Antipyretic role of the NO-cGMP pathway in the anteroventral preoptic region of the rat brain

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Steiner, Alexandre A., Jose Antunes-Rodrigues, Samuel M. McCann, and Luiz G. S. Branco. Antipyretic role of the NO-cGMP pathway in the anteroventral preoptic region of the rat brain. Am J Physiol Regulatory Integrative Comp Physiol 282: R584–R593, 2002; 10.1152/ajpregu.00391.2001.—We tested the hypothesis that nitric oxide (NO) acts in the anteroventral preoptic region (AVPO) modulating fever. To this end, body core temperature (Tc) of rats was monitored by biotelemetry before and after pharmacological modulation of the NO pathway. Nitrite/nitrate and cGMP in the anteroventral third ventricular region (AV3V), where the AVPO is located, were also determined. Intra-AVPO microinjection of the NO synthase (NOS) inhibitor Nω-monomethyl-L-arginine (L-NMMA, 12.5 μg) did not affect basal Tc, but it enhanced the early stage of lipopolysaccharide (LPS) fever, indicating that NO plays an antipyretic role in the AVPO. In agreement, intra-AVPO microinjection of the NO donor sodium nitroprusside (5 μg) reduced Tc. The antipyretic effect of NO seems to be mediated by cGMP because 1) NO has been shown to activate soluble guanylate cyclase, 2) intra-AVPO microinjection of 8-bromo-cGMP (8-BrcGMP) reduced Tc, and 3) the changes in AV3V levels of nitrite/nitrate and cGMP were similar in the course of fever. Additionally, we observed that nitrite/nitrate and cGMP levels decreased in the AV3V after, but not before, the onset of LPS fever, showing that the activity of the NO-cGMP pathway is reduced in the AV3V after intraperitoneal LPS, a mechanism that could contribute to the genesis and maintenance of fever. It was also observed that the efficacy of 8-BrcGMP in reducing Tc in the AVPO is increased after LPS, emphasizing that the NO-cGMP pathway is antipyretic. This response could explain why intra-AVPO L-NMMA enhanced the early stage of LPS fever, even though the activity of the NO pathway before the onset of fever was unchanged. In summary, these data support an antipyretic role of the NO-cGMP pathway in the AVPO.

nitrergic oxide synthase; soluble guanylate cyclase; fever; lipopolysaccharide; hypothalamus; cryogen

IT IS NOW TWO DECADES since the first papers were published suggesting that the labile diffusible free radical gas nitric oxide (NO) could be a signaling molecule in the biological systems (31). Endogenously formed NO arises from the conversion of L-arginine to L-citrulline and NO, a reaction catalyzed by the enzyme NO synthase (NOS) (for a review, see Refs. 15 and 31). It is currently accepted that NO exerts many of its biological effects by interacting with second messenger systems, more specifically by activating the enzyme soluble guanylate cyclase (sGC) and consequently increasing the intracellular levels of cGMP (15, 31, 38, 48). Over the years, NO has been demonstrated to be an important modulator not only of the cardiovascular system but also of other physiological and pathophysiological processes, including thermoregulation and fever (19, 46).

Fever is a multimediated process that is part of the acute-phase reaction to infection, being characterized by a raised thermoregulatory set point, which leads to an elevation in body core temperature (Tc) (26). It is currently accepted that fever results from de novo synthesis of protein mediators, especially the cytokines interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α), and of mediators derived from lipid metabolism, which is the case for prostaglandins (PGs; Refs. 8, 26). Recently, NO has emerged as an important modulator of the febrile response. In this context, and others have provided evidence that NO plays differential thermoregulatory effects by acting in the periphery and in the central nervous system (CNS). This notion is based on the opposite results obtained by injecting pharmacological modifiers of the NO pathway systemically or intracerebroventricularly (for a review, see Ref. 46). Accordingly, it has been reported that systemic administration of nonselective NOS inhibitors impairs fevers evoked by systemic injection of lipopolysaccharide (LPS) and IL-1 in rats (35, 39, 42, 44) and LPS in guinea pigs (41). Differently from what is observed by administering NOS inhibitors systemically, intracerebroventricular injection of the nonselective NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) enhances fever in pyrogen-tolerant rabbits, whereas intracerebroventricular treatment with NO...
donors has been shown to elicit antipyresis (20). Accordingly, intracerebroventricular L-NNAME also enhances fever in response to systemic LPS (2) and psychological stress (16) in rats. Collectively, these results imply that peripherally generated NO contributes to the genesis of fever, whereas brain NO seems to act as an endogenous antipyretic factor, at least in response to systemic inflammation. In the present study, we will focus on the central antipyretic effects of NO.

