Inhibition of nitric oxide synthase in the paraventricular nucleus prevents the hyperthermia-induced reduction of mesenteric blood flow in rats

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Chen F, Wang Y, Cham JL, Badoer E. Inhibition of nitric oxide synthase in the paraventricular nucleus prevents the hyperthermia-induced reduction of mesenteric blood flow in rats. Am J Physiol Regul Integr Comp Physiol 299: R596–R602, 2010. First published May 26, 2010; doi:10.1152/ajpregu.00003.2010.—Increasing body core temperature reflexly decreases mesenteric blood flow (MBF), and the hypothalamic paraventricular nucleus (PVN) plays an essential role in this response. Nitric oxide (NO) is involved in temperature regulation and is concentrated within the PVN. The present study investigated whether NO in the PVN contributes to the cardiovascular responses elicited by hyperthermia. Anesthetized rats were microinjected bilaterally in the PVN (100 nl/side) with saline or N\(^\text{G}\)-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor (100 or 200 nmol/100 nl (n = 5/group)). Body core temperature was then elevated from 37°C to 39°C, and blood pressure (BP), heart rate (HR), MBF, and mesenteric vascular conductance (MVC) were monitored. In separate groups, L-NAME (200 nmol) (n = 5) or saline (n = 5) was microinjected in the PVN, but body core temperature was not elevated. In control rats, increasing body core temperature resulted in no marked change of BP but an increase in HR and significant decreases in MBF (15%) and MVC. Pretreatment with 100 nmol L-NAME did not affect the responses. In contrast, 200 nmol L-NAME prevented the normal reduction in MBF and MVC but did not significantly affect the BP and HR responses. In rats in which body core temperature was not increased, L-NAME reduced MBF by 19%. The present results suggest that endogenous NO in the PVN is important in mediating the reduction of MBF induced by hyperthermia. In the absence of hyperthermia, however, endogenous NO in the PVN may play a role in maintaining mesenteric vasodilation.

hypothalamic nucleus; mesenteric blood flow; increased body core temperature

THE HOMEOSTATIC REGULATION of body temperature, which is important for normal cell function, is achieved through behavioral and autonomic responses that balance the generation and dissipation of heat. In response to hyperthermia, for example, there are increases in splanchic and renal sympathetic nerve activity (18, 20), resulting in vasoconstriction of the respective vascular beds. In the skin vasculature, however, there is vasodilation, and, in the rat, tail vasodilation also occurs (16–17); the tail is a major thermoregulatory organ in the rat. These changes contribute to the redistribution of the blood flow during hyperthermia such that blood is redirected from the warm internal environment in visceral organs to the peripheral vasculature where heat can be dissipated.

The central nervous system contributes to the reflex changes involved in thermoregulation, and the hypothalamus, in particular, plays a key role in the cardiovascular changes that subserve the redistribution of blood flow. We have recently found that the hypothalamic paraventricular nucleus (PVN) is essential for the normal reflex reduction in renal blood flow and mesenteric blood flow (MBF) induced by hyperthermia (6, 8). The PVN is one of the few sympathetic premotor nuclei present in the brain, that is, it contains neurons that project directly to sympathetic preganglionic motor neurons in the intermediolateral (IML) cell column of the thoracolumbar spinal cord (7, 37). These connections underlie the influence of the PVN on sympathetic nerve activity that contributes to the blood flow responses (3).

The neurochemical mediators within the PVN that contribute to its critical influence on visceral vasculature are unknown. However, there is a dense concentration in the PVN of neurons containing nitric oxide synthase (NOS), the enzyme responsible for the production of nitric oxide. Current evidence suggests that nitric oxide in the central nervous system is important in the thermoregulatory pathways mediating heat dissipation (9–11, 33, 35); for example, intracerebroventricular administration of NOS inhibitors to block nitric oxide production has recently been found to elevate body core temperature in the rat (28) and augment the febrile response elicited by endotoxin (12). Nitric oxide can also augment the lipopolysaccharide-induced increase in body core temperature by acting within the anteroventral preoptic area (36).

