Role of nitric oxide in tolerance to lipopolysaccharide in mice

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Dias, Mirela B., Maria C. Almeida, Evelin C. Carnio, and Luiz G. S. Branco. Role of nitric oxide in tolerance to lipopolysaccharide in mice. J Appl Physiol 98: 1322–1327, 2005.—The injection of repeated doses of lipopolysaccharide (LPS) results in attenuation of the febrile response, which is called endotoxin tolerance. We tested the hypothesis that nitric oxide (NO) arising from inducible NO synthase (iNOS) plays a role in endotoxin tolerance, using not only pharmacological trials but also genetically engineered mice. Body core temperature was measured by biotelemetry in mice treated with Nω-monomethyl-L-arginine (L-NMMA, 40 mg/kg; a nonselective NO synthase inhibitor) and aminoguanidine (AG, 10 mg/kg; a selective iNOS inhibitor) and in mice deficient in the iNOS gene (iNOS KO) mice. Tolerance to LPS was induced by means of three consecutive LPS (100 μg/kg) intraperitoneal injections at 24-h intervals. In wild-type mice, we observed a significant reduction of the febrile response to repeated administration of LPS. Injection of L-NMMA and AG markedly enhanced the febrile response to LPS in tolerant animals. Conversely, iNOS-KO mice repeatedly injected with LPS did not become tolerant to the pyrogenic effect of LPS. These data are consistent with the notion that NO modulates LPS tolerance in mice and that iNOS isoform is involved in NO synthesis during LPS tolerance.

body temperature; thermoregulation; fever; nitric oxide synthase

THE FEBRILE RESPONSE is a complex physiological reaction resulting from contact with infectious or inflammatory agents referred to as exogenous pyrogens. Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, is the experimental model best known and most frequently used to induce fever. In response to LPS, a wide variety of cytokines, called endogenous pyrogens, are mobilized to induce, through either humoral or neuronal pathways, the synthesis and release of prostaglandins, especially prostaglandin E2, which acts largely in the preoptic region of anterior hypothalamus, affecting thermoregulatory neurons and resulting in elevation of body core temperature (Tb), which is defined as fever (for review, see Refs. 5, 11, 16).

Repeated administration of LPS at short-term intervals results in attenuation of the febrile response, which is called endotoxin tolerance (4). Development of tolerance to LPS appears to be an important mechanism to produce controlled inflammatory responses. Tolerant animals exhibit less pronounced responses to nonlethal doses of LPS and also survive to LPS doses that would typically be lethal (10, 15). The induction of tolerance represents a highly effective prophylactic mechanism against mortality from endotoxin shock (10).

Therefore, the investigation of LPS tolerance has been of considerable interest.

Among the several mechanisms involved in LPS tolerance, central mechanisms participating in endogenous antipyresis are proposed to contribute to this phenomenon. For instance, it is known that administration of a vasopressinergic V1 receptor antagonist into the ventral septal area enhances the febrile response to LPS in endotoxin-tolerant animals (32). Other important mechanisms involved in endotoxin tolerance include modulation of LPS-binding sites (CD14) (33), alteration of intracellular signaling (inhibition of MAPK activation and impair of NF-κB translocation) (9), and downregulation of the cytokine response to pyrogen (7, 23, 24).

Nitric oxide (NO), which is widely recognized as a prominent neuromodulator, is synthesized from the l-arginine by the enzyme NO synthase (NOS), resulting in the formation of l-citrulline and NO (13). This diffusible gaseous compound has been demonstrated to be an important signaling molecule in a large variety of physiological processes, including cardiovascular, immune, central, and peripheral nervous systems (19). In addition, it has been demonstrated for a variety of species that NO has an important role in thermoregulation and fever (28). In mice, both inducible (iNOS) and neuronal NOS isoforms have been shown to be involved in LPS-induced fever (17). As regards to endotoxin tolerance, NO has been shown to be involved in the development of tolerance to LPS both in rabbits (12) and rats (2). However, the studies investigating endotoxin tolerance and the role of NO in this mechanism in mice are restricted, because most of them have been performed with “in vitro” analysis, measuring the concentration of cytokines and other components in transduction pathways triggered by LPS (7, 8, 18, 34), and have not examined the febrile response.