Efforts have been made to determine the theroregulatory role of NO in specific nuclei of the CNS. At least in rabbits, NO has been inferred to play a role in fever generation by acting on the organum vasculosum laminae terminalis (OVLT; Ref. 28), which is thought to be an important site through which circulating pyrogens signal the brain (9). However, in those experiments fever was induced by direct injection of LPS into the OVLT, a response that may have mechanisms different from those reported for systemic inflammation. Actually, a recent study has reported that fevers induced by intracerebroventricular LPS are impaired by intracerebroventricular treatment with an NOS inhibitor (49), whereas intracerebroventricular NOS inhibitors are known to augment fever when the pyrogens are administered peripherally (2, 20). Whether NO is a pyretic or an antipyretic molecule in the OVLT when LPS is administered peripherally remains to be determined.

Besides the OVLT, a few studies have investigated the theroregulatory effect of NO in the preoptic region of the anterior hypothalamus (POA), located in the anteroventral third ventricular (AV3V) region, which is considered to be the thermosensitive and thermointegrative site of the CNS (10, 12). In 1984, Dascombe (13) reported that intra-POA administration of a cGMP analog reduces the Tc of rabbits. Considering that NO produces many of its effects through a cGMP-dependent pathway (38, 48), one would expect NO to be an antipyretic molecule in the POA. However, the results obtained to date are controversial. Amir et al. (3) demonstrated that intra-POA administration of the nonselective NOS inhibitor Nω-monomethyl-L-arginine (L-NMMA) attenuates intra-POA PGE2-induced fever in urethane-anesthetized rats, suggesting a pyretic action of a local NO pathway in the POA. On the other hand, Gourine et al. (21) reported that intracerebroventricular administration of NO donors opposes the effects of IL-1, an established fever mediator, on the thermosensitive neurons of the POA in anesthetized rats, a fact consistent with an antipyretic role of NO. Nevertheless, because anesthesia impairs thermoregulation (12), the interpretation of these results is difficult. Clearly, there is a need to resolve this issue.

Therefore, the present study aimed to determine the role of the NO-cGMP pathway in the POA in the febrile response to systemic inflammation using unanesthetized rats. We then hypothesized that the POA is a site where NO exerts its antipyretic effect in the CNS. To this end, we evaluated the effects of microinjections of pharmacological modifiers of the NO-cGMP pathway into the POA on LPS-induced fever and also measured the levels of nitrite/nitrate and cGMP in the AV3V region of rats. Based on the fact that the anteroventral preoptic region (AVPO) appears to be the site where PGE2 acts as the proximal mediator of fever and, therefore, the presumed ultimate pathway for the increase in the thermoregulatory set point in the POA (43), we have chosen to perform microinjections into the AVPO. This approach also permitted us to determine that, similarly to PGE2, the AVPO is a site where NO exerts its thermoregulatory effects.

MATERIALS AND METHODS

Animals

Experiments were performed on adult male Wistar rats weighing 230–260 g housed at controlled temperature (26.0 ± 1.0°C) and exposed to a daily 12:12-h light-dark cycle with lights on at 6:00 AM. The animals were allowed free access to water and food. To obviate possible effects of circadian variations, experiments were started between 8:00 and 9:00 AM.

Drugs

All drugs were purchased from Sigma (St. Louis, MO). The nonselective NOS inhibitor L-NMMA and LPS (from Escherichia coli, serotype 0111:B4) were dissolved in pyrogen-free sterile saline on the day preceding the experiment and stored at −20°C. The NO donor sodium nitroprusside (SNP) and the cGMP analog 8-bromo-cGMP (8-BrcGMP) were dissolved in the dark in pyrogen-free sterile saline just before injection. The concentrations of SNP and 8-BrcGMP solutions were 10–50 and 100–400 mg/ml, respectively, and these solutions had a completely clear aspect.

Surgery

Animals were anesthetized with 2,2,2-tribromoethanol (Aldrich) at the dose of 250 mg/kg ip and fixed in a stereotaxic frame. A stainless steel guide cannula (17-mm long, 22 gauge, thin wall; Small Parts) was introduced 2 mm above the skull cavity. The wound was then closed with skin sutures, and a paramedian laparotomy was performed inside the guide cannula to prevent occlusion and infection. The cannula was attached to the bone with stainless steel screws and acrylic cement. A tight-fitting stylet was kept inside the guide cannula to prevent occlusion and infection. Immediately after, each animal was removed from the stereotaxic frame, and a paramedian laparotomy was performed to insert a biotelemetry probe capsule (model ER-4000; temperature/activity; Mini-Mitter, Sunriver, OR) into the peritoneal cavity. The wound was then closed with skin sutures, and the implanted capsule was used to measure Tc. The surgical procedures were performed over a period of 30–40 min. After surgery, animals were treated with 100,000 U benzylpenicillin im and allowed to recover for 1 wk.