Nitric oxide within the PVN can cause a pronounced alteration of sympathetic nerve activity (23, 30), and it is likely that the PVN neurons projecting to the IML of the spinal cord contribute to those responses. Indeed, we have found that hyperthermia induces marked activation of neurons in the PVN and that ~40% of the activated neurons were nitrergic (7), suggesting nitrergic neurons in the PVN were involved in the responses induced by elevations in body core temperature. Because the PVN is essential for the hyperthermia-induced decrease in MBF, the aim of the present study was to determine whether microinjection of L-NAME in the PVN, to inhibit nitric oxide production, altered the normal reflex reduction in MBF that occurs in response to an elevation in body core temperature.

MATERIALS AND METHODS

Animals and Housing

All experimental protocols used in this study were performed in accordance with the Prevention of Cruelty to Animals Act 1986 (Australia) and to the guidelines set out by the National Health and Medical Research Council of Australia (Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 2007). The protocols were approved by the RMIT University Animal Ethics Committee and conform to the Guiding Principles for Research Involving Animals and Human Beings (1). Male Sprague-Dawley rats (obtained from Monash University Animal Services, Victoria, Aus-
tralia) weighing 300–350 grams were housed in the Animal Facility (RMIT University, Victoria, Australia) with free access to rat chow and tap water at a room temperature of 22 ± 1°C with a 12:12-h light-dark regimen.

Surgical Preparations

All animals were anesthetized initially with Equithesin [pentobarbital sodium (0.5 g)-chloral hydrate (2.219 g)/100 ml mixture] administered intraperitoneally (0.6 ml/100 g) to enable the cannulation of the femoral artery and vein. The femoral vein was cannulated for the intravenous delivery of urethane for the maintenance of anesthesia (1–1.4 g/kg iv, initially, followed by supplemental doses of ~0.05 g/kg as required). The depth of anesthesia was monitored every 15 min and was adjusted to ensure the absence of corneal and pedal reflexes. The femoral artery was cannulated for monitoring arterial blood pressure. The signal was recorded using a Mac Lab data-acquisition System (AD Instruments, Colorado Springs, CO). Mean arterial pressure (MAP) and heart rate (HR) were determined electronically from the phasic arterial pressure.

Throughout the surgical procedures, the body temperature was maintained at ~37.0°C with a custom-made water-circulating blanket, through which warm water (48–52°C measured directly at the source) was pumped through at a rate of 16–26 ml/min. The water jacket was also used to gradually raise body core temperature from 37°C to 39°C. A small animal thermometer inserted in the rectum was used to measure body core temperature.

Microinjection in the Hypothalamic PVN

Each animal was placed prone, and the head was mounted in a Stoelting stereotaxic frame such that both bregma and lambda were positioned on the same horizontal plane. A midline reference point was marked 2 mm rostral to bregma. This was necessary because bregma was removed during the subsequent bone-drilling procedure. To expose the dorsal surface of the brain, a hole, ~4 mm in diameter, was drilled in the skull centered 3.5 mm caudal from the reference point. After the drilling procedure, the hole was covered with cotton wool soaked in normal saline to prevent drying of the exposed surface.

Microinjections were made bilaterally using a fine glass micropipette (with a tip diameter of 50–70 μm) in the PVN (stereotaxic coordinates: 1.5–1.8 mm caudal to bregma, 0.5 mm lateral to midline, and 7.5 mm ventral to the surface of the brain). Microinjection volumes were 100 nl/side and were injected over 1 min. After each microinjection, the micropipette was left in place for ~1 min. To mark the injection sites, a small amount of rhodamine-tagged fluorescent microspheres was included in the microinjected solution (LumaFluor, Durham, NC).

MBF Measurement

After the completion of the surgery on the skull, the mesenteric artery was identified using a retroperitoneal approach via an incision on the left flank. The mesenteric artery was dissected free and carefully cleared from the surrounding tissue. A flow probe (IRB449; Transonic System, Ithaca, NY) was positioned around the mesenteric artery near its junction with the aorta and connected to a T206 small animal blood flowmeter (Transonic System) to enable monitoring of the MBF. This procedure lasted ~1 h. After the implantation of the flow probe, ~15–20 min (rest period) were allowed to elapse to ensure that a steady basal blood flow was attained. Mesenteric vascular conductance (MVC) was calculated by dividing MBF (ml/min) by the MAP (mmHg).