Recently, genetically engineered mice have largely been used in studies focusing on the role of NO in various physiological and pathological mechanisms. The use of mutant mice excludes the problems inherent to the injection of pharmacological agents; however, it has been reported that these animals may generate compensatory mechanisms for the lack of a certain gene (17). Therefore, this study employed a double approach to test the hypothesis that NO is involved in LPS tolerance in mice: 1) the use of pharmacological blockade of the NO synthesis with Nω-monomethyl-l-arginine (l-NMMA) and aminoguanidine (AG) and 2) the use of genetically engineered mice deficient in iNOS gene [iNOS knockout mice (iNOS-KO)]. Because the iNOS isoform is the one induced in response to cytokines and exogenous pyrogens (19), we tested...
the hypothesis that iNOS plays a major role in LPS tolerance. Our results are consistent with the notion that NO arising from iNOS does play a key role in LPS tolerance.

MATERIALS AND METHODS

Animals

Experiments were performed on male C57BL/6 [wild type (WT)] mice and iNOS-KO mice weighing 20–25 g. The mutant mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were maintained in individual plastic cages under pathogen-free conditions, controlled temperature (28.0 ± 1.0 °C), and 12:12-h light-dark cycle, with lights on at 6:00 AM. The animals had free access to water and food. Experiments were performed between 8:00 AM. and 4:00 PM. The study was conducted in compliance with the guidelines of the American Physiological Society (3), with the approval of the University of São Paulo Animal Care and Use Committee.

Surgical Preparations

Mice were anesthetized with general anesthesia (70 mg/kg ketamine hydrochloride and 0.4 mg/kg xylazine ip) for the intraperitoneal surgical implantation of the miniature battery-operated temperature-sensitive transmitter (model ER-4000, Mini-Mitter, Sunriver, OR) through a medial laparotomy. Moreover, a 6-cm-long polyethylene tube (PE-10) connected to a 2-cm-long PE-50 (Clay Adams, Parsippany, NJ) was inserted into the peritoneal cavity for injections of drugs. The catheter was tunneled subcutaneously and exteriorized through the back of the neck to be connected to the needle under conscious, freely moving conditions on the moment of the injections.

Tb Measurements

For all protocols, Tb was measured by biotelemetry (Mini-Mitter) at 5-min intervals and plotted at 10-min intervals, during a period of 30 min before and 360 min after the treatments. Data were acquired and fed to an IBM computer by using the Vital View software (Mini-Mitter, Sunriver, OR).

Pyrogens and Drugs

Drugs were injected intraperitoneally. LPS derived from Escherichia coli (0111:B4, Sigma Chemical, St. Louis, MO) was dissolved in sterile 0.9% sodium chloride (saline) and injected at a dose of 100 μg/kg. The NOS nonselective inhibitor L-NMMA (Tocris Cookson, Ellisville, MO) and the iNOS selective inhibitor AG (Sigma Chemical) were dissolved in pyrogen-free sterile saline. The volume of each injection was 0.08–0.1 ml/mouse for all protocols. Pyrogen-free saline was used for control injections.

Experimental Protocols

LPS-induced tolerance in WT mice. Tolerance to LPS was induced by three repeated intraperitoneal injections of LPS (100 μg/kg) at 24-h intervals. Control mice were injected intraperitoneally with pyrogen-free sterile saline.

Effect of intraperitoneal injection of LPS on the circadian Tb rhythm over the day after the injection. This experiment aimed at testing whether the first LPS injection would influence the circadian Tb rhythm over the day after the LPS injection. Starting 24 h after the first injection of LPS (100 μg/kg) or saline, Tb was measured during a period of 24 h.