Microinjection Procedure and Histology

A 5-μl Hamilton syringe and a dental injection needle (Mizzy, 200-μm outer diameter) connected to a PE-10 tube were used to perform the microinjections into the AVPO of conscious rats. The injection needle was 2 mm longer than the guide cannula so that the AVPO was reached by the needle only at the time of injection. Injection was performed in a volume of 100 nl over a period of 1 min, and 1 more min
was allowed to elapse before the injection needle was removed from the guide cannula to avoid reflux. All injections were performed using a microinjector machine (model 310, Stoelting). At the end of each experiment, 100 nl of 2% Evans’ blue solution was microinjected to mark the injected sites for later histological analysis. Animals were then decapitated, and their brains were removed and stored in 10% buffered formalin solution for at least 2 days. Next, the brains were histologically processed, and serial coronal sections (13 μm) were cut and stained by the Nissl method. The animals in which the site of microinjection was located in the AVPO were considered to have been injected intra-AVPO, whereas microinjections located in nuclei other than the AVPO were considered to be peri-AVPO.

\[ T_c \] Measurements

\[ T_c \] was measured continuously by biotelemetry. Fully conscious rats previously implanted with the biotelemetry probes were housed in individual cages for at least 24 h before the experiment. The \[ T_c \] of the animals was recorded at 5-min intervals for the duration of the experiments. Cages were located on a Mini-Mitter receiver (model ER-4000) connected to a personal computer. Using VitalView software (Mini-Mitter), the data were acquired, displayed graphically on a monitor, printed digitally, and stored on the hard and a (Mini-Mitter), the data were acquired, displayed graphically on a monitor, printed digitally, and stored on the hard and a

\[ \text{Determination of Nitrite/Nitrate Levels in the AV3V Region} \]

The animals were decapitated, and their brains were excised. Immediately afterward, the AV3V region was dissected under a stereomicroscope on the basis of the anatomic landmarks previously described (23, 24), frozen under liquid nitrogen, and stored at -70°C until assay. This dissection procedure has already been used in previous studies (5, 7). To determine nitrite/nitrate levels, the samples were homogenized on ice in 100 μl of a 0.1 N acetic acid solution using a digital 600-W microprocessor cell disruptor (The Vir Tris). The resulting homogenates were then centrifuged at 10,000 g for 10 min at 2°C, and the pellets were processed for protein measurement. The pellet was chosen for protein determination on the basis of a previous study (34). Sixty microliters of absolute ethanol was then added to 30 μl of the supernatant, and after 30 min at 0°C, the samples were centrifuged at 10,000 g for 5 min. Five microliters of the supernatant obtained was injected into an NO analyzer (model 280, Sievers Instruments) for the determination of nitrite/nitrate.

For protein determination, the pellets were diluted in 4 ml of sodium hydroxide (0.1 N) using a digital 600-watt microprocessor cell disruptor (The Vir Tris). The solution obtained was assayed for protein determination using the Bio-Rad protein assay (Bio-Rad Laboratories; code number 500–0006).

\[ \text{Determination of cGMP Levels in the AV3V Region} \]

AV3V samples were obtained as described in the previous item and homogenized on ice in 150 μl of a 6% (wt/vol) trichloroacetic acid (TCA) solution using a digital 600-W microprocessor cell disruptor (The Vir Tris). The resulting homogenates were then centrifuged at 10,000 g for 10 min at 2°C, and the pellets were processed for protein determination as described. TCA was extracted with water-saturated diethyl ether, and the samples were lyophilized. The samples were then reconstituted in 2 ml of the assay buffer provided in the kit (Amersham Pharmacia Biotech, code number RPN226), and cGMP was determined by enzyme immunoassay according to manufacturer’s instructions.

\[ \text{Experimental Protocols} \]

\[ \text{Experiment 1: effect of intra-AVPO microinjection of L-NMMA on } T_c \text{ and LPS-induced fever} \]

Animals prepared as described above were injected intra-AVPO with L-NMMA (12.5 μg in 100 nl) or pyrogen-free sterile saline (vehicle, 100 nl), and \[ T_c \] was recorded throughout the experiment.

