INTRODUCTION

Sepsis, the systemic response to severe infection is accompanied by alterations in several physiological parameters in animals and human beings. Endotoxin (or bacterial lipopolysaccharide; LPS), a component of outer membrane of Gram-negative bacteria, is the prime initiator of the sepsis and its complications known as endotoxic or septic shock. Despite major advances in understanding the pathophysiology of septic shock, the knowledge about the prevention of shock by prophylactic treatments as well as therapy is still very limited.

It has long been known that the repeated exposure to low doses of LPS leads to the rapid development of tolerance to pyrogenic, metabolic, hypothermic/cachexic and other effects of bacterial endotoxin (1, 2). Animals with developed endotoxin tolerance have both less pronounced response to nonlethal doses of LPS and the unique ability to survive challenges to lethal doses of endotoxin (3). Moreover, the state of tolerance seems to be a highly prophylactic mechanism against mortality from endotoxin shock (4). Since endotoxin tolerance can occur naturally in humans, the studies on mechanisms responsible for development and maintenance of tolerance to LPS have been considerable interest. Moreover, the incidence of endotoxin tolerance has been reported in several disease settings, including sepsis, trauma, surgery, and pancreatitis, underlining its clinical significance (5).

The involvement of nitric oxide (NO) in tolerance development to endotoxin has been proposed because peripherally administered NG-nitro-L-arginine methyl ester (L-NAME) (NO synthases inhibitor) delays the endotoxin tolerance formation. Since L-NAME is capable of crossing the blood-brain barrier, the question arises of where activity of NO synthases (inside or outside the blood-brain barrier) is crucial for development of endotoxin tolerance. To clarify the role of different NO synthases (NOS) isoforms, acting in the brain, on the tolerance development, effects of highly selective iNOS and nNOS inhibitors on stepwise attenuation of febrile response during tolerance formation were examined in freely moving biotelemetered rats. We monitored changes in febrile response during the development of tolerance to repeated intraperitoneal (i.p.) injections of lipopolysaccharide (LPS) (50 µg/kg) along with intracerebroventricular (i.c.v.) injections of vinyl-L-NIO, a neuronal NOS inhibitor, or aminoguanidine, an inducible NOS inhibitor at a dose of 10 µg/rat. Both inhibitors injected at the selected doses had no effect on normal day-time as well as night-time body temperature. Rats were treated with LPS and NOS inhibitors for three consecutive days. On the fourth day, all rats were injected with LPS alone. Rats repeatedly injected with LPS became tolerant to pyrogenic effect of LPS as early as on the second day of the experiment. The treatment with iNOS or nNOS inhibitors completely suppressed fever due to the first, second and third LPS injection. When rats, which received the third i.c.v. injections of vL-NIO along with i.p. injections of LPS, were then treated the fourth time with LPS alone, they responded with virtually identical changes in body temperature to that of the group of rats that were injected with water i.c.v. and LPS i.p. for three consecutive days. This data indicate that both group of rats became tolerant to pyrogenic effect of LPS. It is, therefore, reasonable to hypothesize that activation of nNOS and iNOS inside the brain is not important for the development of endotoxin tolerance.

**Key words:** lipopolysaccharide, NG-nitro-L-arginine methyl ester, tolerance, fever, neuronal nitric oxide synthase, inducible nitric oxide synthase, aminoguanidine, N-[(1-imino-3-butenyl)-ornithine, biotelemetry, rat
contribute to the development of febrile hyporesponsiveness to repeated injections of LPS (8, 9).

In the last decade researchers have found that nitric oxide (NO) is a mediator/modulator of key importance for many systems, including cardiovascular system, endocrine system and host defense system. Considerable interest has been aroused by the observations (in vivo and in vitro experiments) that endotoxin and proinflammatory cytokines given peripherally were able to induce NO formation in peripheral tissues (10) as well as in the brain (11). In addition, it has been suggested that overproduction of NO in response to high dose of LPS seems to be responsible for some deleterious effects of LPS during septic shock, and therefore, the pharmacological inhibition of NO synthase (NOS) as well as NO-cyclic guanosine monophosphate (NO-cGMP) pathway has been proposed as an adjunct to the septic shock therapy (12, 13).