Experimental Protocol

Upon completion of the surgical procedures, the rats were randomly assigned to four groups. In the first two groups (n = 5/group), rats were bilaterally microinjected in the PVN with 100 nl/side of N(3)-nitro-L-arginine methyl ester (l-NAME; Sigma-Aldrich, St. Louis, MO), a NOS inhibitor, at a dose of either 100 or 200 nmol/side. In a third group (n = 5), saline replaced l-NAME. Immediately after the completion of the microinjections, the water jacket was used to gradually increase the body core temperature of the animal from 37°C to 39°C at a rate of ~0.1°C every 2 min. This was performed over ~40 min. In the last group of animals, l-NAME (200 nmol/side) (n = 5) was microinjected in the PVN, and the effects were followed over time. In this group of rats, body core temperature was maintained between 37.0 and 37.5°C.

Brain Histology

At the completion of the experiment, rats were killed with an overdose of pentobarbital sodium (300 mg/kg) (Lethabar, Virbac, NSW, Australia). The brains were then carefully removed and fixed in 4% paraformaldehyde solution for ~6 days, and then placed in a solution of phosphate buffer containing 20% sucrose overnight. The hypothalamus was cut into sections (40 μm thick) using a cryostat and mounted on gelatin-subbed slides. The sections were then viewed wet under fluorescent microscopy to identify the rhodamine beads at the site of injection. The sections were then dried before being counterstained with cresyl violet and cover slipped with Depex mounting medium (BDH Lab Supplies, Poole, UK). The sections were then reexamined using light microscopy to determine anatomical structure, and the injection sites were subsequently mapped in relation to the anatomical structure.

Statistical Analysis

The basal resting levels of MAP, HR, MBF, and MVC were compared between groups using one-way ANOVA followed by post hoc comparisons of the group means using the Bonferroni modification for multiple comparisons. The changes in MAP, HR, MBF, and MVC from resting levels were compared within groups using a one-way ANOVA with repeated measures followed by Dunnett’s multiple-comparison test. The comparisons between groups were analyzed using a two-way ANOVA with repeated measures followed by post hoc comparisons of the group means using the Bonferroni modification for multiple comparisons. All data are presented as means ± SE.

RESULTS

Resting Cardiovascular Variables. Before drug/vehicle administration, basal resting MAP, HR, MBF, and MVC were not significantly different between groups (Table 1).

Effect of Increased Body Core Temperature on Cardiovascular Variables

Responses in rats microinjected with saline in the PVN. In animals in which saline was microinjected in the PVN (n = 5), raising body core temperature from 37°C to 39.0°C did not significantly affect blood pressure (Fig. 1). HR, however, was significantly increased by the elevation in body core temperature such that it reached a maximum increase of 17 ± 7 beats/min at 39.0°C (P < 0.05 compared with resting basal value at 37.0°C) (Fig. 1). In contrast, elevation of body core temperature elicited a significant reduction in MBF that reached a maximum of ~2.6 ± 0.4 ml/min or ~14 ± 2% (P < 0.001 from the respective resting basal values) and a significant reduction in MVC that reached a maximum of ~0.031 ± 0.009 ml·min⁻¹·mmHg⁻¹ or ~16 ± 4% by the end of the observation period (P < 0.01 from the respective resting basal values) (Fig. 1).
Table 1. Average basal levels of MAP, HR, MBF, and MVC in rats microinjected with the nitric oxide synthase inhibitor (l-NAME) or vehicle in the hypothalamic PVN