Another group of animals was treated in the same way with intra-AVPO L-NMMA or saline, but this time the rats were injected intraperitoneal with LPS (100 μg/kg) or saline 30 min after the intra-AVPO injections. The volume of the intraperitoneal injection was 0.25 ml. The dose of L-NMMA was used because, in pilot experiments, this dose produced the most consistent and repeatable responses and because its peri-AVPO administration produced no effect. The dose of LPS was chosen on the basis of previous studies from our laboratory (47).

\[ \text{Experiment 2: effect of intra-AVPO microinjection of SNP on } T_c \]

Rats were treated with an intra-AVPO microinjection of saline or SNP at the doses of 1 or 5 μg, and \[ T_c \] was monitored throughout the experiment.

\[ \text{Experiment 3: effect of intra-AVPO microinjection of 8-Br-cGMP on } T_c \]

Intra-AVPO microinjections of 8-Br-cGMP (10 or 40 μg in 100 nl) or saline (vehicle, 100 nl) were performed, and \[ T_c \] was monitored throughout the experiment.

\[ \text{Experiment 4: effect of intra-AVPO microinjection of the cGMP analog 8-Br-cGMP on LPS-induced fever} \]

LPS was injected intraperitoneal at the dose of 100 μg/kg, and 2.5 h later intra-AVPO microinjections of 8-Br-cGMP (10 μg in 100 nl) or saline were performed. \[ T_c \] was monitored throughout the experiment.

\[ \text{Experiment 5: effect of intraperitoneal injection of LPS on the levels of nitrite/nitrate and cGMP in the AV3V region} \]

Animals housed in individual cages for at least 24 h were treated intraperitoneal with LPS (100 μg/kg) or saline (vehicle) in a final volume of 0.25 ml. The animals were then decapitated 1 or 4 h later, and the brains were processed as described for the determination of nitrite/nitrate and cGMP levels.

\[ \text{Statistical Analyses} \]

The results are reported as means ± SE. The values of \[ T_c \] (°C) are the changes from the basal values (\( T_{ci} \); the \( T_c \) at 5-min intervals averaged over the last 30 min of the preceding 1-h stabilization period) plotted at 5-min or 10-min intervals. The nitrite/nitrate and cGMP data are expressed as femtomoles per microgram protein of AV3V. Thermal indexes (TI; °C•h) were calculated as the area under the \( T_c \) curves (excluding the stabilization period). Changes in \( T_c \) were evaluated by ANOVA for repeated measurements. The differences between means were assessed by the Tukey-Kramer multiple comparisons test. Ordinary ANOVA followed by the Tukey-Kramer multiple comparisons test was used to assess differences between cGMP and nitrite/nitrate levels and TI. Values of \( P < 0.05 \) were considered to be statistically significant.
RESULTS

No difference in Tc values or control (intraperitoneal saline) levels of AV3V nitrite/nitrate or cGMP was observed among experimental protocols.

Intra-AVPO Microinjection

Figure 1 shows a typical site of microinjection into the AVPO. Figure 1A depicts a photomicrograph adapted from Paxinos and Watson (33) corresponding to the region where the AVPO is located, having as landmarks the anterior commissure, the third ventricle, and the optic chiasm. Figure 1B shows a representative photomicrograph of a typical site of microinjection into the AVPO obtained in the present study, showing, besides the landmarks of Fig. 1A, the guide cannula trajectory. Beyond the guide cannula trajectory, only the injection needle invaded the preoptic tissue. The injection site is indicated by the black arrow and is evident as a cluster of dye staining.

Experiment 1: Effect of Intra-AVPO Microinjection of L-NMMA on Tc and LPS-Induced Fever

As shown in Fig. 2A, microinjection of the nonselective NOS inhibitor L-NMMA into the AVPO (intra-AVPO) produced no change in Tc compared with the groups that received intra-AVPO saline or peri-AVPO L-NMMA. TI data confirm this observation (Fig. 2C). Treatment of the animals with intra-AVPO saline did not affect the intraperitoneal LPS-induced fever (TI = 6.5 ± 1.0 °C·h), which started ~1.5 h after LPS administration and reached a plateau at ~1.5°C above Tci, a response similar to that observed in previous studies from our laboratory without performing microinjections into the AVPO (47). Similarly, peri-AVPO injections of L-NMMA did not affect LPS-evoked fever (TI = 6.7 ± 1.0 °C·h) compared with the group treated with saline. On the other hand, intra-AVPO treatment with L-NMMA significantly enhanced LPS fever (TI = 11.3 ± 1.3 °C·h). More specifically, L-NMMA reduced the onset time for the rise in Tc, which was already evident 30 min after LPS administration, but had little effect on the magnitude of the fever during its plateau phase. In other words, intra-AVPO treatment with L-NMMA initiated more rapidly the febrile increase in Tc in response to intraperitoneal
LPS. These data are depicted in Fig. 2B. In Fig. 2C, TI summarizes these results.