NO seems to be an important mediator of LPS fever in rats and mice. For example, NO inhibitors injected intraperitoneally (i.p.) or intracerebroventricularly (i.c.v.) were able to suppress LPS-induced fever in rats and mice (14-16). Moreover, inducible NOS (iNOS) and neuronal NOS (nNOS) knockout mice (KO) responded with lower fever due to LPS than wild-type mice (17). It is not certain, however, whether NO affects fever due to interference with some peripheral steps involved in fever development or by its participation in thermoregulation. The participation of NO in thermoregulation is based on results showing that NO has a vasodilator properties as well as is able to modulate nonshivering thermogenesis (18).

Because NO is involved in pathogenesis of fever, it is reasonable to consider that this molecule may participate in the progressive attenuation of febrile response during the development of endotoxin tolerance. Indeed, it has been shown that the inhibition of nitric oxide synthase by intraperitoneal injection of L-NAME (NG-nitro-L-arginine methyl ester) delays the tolerance formation due to LPS in rats (19). Moreover, it has been demonstrated that iNOS-KO mice repeatedly injected with LPS did not become tolerant to the pyrogenic effect of LPS (16).

It is important to note that results from experiments with NOS knockout mice have a limited application in understanding the central role of NO in development of endotoxin tolerance, from at least two reasons: firstly - mice are depleted in specific gene in cells on both sides of the blood-brain barrier, and secondly - mutant mice bred for many generations may develop compensatory mechanism for the lack of certain molecule (20).

Accepting that NO is an important molecular mediator for the induction and maintenance of endotoxin tolerance, the question arises of where formation of nitric oxide (inside or outside blood-brain barrier) is crucial for the development of tolerance due to repeated injection of LPS. Since L-NAME is capable of crossing the blood-brain barrier (21), the results of studies with peripherally administered L-NAME do not allow us to determine the site of the action of (i.e. inside or outside the central nervous system) L-NAME on the significant reduction of the febrile response to the repeated administration of LPS. Therefore, we cannot rule out the possibility that some changes inside the nervous system due to L-NAME injected peripherally may participate in delaying of tolerance formation due to LPS injections. Subsequently, the findings that glucocorticoids are required for the induction of tolerance and that blockade of NO formation by L-NAME significantly attenuates the ACTH response to LPS (22) seems to strengthen the concept that NO plays an important role in regulating centrally mediated mechanisms responsible for the development of tolerance.

Thus the purpose of the present study was to assess the effect of two highly selective NOS inhibitors, aminoguanidine hydrochloride (iNOS inhibitor) and vinyl-L-NIO (N-5-(1-imino-3-butenyl)-ornithine; nNOS inhibitor) injected intracerebroventricularly on the development of tolerance to repeated LPS injections in rats.

MATERIALS AND METHODS

Animals and experimental conditions

Specific pathogen-free male Wistar rats weighing 300–320 g were used throughout the study. Before surgery, rats were housed in groups of four per cage and after surgery in individual plastic cages at ambient temperature of 21±1°C with 12:12-h light-dark cycle with lights onset at 6:00 a.m. Rodent diet and water were provided ad libitum. Rats were used only once for each experiment. These experiments have been approved by our University Committee on the Use and Care of Laboratory Animals.

Surgery

All surgeries were made at least 1 week before the start of experimental procedure. For body temperature (Tb) measurement, rats were implanted intraabdominally with a miniature battery-operated temperature-sensitive transmitter (model VM-FH; MiniMitter Co., Bend, OR, USA). Signals for body temperature were collected at 5 min intervals with a peripheral processor (VitalView3000; MiniMitter Co., Bend, OR, USA) connected to an IBM personal computer. For i.c.v. administration of nNOS and iNOS inhibitors or appropriate control, rats were stereotaxically implanted with 5 mm long 26-gauge steel cannulas (Plastic Products Co., Roanoke, VA, USA) into the lateral ventricle. The coordinates were 1 mm posterior to the bregma and 0.5 mm lateral to the midline (coordinates according to the atlas of Pellegrino et al. (23). Before surgeries rats were anesthetized with an intramuscular injection of ketamine hydrochloride (87 mg/kg body weight) and xylazine (13 mg/kg). At the end of experiments, each rat was sacrificed and injected with 5 µl of Evans Blue dye into the cannula. The brain tissue was removed and soaked in 10% formalin overnight. Cannula placement was confirmed by locating the Evans Blue dye in the fixed brain.

