Suppression of endotoxin-induced fever in near-term pregnant rats is mediated by brain nitric oxide

Denovan P. Begg,1 Stephen Kent,1 Michael J. McKinley,2 and Michael L. Mathai2

1 School of Psychological Science, La Trobe University, Bundoora, Victoria; and 2 Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Melbourne, Victoria, Australia

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Begg DP, Kent S, McKinley MJ, Mathai ML. Suppression of endotoxin-induced fever in near-term pregnant rats is mediated by brain nitric oxide. Am J Physiol Regul Integr Comp Physiol 292: 2174–2178, 2007. First published March 1, 2007; doi:10.1152/ajpregu.00032.2007.—Over the last three decades, experiments in several mammalian species have shown that the febrile response to bacterial endotoxin is attenuated late in pregnancy. More recent evidence has established that the expression of nitric oxide synthase (NOS) enzymes is increased in the brain late in pregnancy. The current study investigated the possible role of brain nitric oxide in mediating the phenomenon of fever suppression. Core body temperature (Tb) of near-term pregnant rats (day 19 and 20) was measured following injection of brain NOS and intraperitoneal injection of LPS (50 μg/kg); they were compared with both day 15 pregnant and virgin animals. Intracerebroventricular injection with an inhibitor of NOS, Nω-monomethyl-l-arginine citrate (l-NMMA; 280 μg), in near-term pregnant rats restored the febrile response to LPS. As expected, near-term dams that received intracerebroventricular vehicle + IP LPS did not increase Tb, in contrast to the 1.0 ± 0.2°C rise in Tb in dams treated with ICV l-NMMA + IP LPS (P < 0.01). In virgin females and day 15 pregnant controls receiving this treatment, the increases in Tb were 1.5 ± 0.3°C and 1.6 ± 0.4°C, respectively. Thus, blockade of brain NOS restored the febrile response to LPS in near-term dams; at 5 h postinjection, Tb was 60–70% of that observed in virgins and day 15 pregnant animals. Intracerebroventricular l-NMMA alone did not induce a significant change in Tb in any group. These results suggest that the mechanism underlying the suppression of the febrile response in near-term pregnancy is mediated by nitric oxide signaling in the brain.

nitric oxide synthase; Nω-monomethyl-l-arginine citrate; lipopolysaccharide; core body temperature; thermoregulation

FEVER IS A REGULATED INCREASE in core body temperature (Tb) caused by initiation of heat-conserving and heat-producing systems. It plays a crucial role in the physiological response to infection by pathogens, improving the efficiency of lymphocytes and reducing the replication of many microorganisms (23). Fever can be caused by a number of different stimuli, such as infection with bacteria and viruses (27) or by stress (13, 17). It has been widely documented that these factors result in the release of proinflammatory cytokines from macrophages (11). This results in the stimulation of central and peripheral cyclooxygenase (COX) enzymes that catalyze the synthesis of PGs (10). The enzymes COX-1 and COX-2 are the rate-limiting step in the production of PGs, and inhibition of COX blocks the febrile response (39). PGE2 has been seen traditionally as the final step in the process of fever generation. The febrile response is integral to the body’s defense against infection; however, if Tb becomes excessively high, it endangers the host, as these temperatures can result in cellular injury, seizures, and in extreme cases death (23).

Kasting et al. (26) were the first to report that ewes show a reduction in the febrile response to infectious stimuli late in pregnancy. This finding has since been extended to guinea pigs (49) and rats (30); however, support has not been universal (4). It has further been established that in some cases, infectious stimuli can, in fact, lead to hypothermic responses (45). Several mechanisms have been proposed to account for the suppression of fever in pregnant mammals. Animals injected with LPS late in pregnancy show lower levels of COX-2 in hypothalamic tissue (34), as well as decreased concentrations of PGE2 in their CSF compared with nonpregnant controls (24). The mechanism responsible for this suppression has not been identified; however, one molecule that may be involved is nitric oxide (NO). When administered centrally, nitric oxide synthase (NOS) inhibitors such as N-nitro-l-arginine methyl ester (l-NNAME) increase blood pressure (25) and Tb (31). However, these increases can be blocked by the nonspecific COX inhibitor indomethacin (31, 44), indicating interactions between COX and NO activity. Oestrogen and progesterone, secreted during pregnancy, increase expression of endothelial NO synthase (eNOS) within cerebral blood vessels (33), and the neuronal NO isoform is also increased in the hypothalamus during the late stages of pregnancy (38, 47, 48).