Experiment 2: Effect of Intra-AVPO Microinjection of SNP on $T_c$

Microinjection of SNP into the AVPO at the dose of 1 $\mu$g caused no change in $T_c$ compared with the animals that received saline (data not shown). However, intra-AVPO microinjection of SNP at 5 $\mu$g produced a significant decrease in $T_c$, which started ~40 min after the injection, peaked at 1 h at $-1^\circ C$ below $T_{ci}$, and returned toward baseline. The overall response lasted 2.5 h. The response observed after peri-AVPO administration of the same dose of SNP did not differ from that obtained with intra-AVPO microinjection of saline. These results are illustrated in Fig. 2.

Experiment 3: Effect of Intra-AVPO Microinjection of the cGMP Analog 8-BrcGMP on $T_c$

Figure 4 shows that intra-AVPO microinjection of 8-BrcGMP at the dose of 40 $\mu$g significantly decreased the $T_c$ of rats compared with the group that received intra-AVPO saline. The latency for the onset of this response was ~20 min. Neither the same dose of 8-BrcGMP microinjected peri-AVPO nor the lower dose (10 $\mu$g) administered intra-AVPO affected $T_c$.

Experiment 4: Effect of Intra-AVPO Microinjection of the cGMP Analog 8-BrcGMP on LPS-Induced Fever

Animals pretreated with LPS (100 $\mu$g/kg ip) developed a fever of ~1.5°C, in agreement with the present results (Fig. 2B) and with previous studies (47). Two and one-half hours after LPS administration, 8-BrcGMP was microinjected into the AVPO. This period of time (2.5 h after LPS) was chosen because it was the time when the plateau phase of fever was reached in our experimental model. Intra-AVPO microinjection of 8-BrcGMP at a dose that caused no change in the $T_c$ of eutheranic animals (10 $\mu$g; Fig. 4) caused a significant decrease in the $T_c$ of febrile rats, a response that was already evident 10 min after microinjection and lasted 30 min (Fig. 5). Intra-AVPO administration of saline or peri-AVPO injection of 8-BrcGMP (10 $\mu$g) did not affect the course of the febrile response (Fig. 5).

Experiment 5: Effect of Intraperitoneal Injection of LPS on the Levels of Nitrite/Nitrate and cGMP in the AV3V Region

The levels of nitrite/nitrate in the AV3V region of rats injected intraperitoneally with pyrogen-free sterile saline were ~6 fmol/µg protein. Compared with the group treated with saline, the levels of nitrite/nitrate in the AV3V region were significantly reduced at 4 h, but not at 1 h, after intraperitoneal injection of LPS (100 $\mu$g/kg). These data are plotted in Fig. 6A. Similarly, intraperitoneal administration of LPS also decreased the levels of cGMP in the AV3V region, a response that was evident and significant at 4 h, but not at 1 h, after LPS injection. AV3V basal levels of cGMP in the saline-treated group were ~1.6 fmol/µg protein. Figure 6B illustrates these results.

DISCUSSION

The present study provides evidence that NO plays an antipyretic role in the AVPO region of the rat brain during LPS-induced fever because intra-AVPO treatment with the nonselective NOS inhibitor L-NMMA enhanced LPS fever (Fig. 2). Moreover, intra-AVPO administration of the NO donor SNP resulted in a drop in $T_c$ (Fig. 3). This thermoregulatory effect of NO seems to be mediated by activation of sGC and a consequent rise in cGMP levels, a suggestion supported by the following evidence: 1) NO has been reported to activate sGC in several tissues (15, 31, 38, 48); 2) intra-AVPO
microinjection of the cGMP analog 8-BrcGMP produced a decrease in Tc, an effect similar to the effect of SNP, except that it started 20 min earlier (Fig. 4); and 3) the changes in AV3V levels of nitrite/nitrate and cGMP were similar in the course of LPS fever (Fig. 6).

**Methodological Considerations**

Microinjection techniques have been extensively used to assess the actions of several substances in specific nuclei of the CNS. In the present study, we performed microinjections of pharmacological modulators of the NO-cGMP pathway into a specific region of the POA, the AVPO (Fig. 1), to determine the role of the NO-cGMP pathway into the AVPO in the febrile response to LPS. The POA is a region that has attracted much attention over the years in the study of the mechanisms of fever because it is considered to be the thermointegrative and thermosensitive site of the CNS, containing warm- and cold-sensitive neurons, a balance of which determines the thermoregulatory set point (10, 12). Several substances have been shown to act in the POA to increase Tc, among which PGE2 has received special attention, being considered the proximal mediator of fever (8). More recently, Scammell et al. (43) have shown that the AVPO region is the “pyrogenic zone” for the thermoregulatory effects of PGE2 into the POA and is the site of the POA that is activated by administration of intravenous LPS (18) and intra-POA PGE2 (43). Because the AVPO seems to be the site of the POA involved in fever, we decided to investigate the thermoregulatory role of NO in the AVPO.