Determination of the effect of the L-NMMA or AG on LPS tolerance in WT mice. To verify the effect of L-NMMA or AG on Tb of mice, animals were injected either with L-NMMA (40 mg/kg) or AG (10 mg/kg) and Tb was measured; control animals were injected with saline. To test the effect of pretreatment with the NOS blockers on endotoxin tolerance, WT mice were injected in the first and second days, with LPS (100 μg/kg ip). On the third day, the animals received an intraperitoneal injection of L-NMMA (40 mg/kg) or AG (10 mg/kg) 30 min before the last injection of LPS. Control mice received an intraperitoneal injection of pyrogen-free sterile saline. L-NMMA and AG doses were chosen on the basis of a previous study (17) and pilot experiments from our laboratory.

Determination of the effect of the iNOS deficiency on LPS tolerance in mice. iNOS-KO mice received an intraperitoneal injection of LPS at a dose of 100 μg/kg for 3 consecutive days. Control iNOS-KO mice were injected intraperitoneally with pyrogen-free sterile saline. Other two groups of WT mice were used as control. The first group was injected with LPS (100 μg/kg ip) and the second group with pyrogen-free sterile saline.

Statistical Analysis

All values in this study are reported as means ± SE. The thermal response to administration of LPS alone or along with pharmacological treatment or vehicle was compared by two-way ANOVA for repeated measures, followed by Duncan’s post hoc test. Values were considered significantly different when P < 0.05.

RESULTS

LPS Tolerance in WT Mice

Figure 1 shows the development of endotoxin tolerance in mice after three consecutive injections of LPS. On the first day, LPS injection (100 μg/kg) evoked a significant febrile response [F(1,15)= 25.28, P < 0.001; compared with saline-treated controls] with a peak occurring at 100 min postinjection and Tb remaining elevated until the end of the experiment. On the second day, the mice treated with LPS presented a significant increase in Tb [F(1,15) = 14.46, P = 0.002] compared with the Tb response of the corresponding saline controls. However, the peak temperature occurred earlier and was greater but less prolonged, occurring at 40 min postinjection. After the third day of LPS injection, fever response was significantly attenuated [F(1,7) = 13.71, P = 0.008; vs. the fever response on the first day]. There was no significant difference in Tb on the third day among LPS-injected and saline-injected groups.

Circadian Rhythm of Tb on the Day After the First Injection of LPS

Figure 2 illustrates the effect of intraperitoneal injection of LPS (100 μg/kg) or saline on the circadian Tb rhythm over the day after the injection. As shown, the intraperitoneal injection of LPS did not affect the circadian rhythm of Tb of WT mice.

Effect of the NOS blocker on LPS Tolerance in WT Mice

Figure 3A shows the effect of intraperitoneally administered L-NMMA (a nonspecific NOS inhibitor) on Tb. No statistically significant difference was found between the Tb responses of the animals treated with L-NMMA or saline, indicating that L-NMMA, at the dose employed (40 mg/kg), does not affect daytime body temperature in afebrile mice.

The effect of intraperitoneal pretreatment with L-NMMA (40 mg/kg) on LPS response of endotoxin-tolerant mice is shown in Fig. 3B. LPS-tolerant animals, pretreated with L-NMMA 30 min before the third LPS injection, showed an enhanced febrile response. This response was significantly different from that of vehicle-treated mice [F(1,12)= 5.18, P = 0.04].
Effect of the iNOS Blocker on LPS Tolerance in WT Mice

The effect of intraperitoneally administered AG (a specific iNOS inhibitor) on Tb is depicted in Fig. 4A. We observed that intraperitoneal injection of AG (10 mg/kg) caused no significant changes in Tb. Similarly, animals injected with saline did not show any significant change in Tb.

Endotoxin-tolerant animals pretreated with AG (10 mg/kg, ip), 30 min before the third LPS injection, showed a significantly febrile response \([F(1,15) = 4.85, P = 0.044]\) compared with the animals pretreated with saline (Fig. 4B).