There is growing evidence that NO is involved in thermoregulation through neural signaling (31, 42). Neurons containing NOS have been identified in the medial preoptic area (3), a key integrative site for thermoregulatory signals. There is some evidence that intravenous injection of NO donors leads to hypothermia through increases in respiratory frequency, evaporative water loss, and cutaneous heat dissipation and may reduce the febrile response to LPS (21, 32). Others, however, have found central NO donors to be pyrogenic (29). When NOS is inhibited by central administration of l-NNAME, the febrile response to LPS and stress-induced hyperthermia is potentiated (42). Similarly, central inhibition of NOS blocks the hypothermic effects of AVP and insulin (1). Interestingly, peripheral NOS inhibition has very different effects and can attenuate fever caused by increased thermogenesis (43). Although the effect of NOS inhibitors on febrile mechanisms varies between the periphery and the brain, it is clear that central inhibition of NOS augments fever in response to endotoxic stimuli and prevents the cooling responses of hypother-

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mic stimuli. The aim of the current study was to test whether the attenuated febrile response in near-term pregnant rats is reversed following NOS inhibition. It was postulated that inhibiting central NOS would restore LPS-induced fever in near-term pregnant rats.

MATERIALS AND METHODS

Animals and Housing

A total of 76 female Sprague Dawley rats were used. The animals were obtained from Monash SPF animal services (Clayton, Victoria, Australia) and housed in the Central Animal House of La Trobe University. Animals arrived in the laboratory at 8 wk of age and were given at least 1 wk to acclimatise before experiments commenced. They were individually housed, fed standard laboratory rat pellets, and these, along with water, were available ad libitum. The animals were kept in a 12:12-light-dark cycle, with the ambient temperature maintained at 25–27°C, the rat thermoneutral zone. All testing was approved by the La Trobe University Animal Ethics Committee.

Surgery

Animals were anesthetized with an intraperitoneal injection of ketamine (61 mg/kg) and xylazine (9 mg/kg); carpofen (7 mg/kg) was administered subcutaneously to reduce postoperative discomfort. A 23-gauge cannula was aligned with bregma and positioned 1.5 mm to the right and 0.2 mm caudally. A hole was drilled at this point through the skull; the cannula was positioned 3 mm below the dura mater into the ventricle. The alignment of the cannula tip was confirmed by flow of saline under gravity. The cannula was secured with dental acrylic and screws in the skull and capped to prevent blockage. Anesthetized animals also had a biotelemetry device (E-4000, Mini-Mitter, Bend, OR) implanted into the peritoneal cavity via a 1-cm incision made into the right and 0.2 mm caudally. A hole was drilled at this point through the skull; the cannula was positioned 3 mm below the dura mater into the ventricle. The alignment of the cannula tip was confirmed by flow of saline under gravity. The cannula was secured with dental acrylic and screws in the skull and capped to prevent blockage. Anesthetized animals also had a biotelemetry device (E-4000, Mini-Mitter, Bend, OR) implanted into the peritoneal cavity via a 1-cm incision made into the skin and muscle wall. The muscle and skin were sutured, and antibacterial solution was used to clean the area. A recovery period of at least 7 days followed the surgery.

Chemicals

\(L^n\)-monomethyl-L-arginine citrate (L-NMMA) (molecular weight 756.8) was purchased from Cayman Chemical (Ann Arbor, MI). LPS from **Escherichia coli** (serotype 0111:B4) was purchased from Sigma (Castle Hill, NSW, Australia). Ketamine and xylazine were purchased from Ilium Laboratories (Smithfield, NSW, Australia) and carpofen from Allhank Trading (South Melbourne, Victoria, Australia).

Procedure

Two-thirds of the animals were mated when they were 8–10 wk old. Pregnancy was confirmed by the presence of vaginal sperm plugs. After mating, pregnant animals and age-matched virgin females were surgically prepared with a ventricular cannula and a Mini-Mitter. On day 15 of gestation (mid-term pregnant), day 19 or 20 of gestation (near-term pregnant), or the equivalent time in age-matched controls, animals received one of the following treatments: L-NMMA (280 \(\mu\)g icv) and LPS (50 \(\mu\)g/kg ip), intracerebroventricular artificial cerebrospinal fluid (aCSF) and intraperitoneal LPS (50 \(\mu\)g/kg), L-NMMA (280 \(\mu\)g icv) and intraperitoneal saline; and intracerebroventricular aCSF and intraperitoneal saline. All intracerebroventricular injections were in 3 \(\mu\)l of aCSF. The injections were given 2 h after lights on (time 0), and intracerebroventricular injections were given immediately preceding intraperitoneal injections. \(T_b\) was continuously monitored for 6 h after the injections using the VitalView (Bend, OR) computer software system.