We then considered microinjections located into the AVPO as intra-AVPO, and microinjections located in nuclei other than the AVPO as peri-AVPO. It is known that once a drug is administered into a nucleus, it diffuses proportionally to its dose and the volume injected. Accordingly, theoretical calculations by Nicholson (32) demonstrate that, based on a 10-nl injection into the brain tissue, the concentration of the injectate 300 μm from the injection site never exceeds 20% of the initial dose. Additionally, Lipski et al. (29) estimated that in a 30-nl microinjection, the drug diffused ~325 μm, whereas Mitra et al. (30) reported that microinjections performed in a volume of 100 nl could spread as far as 1 mm. On the basis of these notions, in our experiments we used threshold doses, i.e., doses that elicited a thermoregulatory action when microinjected.
intra-AVPO, but not when administered peri-AVPO (Figs. 2–5). This approach permitted us to consider peri-AVPO injections as a control for the intra-AVPO injections. In other words, when the drug was injected outside the AVPO (peri-AVPO), the amount of drug that reached the AVPO was not high enough to produce a thermoregulatory response. For example, in pilot experiments, we injected L-NMMA into the AVPO at 25 μg, but at this dose peri-AVPO microinjections had the same thermoregulatory actions as did intra-AVPO. For that reason, in the present study we administered L-NMMA into the AVPO at the threshold dose of 12.5 μg, which is 20-fold lower than that used in previous experiments to verify the thermoregulatory effects of intracerebroventricularly administered NO inhibitors in rats (2, 16).

In agreement with previous studies, we observed an increase in Tc of ~0.5°C after insertion of the injection needle into the preoptic tissue (data not shown) or microinjection of saline alone into the AVPO (Fig. 2A), but the magnitude of this response was small compared with previous studies that injected higher volumes (36, 51). In fact, local injury due to microinjection procedures into the POA has been shown to increase Tc in rats (1, 14), cats (17), guinea pigs (40), and rabbits (13), a response that has been attributed to the production of inflammatory mediators by damaged tissue and, thus, has been shown to be attenuated by treatment with paracetamol (13) and indomethacin (40). Although in the present experiments a slight increase in Tc was observed after all microinjections, it should be emphasized that this effect was minimized by injecting a small volume (100 nl) over a relatively long period of time (1 min).

Fever After Inhibition of the NO Pathway in the AVPO

As shown in Fig. 2, intra-AVPO administration of the nonselective NOS inhibitor l-NMMA (31) at the dose of 12.5 μg did not affect basal Tc, but enhanced LPS-induced fever. These results imply that NO plays no tonic role in Tc control but has an antipyretic action during LPS fever by acting in the AVPO. It should be emphasized that intra-AVPO injection of l-NMMA actually initiated more rapidly the febrile increase in Tc than did intracerebroventricular administration of NO donors (2, 20). However, we believe the POA is not the only brain site involved because intracerebroventricular injection of NOS inhibitors, differently from intra-AVPO microinjections, not only initiated fever more rapidly, but also augmented the peak Tc reached during fever (2, 20). In this context, another region that might be potentially involved in the antipyretic effect of NO in the brain is the paraventricular nucleus (PVN) of the hypothalamus because Yang and Krukoff (54) have reported that NO-producing neurons in the PVN are activated after intravenously injected LPS and that intracerebroventricular injection of NO donors inhibits the activation of the PVN in response to systemic LPS. Considering that the PVN plays a major role in the genesis of fever (43) by increasing sympathetic activity, inhibition of PVN neurons by NO is likely to result in antipyresis.