Substances and injections

Bacterial LPS (derived from Escherichia coli, 0111:B4, Sigma, St. Louis, MO, USA) was dissolved in pyrogen-free 0.9% saline at a concentration of 1 mg/ml and stored at −20°C as a stock solution. On the day of experiment, LPS was diluted to 100 µg/ml in saline, and injected intraperitoneally (i.p.) at a dose of 50 µg/kg. Pyrogen-free saline was used for control injections.

Vinyl-L-NIO (N5-(1-imino-3-butenyl)-ornithine; Alexis Biochemicals, USA), a neuronal nitric oxide synthase (nNOS) inhibitor, and aminoguanidine hydrochloride (Sigma, St. Louis, MO, USA), an inducible nitric oxide synthase (iNOS) inhibitor, were dissolved in pyrogen-free water. The rationale for using the pyrogen-free water instead of aCSF (artificial cerebro-spinal fluid) as a solvent was due to the fact that both inhibitors did not dissolve in aCSF as well as in pyrogen-free saline. Both inhibitors were prepared freshly on the day of experiment and injected at a dose of 10 µg/kg. Rats were restrained in a towel but not anesthetized during i.p. and i.c.v. injections. For i.c.v. injection, the dummy cannula was removed and the injection cannula inserted into the guide cannula. Injections were made using a Hamilton syringe. The final volume for each i.c.v. injection was 5 µl. After i.c.v. injection, the injection cannula was removed and the dummy cannula replaced. Control rats were injected i.c.v. with an equal volume of pyrogen-free water. To avoid any effect of the circadian variation on body temperature all injections were made between 8:00 and 9:00 a.m. (8:30 for L-NIO experiment and 9:00 for aminoguanidine experiment).
Experimental protocols

Experiment 1: effect of i.c.v. administration of neuronal nitric oxide synthase inhibitor on changes of $T_b$ during the development of tolerance to daily repeated injections of lipopolysaccharide

Because vL-NIO given i.c.v. at a dose of 10 µg/rat was able to prevent LPS-induced fever and did not change the normal $T_b$ in rats (15), this dose was used for i.c.v. injection of vL-NIO. Moreover, it is well established that LPS at an i.p. dose of 50 µg/kg reproduces characteristic febrile rise in $T_b$ in rats (15, 16). Four groups of rats were used to test the role of nNOS in the development of tolerance to LPS. Four groups of rats were injected for three consecutive days with:

1. Water i.c.v. along with 50 µg/kg LPS i.p. (WATER i.c.v. + LPS i.p.);
2. Water i.c.v. along with equivalent volume of saline i.p. (WATER i.c.v. + SALINE i.p.);
3. 10 µg/rat vL-NIO i.c.v. along with equivalent volume of saline i.p. (vL-NIO i.c.v. + SALINE i.p.);
4. 10 µg/rat vL-NIO i.c.v. along with 50 µg/kg LPS i.p. (vL-NIO i.c.v. + LPS i.p.).

On the fourth day of experiment rats from Groups 1 and 4 were injected i.p. with LPS, whereas rats from Groups 2 and 3 were i.p. treated with saline.