Effect of the iNOS Deficiency on Tolerance in Mice

Figure 5 shows the effects of three repeated intraperitoneal injections of LPS (100 µg/kg) at 24-h intervals on iNOS-KO and WT mice. We observed that iNOS-KO mice failed to demonstrate attenuation of the febrile response to repeated doses of LPS. The LPS response in iNOS-KO mice was similar to WT mice on the first and second days but was markedly higher on the third day of treatment \([F(1,24) = 12.91, P = 0.001]\) vs. WT mice. The rise in Tb was significantly higher in the iNOS-KO mice treated with LPS in the 3 days of treatment compared with the iNOS-KO mice injected with saline.

DISCUSSION

The present study shows that the attenuation of the febrile response to repeated injection of LPS, i.e., endotoxin tolerance, develops in mice and is modulated by NO, because the intraperitoneal injection of the NOS inhibitor L-NMMA abolished the endotoxin tolerance. Moreover, we demonstrated that iNOS...
is involved in LPS tolerance, because the treatment with the iNOS inhibitor AG resulted in an enhanced febrile response to LPS in tolerant mice. In agreement, iNOS-KO mice repeatedly injected with LPS did not become tolerant to the pyrogenic effect of LPS.

In this study, tolerance to LPS was expressed through an attenuation of the febrile response. Tolerance to LPS was already observed on the second day, but it was more pronounced on the third day of LPS treatment. Our results add to previous studies that assessed LPS tolerance not by measurements of $T_b$ but by analyzing its immunological aspects through measurements of cytokines and other mediators involved in the tolerance mechanism (7, 8, 18, 34). To our knowledge, no study has investigated the thermoregulatory aspect by measurements of $T_b$ in mice so far.

It is known that mice show a very pronounced circadian rhythm with regard to $T_b$. Thus one could argue that the reduced febrile response to LPS over the days is a consequence of an altered circadian rhythm. However, we demonstrated that animals injected with LPS show a similar pattern of circadian $T_b$ rhythm over the day after the injection compared with saline-injected animals. Moreover, the basal $T_b$ previous to the second and third LPS injections of mice treated with LPS did not differ from the basal $T_b$ of animals injected with saline, indicating that the reduced febrile response to LPS over the days is a consequence of the development of endotoxin tolerance, rather than any alteration in the circadian rhythm of $T_b$ in mice.

![Fig. 4](http://jap.physiology.org/)

A: thermal responses of mice to intraperitoneal injection of aminoguanidine (AG; 10 mg/kg) or saline (arrow, time 0). B: effect of pre-treatment with AG or saline (arrow, time 0) on the febrile response to LPS in endotoxin-tolerant mice. Values are means $\pm$ SE. Nos. in parentheses are no. of animals. *$P < 0.05$ vs. saline/LPS.

![Fig. 5](http://jap.physiology.org/)  

Fig. 5. Effect of repeated intraperitoneal injection of LPS (100 μg/kg) or saline (arrow) on body temperature of wild-type control (WT) mice on the first (A), second (B), and third (C) day of injections and on body temperature of mice deficient in inducible nitric oxide synthase (iNOS-KO) on the first (D), second (E), and third (F) day of injections. Values are means $\pm$ SE. Nos. in parentheses are no. of animals. *$P < 0.05$ vs. saline.
Studies with rats, guinea pigs, and rabbits have demonstrated that the febrile response to LPS in these animals is usually polyphasic. Furthermore, the LPS tolerance is described mainly through alteration of the biphasic response to a monophasic response (2, 27, 32). The \( T_b \) pattern response to LPS in mice varies among different studies. The use of different injection procedures, LPS doses, and ambient temperatures are some of the reasons that may explain these discrepancies. In this study, \( T_b \) started to increase within the first 10 min after LPS injection and remained elevated throughout the 360-min assessment period. This pattern differs from the responses observed by Alheim et al. (1), who showed that intraperitoneal injection of LPS at a dose of 100 \( \mu \)g/kg (the same as the present study) caused hypothermia followed by a delayed fever. The difference in the stress levels caused by a painful intraperitoneal injection vs. an intraperitoneal injection through a preimplanted intraperitoneal polyethylene catheter (present study) may have accounted for the difference between the two studies.