Thermoregulatory Effects of SNP and 8-BrcGMP in the AVPO

In agreement with the current hypothesis that NO reduces Tc by acting in the AVPO, we also observed that intra-AVPO injection of the NO donor SNP at the dose of 5 μg evoked a marked drop in the Tc of rats (Fig. 3). Consistent with this finding, intracerebroventricular injection of NO donors has been reported to impair fever in rabbits (20), an observation that, according to our results, has the AVPO as at least one putative site of action. A growing number of studies have supported the idea that NO acts as a neurotransmitter and/or neuromodulator in the brain (15). Actually, intracerebroventricular administration of NO donors has been shown to oppose the effects of IL-1, an established fever mediator, on the thermosensitive neurons of the POA in anesthetized rats (21), a fact consistent with the antipyretic role of NO.
NO has been shown to activate sGC by binding to the iron in the heme group of this enzyme, a fact that promotes an increase in intracellular cGMP levels (15, 31, 38, 48). Therefore, we hypothesized that the thermoregulatory effect of NO in the AVPO is mediated by cGMP. To test this hypothesis, we first microinjected the inhibitors of sGC, 1H-(1,2,4)oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) and methylene blue, or their vehicles (1% DMSO in saline or saline alone, respectively) into the AVPO followed by administration of SNP. However, prior microinjections of the vehicles only abolished the thermoregulatory effect of SNP into the AVPO, a fact that precluded any further experiment using two consecutive intra-AVPO microinjections (data not shown). We then assessed the effect of intra-AVPO microinjection of the cGMP analog 8-BrcGMP, which crosses the cell membrane to reach the intracellular targets of cGMP (see Refs. 13, 14), e.g., protein kinase G (4), on Tc. As a result, we observed that intra-AVPO 8-BrcGMP at 40 μg decreased the Tc of rats (Fig. 4) similarly to SNP (Fig. 3), with an important difference in the latency for the onset of the response. Accordingly, the drop in Tc after administration of SNP took 40 min to start, whereas Tc began to decrease already 20 min after 8-BrcGMP administration, indicating that cGMP is likely to be downstream from NO in the cascade of events that culminate in the reduction in Tc. This evidence, together with the facts that NO increases cGMP levels (15, 31, 38, 48) and that NO donor SNP and 8-BrcGMP produce hypothermia with similar curve shapes when microinjected into the AVPO, supports the idea that cGMP mediates the thermoregulatory effect of NO in the AVPO. In agreement with this finding, a previous report has shown that intra-POA administration of a cGMP analog at doses ranging from 100 to 200 μg reduces the Tc of rabbits (13). Similar doses of cyclic nucleotide analogs have been shown to produce thermoregulatory effects in rats (14).

Moreover, Fig. 5 shows that intra-AVPO administration of 8-BrcGMP even at a dose (10 μg) that does not affect Tc of euthemic animals did attenuate systemic LPS-induced fever. The latency for the decrease in Tc after intra-AVPO microinjection of 8-BrcGMP was also further reduced in febrile rats, i.e., ~10 min (Fig. 5). These results indicate that the thermoregulatory effect of the NO-cGMP pathway in the AVPO is antipyretic rather than hypothermic because an antipyretic agent is defined as a substance that can reduce Tc of febrile animals but has no effect on the Tc of euthemic animals, unless the dosage is excessive (6). In agreement with this notion, a previous study (20) has shown that intracerebroventricularly administered NO donors may attenuate fever in rabbits, even at doses that have no effect on Tc of euthemic animals.