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>MAP, mmHg</th>
<th>HR, Beats/min</th>
<th>MBF, ml/min</th>
<th>MVC, ml·min⁻¹·mmHg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>5</td>
<td>99 ± 4</td>
<td>386 ± 19</td>
<td>18.8 ± 1.7</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>l-NAME (100 nmol)</td>
<td>5</td>
<td>107 ± 6</td>
<td>361 ± 10</td>
<td>17.6 ± 1.5</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>l-NAME (200 nmol)</td>
<td>5</td>
<td>103 ± 6</td>
<td>373 ± 14</td>
<td>18.4 ± 0.9</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>l-NAME (200 nmol, no change in body core temperature)</td>
<td>5</td>
<td>108 ± 4</td>
<td>367 ± 14</td>
<td>21.3 ± 1.1</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>Vehicle (no change in body core temperature)</td>
<td>5</td>
<td>89.0 ± 1.7</td>
<td>338 ± 4</td>
<td>20.2 ± 2.6</td>
<td>0.23 ± 0.03</td>
</tr>
</tbody>
</table>

All data are expressed as means ± SE; n, no. of rats. MAP, mean arterial blood pressure; HR, heart rate; MBF, mesenteric blood flow; MVC, mesenteric vasculature conductance; l-NAME, Nω-nitro-l-arginine methyl ester; PVN, paraventricular nucleus. Rats had their body core temperature maintained at ~37°C or it was raised to 39°C.

Responses in rats microinjected with l-NAME in the PVN. In the rats treated with bilateral microinjections of l-NAME (100 or 200 nmol) in the PVN, the MAP and HR responses elicited by increasing body core temperature were not significantly different from the respective responses observed in the saline-treated control group (Fig. 1). In contrast, MBF and MVC responses were significantly different between the three groups [MBF, F(3,95) = 10.579, P < 0.001; MVC, F(3,95) = 12.325, P < 0.001]. After 200 nmol l-NAME, the normal reduction in MBF that accompanied the increasing body core temperature was prevented (P < 0.001 compared with the control group), and the average change observed was only 2 ± 4% (Fig. 1). This effect was dose dependent, since 100 nmol of l-NAME in the PVN did not significantly affect the fall in MBF compared with the control group (Fig. 1).

Similarly, after microinjection of 200 nmol of l-NAME in the PVN, there was no reduction in MVC induced by the elevation in body core temperature, which was significantly different from the control group (P < 0.05) (Fig. 1). However, after the administration of the lower dose of l-NAME, MVC fell to a maximum of −21 ± 3%, as body core temperature increased, and this was not significantly different from the response observed in the control group but was significantly different from the response observed with 200 nmol l-NAME (P < 0.001) (Fig. 1).

Effect of l-NAME in the PVN, Without a Change in Body Core Temperature, on Cardiovascular Variables

In two separate group of rats, either saline (100 nl/side) (n = 5) or l-NAME (200 nmol) was microinjected in the PVN (n = 5) while body core temperature was kept at 37.0–37.5°C, and the cardiovascular variables were monitored. Saline in the PVN did not significantly affect MAP, HR, MBF, or MVC. After l-NAME, MAP increased by a maximum of 8 ± 3 mmHg, from 108 ± 4 mmHg, within 15–25 min, which was significantly different from the resting preinjection level (P < 0.05) and from the control group that received saline in the PVN (P < 0.01) (Fig. 2). HR before l-NAME was 367 ± 14 beats/min and was not significantly altered by l-NAME treatment (Fig. 2). MBF fell from 21.3 ± 1.1 ml/min by a maximum of 19% within 20 min, which was significantly different compared with the preinjection level and from the control group (P < 0.05) (Fig. 2). Similarly, MVC fell from 0.198 ± 0.001 ml·min⁻¹·mmHg⁻¹ by 23% within 20–25 min (P < 0.05 compared with resting level; P < 0.01 compared with the saline group) (Fig. 2).