Statistical Analyses

\(T_b\) data were collected at 1-min intervals on the VitalView system. Baseline \(T_b\) was taken as the average for 2 h before injection, and changes from baseline were calculated for 30-min averages. Thus, 12 half-hour averages were calculated postinjection. The groups were compared from time point 3 (60 min) to time point 12 (360 min) using a repeated-measures ANOVA, with post hoc least significant difference comparisons performed to elucidate differences between groups. Results are presented as means ± SE.

RESULTS

There was a significant effect of treatment group (\(F_{11.61} = 4.95, P < 0.001\)). As reported in previous studies (18, 20, 33), systemic injection with LPS in day 19 and 20 dams resulted in an attenuation of the febrile response. However, central NOS inhibition in conjunction with LPS injection induced fever in day 19 and 20 pregnant dams (see Fig. 1), closely resembling that observed in day 15 dams (Fig. 2) and virgin females (Fig. 3). There was a significant increase in \(T_b\) in day 19 and 20 dams treated with L-NMMA and LPS compared with those treated with LPS alone (\(P = 0.02\)) or controls (\(P = 0.03\)). As expected, systemic injection with LPS alone induced a febrile response in both the virgin and day 15 pregnant dams. The mean rise in \(T_b\) in NOS-inhibited day 19 and 20 dams treated with LPS was 1.0 ± 0.2°C compared with a 1.5 ± 0.2°C rise observed in virgins and a 1.6 ± 0.3°C increase in day 15 pregnant controls. Thus, the fever in 19- and 20-day pregnant rats was 60–70% of that seen in the other groups. Nonetheless, the difference between day 19 and 20 pregnant and virgin or day 15 pregnant animals treated with L-NMMA and LPS was not significant (\(P = 0.86\) and \(P = 0.65\), respectively). In contrast, there was a significant difference between day 19 and 20 pregnant and virgin or day 15 pregnant animals treated with LPS alone (\(P = 0.014\) and \(P < 0.001\), respectively).

There was a small transient increase in \(T_b\) in day 19 and 20 dams, after central NOS inhibition, but this was not significantly different from day 19 and 20 aCSF-treated controls. Significant febrile responses occurred in response to LPS compared with saline-treated controls in virgins (\(P = 0.015\)) and 15-day pregnant dams (\(P < 0.001\)). This febrile response was also seen in LPS-treated virgins (\(P < 0.001\)) and 15-day pregnant dams (\(P = 0.004\)) with NOS inhibition. There was no significant difference between NOS inhibition with LPS and LPS alone in either virgins or 15-day dams (\(P = 0.27\) and \(P =
no nonspecific response to L-NMMA. due to a restoration of the neuroimmune response to LPS and demonstrating that the febrile response in near-term dams was in these groups.

15 febrile response that was restored by blockade of NO synthesis in near-term pregnancy (38, 47, 48) and that central NO donors can inhibit LPS-induced fever (21). The dose of L-NMMA that we used did not cause significant hyperthermia by itself in either day 19 and 20 pregnant, day 15 pregnant, or virgin female rats, demonstrating that the febrile response in near-term dams was due to a restoration of the neuroimmune response to LPS and not a nonspecific response to L-NMMA.

The suppression of the febrile response to systemic endotoxin in near-term pregnancy is an established phenomenon that has been widely studied. This is the first study to show that this febrile response can be restored and that brain NO plays a determining role in the mechanism of fever suppression. This NO-mediated mechanism is consistent with previous reports that the expression of NOS within the brain is increased during late pregnancy (38, 47, 48) and that central NO donors can inhibit LPS-induced fever (21). The dose of L-NMMA that we used did not cause significant hyperthermia by itself in either day 19 and 20 pregnant, day 15 pregnant, or virgin female rats, demonstrating that the febrile response in near-term dams was due to a restoration of the neuroimmune response to LPS and not a nonspecific response to L-NMMA.