Activity of the NO Pathway in the AV3V After Systemic LPS

To investigate whether the NO pathway in the POA is activated or inactivated during fever, we determined the levels of nitrite/nitrate, which are NO metabolites and have been extensively used to quantify NO production (15, 31), and of cGMP in the AV3V region, where the POA is located (23, 24). Interestingly, intraperitoneal injection of LPS at a dose that causes fever (Figs. 2 and 5) did not affect AV3V levels of nitrite/nitrate or cGMP 1 h after injection but significantly reduced the levels of these substances 4 h after LPS administration (Fig. 6). Taken together, these data show that the activity of the NO-cGMP pathway in the AV3V region is not affected before the onset of fever, i.e., 1 h after LPS, but is reduced during the course of fever, i.e., 4 h after LPS. The similarity in the time course of changes in nitrite/nitrate and cGMP reinforces the notion that NO exerts its antipyretic effects in the AVPO through a cGMP-dependent pathway. Immunolocalization studies have demonstrated that the constitutive isoforms of NOS, especially neuronal NOS, are expressed in the POA of rats (11, 37, 53) and guinea pigs (50). Regarding the inducible isoform of NOS (iNOS), a recent study has shown that iNOS is not overexpressed in the POA after peripheral administration of LPS (25), a fact that is consistent with our results because it supports the possibility of a reduction in NO production by inhibition of the activity of a constitutive NOS isoform. It should be pointed out that the AV3V region also includes another region of relevance for fever, i.e., the OVLT, a site where iNOS has been shown to be overexpressed ~3 h after intraperitoneal LPS administration (25). However, because in that study a higher dose of LPS (250 μg/kg) was used, it is not certain if in the present experiments iNOS had been induced in the OVLT. Another aspect that deserves comment is that if iNOS was being overexpressed in the OVLT in our experimental model, it would counteract the observed decrease in nitrite/nitrate and cGMP levels in the AV3V, suggesting that the inactivation of the NO-cGMP pathway in the POA could be even more pronounced than it appears from our data. Taking into consideration that NO appears to be an antipyretic molecule in the AVPO, it is appropriate to speculate that the inactivation of the NO-cGMP pathway in this region after intraperitoneal LPS could contribute to the development of LPS fever. Moreover, the observed reduction in the activity of the NO-cGMP pathway 4 h after intraperitoneal LPS is consistent with the fact that intra-AVPO microinjection of L-NMMA did not affect the plateau phase of LPS fever. If so, one question arises: how may intra-AVPO administration of L-NMMA reduce the onset time of fever if the NO-cGMP pathway is not activated 1 h after LPS injection? A possible explanation for this question is that after neuroimmune signaling to the brain in animals treated with LPS, an increase in the efficacy of preoptic cGMP in reducing Tc may occur, which in turn would counteract the rise in Tc evoked by pyrogens, resulting in no change in Tc. When L-NMMA was administered intra-AVPO, NO and cGMP levels probably fell, and a reduction in the onset time of fever was observed. Corroborating this hypothesis, we observed that 8-BrcGMP at a dose (10 μg) that caused no change in the Tc of...
euthemic animals significantly reduced the $T_c$ of febrile animals (Fig. 5). This increased efficacy of cGMP to reduce $T_c$ in febrile rats is not surprising because, as discussed previously in this report, it would be in agreement with the antipyretic effect of the NO-cGMP pathway in the AVPO. Although it seems that the efficacy of the NO-cGMP pathway in reducing $T_c$ in the AVPO increases after an immune challenge, the mechanisms underlying this response still remain obscure and require future studies. However, a possible explanation may reside in the observation of Soff et al. (45). These authors reported that CAMP suppresses the activity of cGMP-dependent protein kinase (PKG). Because in a recent study (Steiner, Antunes-Rodrigues, and Branco, unpublished data), we have demonstrated that a reduction in preoptic levels of cAMP by the action of PGE$_2$ is associated with fever, it is possible that a reduction in cAMP in the AVPO leads to increased activity of PKG, resulting in an increased efficacy of cGMP to reduce $T_c$. More studies are needed to firmly establish this issue.

**Conclusion**

In summary, the present results indicate that the NO-cGMP pathway plays an antipyretic role in the AVPO region of the rat brain. Taken together, our results indicate that the actions of the NO-cGMP pathway in the AVPO in the course of fever, at least in our experimental model, occur as follows: 1) when LPS is administered intraperitoneally, an array of systemic reactions takes place and culminates in the transfer of the signals from peripherally generated pyrogens to the brain; 2) the efficacy of the NO-cGMP pathway in reducing $T_c$ in the AVPO is then increased and counteracts the effects of pyrogens, resulting in no change in $T_c$; and 3) later on, the activity of the NO-cGMP pathway in the AVPO is reduced, permitting pyrogens to act in the brain, especially in the AVPO, to evoke fever.

**Perspectives**

A growing body of evidence supports that NO plays a role in thermoregulation under euthemic conditions and is an important modulator of fever. In fact, NO seems to participate in several levels of integration in the control of $T_c$. In this context, we and others (46) have provided evidence that NO plays different thermoregulatory effects by acting in the periphery and in the CNS. The actions of NO are even expanded during fever because when a pyrogen enters into an organism, several pathways at which NO might be involved are activated. Evidence has accumulated that peripherally acting NO is a pyretic molecule. On the other hand, intracerebroventricular administration of NOS inhibitors enhances fever, suggesting that NO is an antipyretic molecule by acting in the CNS (46). The present study provides evidence that the POA is likely to be one of the sites where NO exerts its antipyretic action in the brain. Moreover, we believe that the integration of several approaches such as microinjection techniques, immunocytochemistry, microdialysis, and slice preparations, as well as the study of other brain regions involved in the regulation of $T_c$, will be necessary to firmly establish how NO acts in the brain modulating fever.

In the present study, we also reported that a reduction in cGMP levels in the POA could play an important role in the development and maintenance of fever. This finding may be of particular interest in understanding the cellular mechanisms responsible for the determination of the thermoregulatory set point, which is believed to be given as a balance between the activity and sensitivity of warm- and cold-sensitive neurons in the POA (10, 12). In fact, hypoxia, which is a stimulus that reduces the thermoregulatory set point (52), different from fever is accompanied by a rise in cGMP levels in the brain (22). However, even though progress has been made in the understanding of the role of NO in fever, it is important to keep in mind that it still remains little explored and represents a field that needs urgent research.

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