It has been demonstrated for a variety of species that NO has an important role on thermoregulation and fever (for review, see Ref. 28). In the central nervous system, NO has been shown to decrease \( T_b \) and act as an antipyretic molecule (29). On the other hand, systemic administration of NO inhibitors causes hypothermia and inhibits LPS fever (25). However, the peripheral effects of NO on fever seem to be related to its direct effect on thermoregulatory tissues rather than the febrigenic signaling to the brain (30). Indeed, NO can alter thermoregulation by acting either in the brown adipose tissue, where it is needed for heat production (20, 22) or vascular smooth muscle (31) where it can alter heat loss and conservation through the skin.

Previous studies have verified the participation of NO on LPS tolerance in rats (2, 26) and rabbits (12). However, data about the role of NO on tolerance to LPS in mice are limited to in vitro analysis. Fahmi et al. (8) used peritoneal macrophages of mice to study the participation of NO on LPS tolerance. The state of LPS hyporesponsiveness was suggested to result from the reduction of TNF-\( \alpha \) production in response to repeated exposure to LPS. Furthermore, the addition of L-NMMA partially inhibited LPS tolerance, and the exposure of cells to the NO donors mimicked LPS-induced desensitization, suggesting that NO takes part in LPS tolerance in mice. These data corroborate the present results showing that NOS inhibition can reverse the tolerance state: tolerant animals when treated with L-NMMA presented an increased febrile response induced by LPS compared with the control group (Fig. 2), suggesting that NO is important to the development of endotoxin tolerance in mice.

To verify whether iNOS was involved in tolerance to LPS, we used AG, which has been used as a selective inhibitor of iNOS (6, 14). We observed that intraperitoneal administration of AG at a dose of 10 mg/kg did not affect the \( T_b \) of euthermic mice, showing that iNOS does not seem to be important to the maintenance of \( T_b \) under euthermic conditions. Interestingly, intraperitoneal AG in tolerant mice caused an increase in the febrile response after LPS injection compared with the control group, suggesting that iNOS isoform is responsible for NO synthesis during the development of LPS tolerance in mice. It is important to emphasize that AG was injected intraperitoneally, causing a reduction of iNOS activity not only in the periphery but also in the central nervous system. Because NOS inhibition in the central nervous system causes an increase in \( T_b \) (2, 28), it is reasonable to speculate that the main site of action of AG to restore LPS-induced fever is the central nervous system. Additionally, our results show that iNOS-KO mice repeatedly treated with LPS demonstrated significant febrile response in the 3 days of treatment, indicating the inability of these animals to develop LPS tolerance. These data corroborate the results obtained with pharmacological approach and further confirm our hypothesis that iNOS isoform is involved in tolerance to LPS in mice. In agreement, Mustafa and Olson (21) have demonstrated that the expression of iNOS mRNA demands 3–6 h to be induced. Because LPS tolerance is an event observed 24 h after the first exposure to LPS, this is an appropriate time for iNOS expression. It is important to point out that mice depleted in specific NOS genes do not express the gene on both sides of the blood-brain barrier. Therefore, the response in iNOS-KO mice can be due to peripheral and central action of NO. Further studies (using intracerebroventricular injections, for instance) might reveal the relative contribution of central and peripheral NO in LPS tolerance in mice.

In conclusion, our results show that NO plays an important role in the development of LPS tolerance in mice. The present data indicate that NO synthesized by iNOS mediates tolerance to LPS in mice based on the fact that either pharmacology inhibition or genetic deficiency of iNOS abolished endotoxin tolerance.

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