In our experiments, we noted that the time course of the febrile response that was restored by blockade of NO synthesis was similar in day 19 and 20 pregnant rats compared with day 15 pregnant rats and virgins, although the magnitude of the peak fever was 60–70% that of the latter. Recent work has shown that following a higher dose of the same serotype of LPS (160 µg/kg) as used in the current study, the increases in plasma levels of the pyrogenic cytokines, IL-1β, and IL-6 in near-term pregnant rats were inhibited compared with nonpregnant rats (18). Furthermore, levels of the endogenous antipyretic molecule IL-1 receptor antagonist, which is readily secreted from macrophages, particularly during disease, have been shown to increase near term (2, 18). Thus, it is possible that the lower fever peak that we observed in near-term pregnant rats may have been related to reduced cytokine-mediated activation of COX-2 and PGE2 synthesis. However, other workers observed no differences between the levels of IL-1β, IL-6, TNF-α, and IFN-γ in day 15 pregnant, near-term, and lactating rats when a lower dose of a different serotype of LPS was used (50 µg/kg) (35). Furthermore, the same group found that the IL-6 STAT3 signaling pathway remained unchanged in near-term pregnant animals and is therefore not related to the febrile response (22). Consequently, the differences in plasma levels of cryogenic and pyrogenic cytokine signaling molecules observed in near-term pregnant rats compared with nonpregnant rats (18) could be due to differences in the dose and serotype of LPS used or possibly elevated peripheral NOS that would have remained unaffected by central L-NMMA administration and may not directly relate to the mechanism of fever suppression in near-term pregnancy.

The failure of intracerebroventricular L-NMMA to elicit a hyperthermic response in the current study, as seen previously in male rats, may be due to fundamental differences in temperature signaling between the sexes (2). One example of a fundamental sex difference is the role of AVP in thermal signaling. AVP has been reported to have antipyretic properties in male rats (5, 46). However, females do not have the same response to AVP, and they may rely on different antipyretic mechanisms (37). Another possibility is that different antipyretic mechanisms may reduce the febrile response caused by different pyrogens. For example, AVP antagonist restores the febrile response to intracerebroventricular administration of PGE1 (15, 16), but AVP does not mediate the attenuated febrile response to intravenous IL-1β in pregnant dams (14). Furthermore, it has been reported that AVP antagonism does not restore the febrile response to PGE2-treated rats (9).

Furthermore, NOS inhibition alone had no significant effect on Tb (P = 0.32 and P = 0.82, respectively) in these groups.

**DISCUSSION**

The suppression of the febrile response to systemic endotoxin in near-term pregnancy is an established phenomenon that has been widely studied. This is the first study to show that this febrile response can be restored and that brain NO plays a determining role in the mechanism of fever suppression. This NO-mediated mechanism is consistent with previous reports that the expression of NOS within the brain is increased during late pregnancy (38, 47, 48) and that central NO donors can inhibit LPS-induced fever (21). The dose of L-NMMA that we used did not cause significant hyperthermia by itself in either day 19 and 20 pregnant, day 15 pregnant, or virgin female rats, demonstrating that the febrile response in near-term dams was due to a restoration of the neuroimmune response to LPS and not a nonspecific response to L-NMMA.

The preoptic area of the hypothalamus is implicated in the generation of fever, and both NOS and COX-2 enzymes are present in this area (6, 42). There is evidence that NO can inhibit both the expression (12, 35) and activity of COX-2 (20); conversely, others have shown an upregulation (41). First, elevated NO-cGMP signaling increases expression of IκB (36), an inhibitor of NFkB, thereby causing an inhibition of NFκB-mediated induction of COX-2 (12). However, IκB levels, and hence NF-κB activity, have been found to be similar in 15-day and near-term pregnant rats in response to LPS (35). These findings indicate that the decrease in COX-2 activation seen in near-term rats in response to pyrogenic stimuli is unlikely to be mediated by the NF-κB pathway. Second, there is evidence that NO can directly inhibit the activity of COX-2, by nitrosylating a tyramine in the COX catalytic site (20). This proposed inhibition of COX-2 is consistent with previous work showing that brain COX-2 and PGE2 synthesis following LPS injection were reduced in near-term pregnant rats (24, 34).

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indicates that while AVP may attenuate PGE1-induced febrile responses, the increase in AVP seen in near-term pregnant animals (28) is unlikely to mediate antipyretic responses to proinflammatory cytokines, bacterial endotoxin, or PGE2-induced fever.

**Perspectives**

The suppression of fever by increased brain NO may be an important mammalian adaptation to prevent maternal inflammation and hyperthermic stress to the young during the birth process. Maternal LPS treatment has been shown to induce inflammatory responses associated with white matter injury in the offspring (40), similar to the damage seen in the brains of neonatal rats injected with LPS (8). Suppression of the febrile process. Maternal LPS treatment has been shown to induce inflammatory responses associated with white matter injury in the offspring (40), similar to the damage seen in the brains of neonatal rats injected with LPS (8). Suppression of the febrile process. Maternal LPS treatment has been shown to induce inflammatory responses associated with white matter injury in the offspring (40), similar to the damage seen in the brains of neonatal rats injected with LPS (8). Suppression of the febrile

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