thermoregulatory effect on the initial (acute) response to hypoxia rather than on the latter phase of the response.

It is important to point out that the participation of nNOS in the thermoregulatory response to hypoxia could be more pronounced than it looks from our results because of the incomplete inhibition of the enzyme by the dose of 7-NI used (26, 28, 29). Accordingly, central injection of L-NAME at the dose of 250 $\mu$g, which is known to inhibit 100% of the NOS activity in the central nervous system (2), completely abolishes hypoxia-induced anapyrexia, whereas 7-NI (25 mg/kg), which inhibits ~55% of nNOS activity (28, 29), only attenuates the response.
In the present study, we were unable to centrally administer 7-NI because it requires an oil vehicle and therefore it was impossible to identify the site of nNOS action. However, because the nNOS isoform has been found in the periphery at the thermogenic tissues (sympathetic ganglia of brown adipose tissue and skeletal muscle; 24, 25, 36), whereas NO in the central nervous system has been shown to mediate hypothemic stimuli such as hypoxia (8), systemic AVP (37), and 2-deoxy-D-glucose (11), it is likely that 7-NI attenuates hypoxia-induced anapryrexia by inhibiting NO synthesis by nNOS in the central nervous system. Yet, supporting the effect of NO reducing Te by acting on the central nervous system, experiments performed on febrile animals also demonstrated that intracerebroventricular administration of NO donors elicits antipyresis, whereas intracerebroventricular L-NAM Enhances fever (1, 19). Although more research is necessary to identify the site of nNOS action to produce anapryrexia, some speculation is natural.

Taken as a whole, our data indicate that the nNOS isoform contributes to the production of hypoxia-induced anapryrexia.

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