Experiment 2: effect of i.c.v. administration of inducible nitric oxide synthase inhibitor on changes in $T_b$ during the development of tolerance to daily repeated injections of lipopolysaccharide

Since i.c.v. administration of aminoguanidine at a dose of 10 g/rat completely prevented fever induced by LPS in rats (15), this dose was utilized to test the role of iNOS in development of tolerance to LPS. Four groups of rats were injected for three consecutive days with:

1. Water i.c.v. along with 50 µg/kg LPS i.p. (WATER i.c.v. + LPS i.p.);
2. Water i.c.v. along with equivalent volume of saline i.p. (WATER i.c.v. + SALINE i.p.);
3. 10 µg/rat aminoguanidine i.c.v. along with equivalent volume of saline i.p. (AMINOGUANIDINE i.c.v. + SALINE i.p.);
4. 10 µg/rat aminoguanidine i.c.v. along with 50 µg/kg LPS i.p. (AMINOGUANIDINE i.c.v. + LPS i.p.).

On the fourth day of experiment rats from Groups 1 and 4 were injected i.p. with LPS, whereas rats from Groups 2 and 3 were i.p. treated with saline.

The $T_b$ was recorded for 3 hours before the injection and 21 hours afterwards. Data collected for $T_b$ at 5-min intervals were divided into 15-min averages before statistical analysis and graphical presentation. To assess a difference in the magnitude of febrile response during the development of tolerance, changes of $T_b$ from 0 to 7 h after injection were averaged and 7-h fever index was calculated. Fever indexes, the integrated areas under the fever curves were expressed as a product of degrees Celsius and hour. The mean values of body temperature 1 h before the injections were designated as baseline temperatures. To examine temperature changes over several days, temperature data were averaged over 12-h intervals beginning at the onset of light.

Data analysis

All values in the figures and text are expressed as mean ±S.E.M. of $n$ observations, where $n$ represents the number of animals. ANOVA with repeated measures was used to determine differences among groups in patterns of temperature changes over time. ANOVA followed by Scheffe's pairwise comparisons was used to test for statistical differences among groups at individual time points. A $p$ value of $<0.05$ was considered to be significant.

RESULTS

Effect of i.c.v. administration of neuronal nitric oxide synthase inhibitor on changes of $T_b$ during development of the tolerance to daily repeated injections of lipopolysaccharide

Fig. 1 shows the mean abdominal temperature of four group of rats injected intraperitoneally with LPS at a dose of 50 g/kg along with intracerebroventricular administration of pyrogen-free water or vL-NIO (10 g/rat), and injected i.p. with saline along with i.c.v. administration of vL-NIO or pyrogen-free water. Rats disturbed by handling due to i.p. and i.c.v. injections responded with a sharp increase of $T_b$ that lasted about 60 min, which is regarded as stress-induced rise in body temperature (Fig. 1). This elevation in $T_b$ was observed in rats injected i.c.v. with pyrogen-free water along with i.p. administration of LPS or saline. In contrast to these groups of rats, animals injected i.c.v. with vL-NIO and then treated i.p. with LPS or saline did not respond with stress-induced rise in $T_b$ due to handling and injections. These observations may suggest that activity of iNOS in the brain is involved in mechanisms responsible for stress-induced rise in body temperature.

As can be seen in Fig. 1, i.c.v. water-pretreated rats injected i.p. with LPS developed a typical biphasic febrile response with temperature peaks occurring at ca. 120 and 225 min postinjection (38.61±0.31 and 38.77±0.29°C for the first and second peak of fever, respectively). In contrast to Day 1 of LPS treatment, the injection of LPS on Days 2 and 3 evoked only monophasic rise in $T_b$, indicating that rats developed pyrogenic tolerance to LPS (Fig. 1). A significant decrease of fever index was observed as early as on the second day of LPS treatment: 8.00±1.02°C × h after the first injection of LPS and 3.51±0.79°C × h after the second injection of LPS ($p<0.01$) (Fig. 2). Third injection of LPS further reduced the response of rats to endotoxin, however, there was no significant difference between LPS- or saline-injected rats in fever index (1.44±0.57 versus 0.91±0.38°C × h for LPS- or saline-treated rats, respectively) (Fig. 2). Compared with the first LPS-induced fever, the febrile response to the fourth injection of LPS was abrogated almost completely.