Neuroanatomical Location of Injection Sites

The anatomical locations of the microinjection sites within the brain were determined histologically at the conclusion of

![Fig. 1. Blood pressure, heart rate, mesenteric blood flow (MBF), and mesenteric vascular conductance (MVC) effects induced by changes in body core temperature in anesthetized rats in which saline vehicle (n = 5, ○), 100 nmol Nω-nitro-l-arginine methyl ester (l-NAME) (n = 5, □), or 200 nmol l-NAME (n = 5, △) was microinjected bilaterally in the hypothalamic paraventricular nucleus (PVN). ***P < 0.001 between groups (2-way ANOVA with repeated measures corrected with Bonferroni’s post hoc test). *P < 0.05 compared with basal level, within groups, before the increase in body core temperature (1-way ANOVA with repeated measures corrected with Dunnett’s post hoc test). Data are presented as means ± SE.](http://ajpregu.physiology.org/Downloaded from)
the experiments and are shown in Fig. 3. The microinjection sites of L-NAME in the PVN were centered at rostral-caudal levels that encompassed the PVN and ranged from ~1.8 to 2.2 mm caudal to bregma (Fig. 3). The rostral-caudal distribution of the saline microinjection sites in the PVN was similar to the distribution of L-NAME microinjection sites (Fig. 3).

**DISCUSSION**

The most important finding of the present study was that inhibition of NOS within the PVN, using local administration of L-NAME (200 nmol), prevented the reflex reduction in MBF that normally occurs when body core temperature is elevated. The effect of L-NAME was dose dependent, since 100 nmol of L-NAME in the PVN did not affect the responses observed following an elevation in body core temperature.

In the present study, we observed that an elevation in body core temperature had no significant effect on blood pressure but significantly increased HR and significantly reduced MBF and MVC; the latter are critical in the redistribution of blood flow to the skin vasculature to help dissipate heat. These responses were similar to those that have been reported previously (18–19, 22). The PVN is essential in the vasoconstriction of the mesenteric vascular bed during hyperthermia; it has been well described that neurons in the PVN are activated by increases in body core temperature (2, 4, 7, 13, 15, 21), and inhibition of neuronal activity in the PVN abolishes the normal reduction in MBF induced by hyperthermia (8). The efferent pathways from the PVN involved in the responses are unknown; however, the PVN has direct projections to sympathetic preganglionic motor neurons in the spinal cord, and it can indirectly influence sympathetic nerve activity through its projections to the pressor region of the rostral ventrolateral medulla, and a proportion of both of these populations of neurons can be activated by elevations in body core temperature (5, 7, 34). Interestingly, the distribution of these activated neurons coincides with regions in the PVN containing nitrergic neurons (38). Indeed, we have found previously that ~40% of nitrergic neurons in the PVN were activated by hyperthermia. Some of the spinally projecting neurons in the PVN activated by an elevation in body core temperature were nitrergic, but this was not so for the activated PVN neurons projecting to the rostral ventrolateral medulla (5, 7). Taken together, these findings suggest that nitrergic neurons in the PVN, some of which project to the spinal cord, may contribute to the cardiovascular responses induced by hyperthermia.

The present study supports this suggestion by providing direct evidence for a role of nitric oxide in the reflex MBF response induced by hyperthermia. We found that microinjections of 100 nmol L-NAME to inhibit NOS, the enzyme responsible for the production of nitric oxide, locally in the PVN did not affect the responses normally induced by increasing body core temperature. By contrast, raising the dose of L-NAME to 200 nmol resulted in the inhibition of the changes in MBF and MVC without significantly affecting the blood pressure and HR responses following the increase in body core temperature. The present findings suggest that nitric oxide within the PVN is an important mediator of the reflex reduction in MBF induced by hyperthermia. This does not mean that nitric oxide in other brain regions could not influence the cardiovascular responses to hyperthermia.

Nitric oxide in the brain is believed to contribute to thermoregulation, including a key role in increasing heat dissipation. In the rabbit, central administration of nitric oxide donors decreases body core temperature accompanied by increased respiratory frequency, evaporative heat loss, and increased ear skin temperature; conversely, inhibition of nitric oxide production elicited opposite effects (29). In the rat, blockade of central nitric oxide production has been found to elevate body core temperature at rest and during exercise (24, 28) and was accompanied by an increased tail skin temperature (24). The mechanisms and sites of action of nitric oxide involved in these thermoregulatory effects are unknown but are likely to involve the anterior hypothalamus (36). The results of the present findings suggest that, in conditions in which there are increased ambient environmental temperatures and a resultant elevation in body core temperature, nitric oxide can also facilitate heat dissipation by acting within the hypothalamic PVN to redirect
blood flow from the warm internal core organs to the cooler peripheral skin vasculature.

In the present study, we found that inhibition of NOS in the PVN without increasing body core temperature resulted in an acute increase in blood pressure that lasted 40–50 min. This suggests that tonic production of nitric oxide in the PVN lowers blood pressure, which is consistent with previous reports from several laboratories (14, 26, 40). We did not observe a significant change in HR, although others have (14, 40). The acute reduction in MBF induced by L-NAME microinjected in the PVN of rats whose body core temperature was maintained at 37–37.5°C suggests that nitric oxide is tonically active in the PVN and important in maintaining MBF via a sympathoinhibitory action. This is in addition to previous reports suggesting that nitric oxide has sympathoinhibitory effects on renal nerve activity (39–40, 42).

The acute effects on MBF following the administration of L-NAME in the PVN suggest that we should have seen an enhancement of the normal reduction in MBF elicited by the elevation of body core temperature; however, this was not observed. The results suggest that nitric oxide tonically produced in the PVN is sympathoinhibitory, whereas nitric oxide produced in response to an elevation in body core temperature is sympathoexcitatory, at least on sympathetic nerves supplying the mesenteric vasculature. The mechanisms accounting for these opposing effects of nitric oxide are unknown; however, nitric oxide has been reported to have excitatory effects on some neurons and inhibitory effects on others (12, 32). Additionally, nitric oxide has been observed to enhance the responses to glutamate, acting on the 3-hydroxy-5-methyl-4-isoxazole propionate receptor subtype, in magnocellular neurons of the PVN (31) but has been found to attenuate the cardiovascular actions elicited following activation of the N-methyl-D-aspartate subtype of glutamatergic receptors in the PVN (25). Clearly, further study into the mechanisms of action of nitric oxide in the PVN will be required to shed further light into the complex roles of nitric oxide in cardiovascular regulation during hyperthermia.

Methodological Considerations

The present studies were performed under general anesthesia and after surgical procedures were performed to enable 1) intracerebral injections and 2) direct measurement of MBF. Anesthesia will affect the normal regulation of body core temperature; however, the reflex changes in MBF elicited by hyperthermia in the present study are part of the normal reflex response elicited by hyperthermia in conscious animals. In the present work, we used urethane for the maintenance of anesthesia. This anesthetic is commonly used for in vivo studies, since it produces
a long-lasting surgical plane of anesthesia and has minimal effects on cardiovascular regulation and sympathetic nerve activity (27), and we have used it in previous studies in which we investigated the cardiovascular responses to hyperthermia (6, 8).

The doses of L-NAME chosen in the present work are based on the study of Zhang and Patel (41) who used 50–200 nmol to inhibit NOS in the PVN. They found that that 200 nmol L-NAME produced approximately double the increase in renal sympathetic nerve activity compared with 100 nmol (i.e., 90% vs. 45% increase). The lower dose of L-NAME elicited similar effects to a 50 nmol dose of L-NAME, suggesting a steep dose-response curve for renal nerve activity. In the present study, there was no inhibition of the MBF response elicited by hyperthermia with 100 nmol L-NAME, but, given we saw significant inhibition of the response with 200 nmol of L-NAME, we suspect that the dose-response curve for inhibition of the MBF response may also be steep.

Perspectives and Significance

One of the essential factors in temperature regulation is the mechanism involved in the dissipation of heat when body core temperature is elevated. The PVN appears to be a critical central nucleus mediating cardiovascular responses elicited by hyperthermia. The present study suggests that the hyperthermia-induced mesenteric vasoconstriction is mediated via nitric oxide in the PVN. The constriction of the mesenteric vascular bed as body core temperature rises is important in maintaining blood pressure in the face of vasodilation of the large skin vasculature. Thus we suspect that any dysfunction to the nitrogentic system within the PVN that prevents the normal vasoconstriction induced by the increased body core temperature may predispose an individual to heat stroke.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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