Injection of vL-NIO at an intracerebroventricular dose of 10 µg/rat significantly suppressed the LPS-induced fever. Rats pretreated with vL-NIO and LPS-injected evoked only monophasic rise in $T_b$ with temperature peak occurring at ca. 120 min postinjection (38.18±0.09°C - early phase of febrile response) (Fig. 1). This peak of fever was significantly lower compared with that of water-LPS group (38.61±0.31°C). As a result, fever index calculated for the period of 7 hours post-LPS was significantly lower ($p<0.001$, reduction by ~62%) in rats with vL-NIO (3.04±0.65°C × h) compared with that of water-LPS group (8.00±1.02°C × h) (Fig. 2). As can be seen on Fig. 1, on the second day of experiment, the vL-NIO-pretreated rats developed markedly lower monophasic fever than water-pretreated and LPS-injected rats. The temperature peak of febrile response for water-pretreated and LPS-injected rats was 38.38±0.18°C at 90 min postinjection, whereas for vL-NIO-pretreated and LPS-injected rats the mean $T_b$ at the same time point was 37.85±0.10°C ($p=0.001$). However, there was no significant difference in fever index between vL-NIO-pretreated LPS-injected rats (1.30±0.48°C × h) and water- or vL-NIO-pretreated saline-injected rats (1.27±0.50 and 0.55±0.65°C × h, respectively) (Fig. 2), suggesting that fever due to second
Fig. 1. Effect of vL-NIO, a selective inhibitor of nNOS, injected intracerebroventricularly at a dose of 10 µg/animal on time course of $T_b$ during development of tolerance to LPS from *E. coli* (50 µg/kg i.p.). On fourth day of experiment, rats from groups "WATER+LPS" and "vL-NIO+LPS" were injected with LPS alone. Rats were injected at the same time with LPS and nNOS inhibitor or with saline and pyrogen-free water as controls. Values are means ± S.E.M. Arrowhead indicates the time of injection. Sample sizes are indicated in parentheses.
injection of LPS was almost completely suppressed in rats pretreated i.c.v. with vL-NIO. No further reduction of febrile response due to third LPS injection was observed in vL-NIO-pretreated rats (1.30±0.48°C × h versus 1.30±0.47°C × h after second and third LPS injection, respectively) (Fig. 2). When rats, which received three i.c.v. injections of vL-NIO along with i.p. injections of LPS, were then treated the fourth time with LPS alone, they responded with virtually identical changes in $T_b$ to that of the group of rats that were given three injections of water i.c.v. and LPS i.p. (Fig. 1). There was no significant difference between this and the control group of rats in calculated fever index (0.12±0.64 versus 0.19±0.63°C × h for vL-NIO-pretreated and water-pretreated LPS-injected rats, respectively) (Fig. 2).

Circadian rhythmicity in thermoregulation over several days is shown in Fig. 3. Before injections, rats from all groups displayed the normal circadian pattern of $T_b$ changes, with lower daytime $T_b$ (12-h averages) and higher nighttime $T_b$. During the day of injection (Day 0), rats injected i.p. with LPS along with i.c.v. vL-NIO had significantly lower 12-h average $T_b$ than rats injected with LPS along with water (p<0.01), indicating that vL-NIO suppressed the rise in $T_b$ due to LPS injection during this time. There was however, no significant difference between both groups of rats in 12-h averages of $T_b$ calculated for the first post-injection night (Night 0). Twelve-hour average $T_b$ values for both LPS groups calculated for Day 1 (second injection of LPS) were 37.73±0.11 and 37.52±0.08°C, respectively for rats pretreated with water and vL-NIO (p<0.05). Starting with Night 1, $T_b$ recovered to normal circadian rhythm, indicating that the following injections did not further affect daytime and nighttime $T_b$. Moreover, rats treated i.c.v. with vL-NIO or pyrogen-free water and then injected i.p. with saline (vehicle for LPS) displayed normal circadian rhythm in $T_b$ postinjection, with lower daytime $T_b$ (12-h averages) and higher nighttime $T_b$. There was no significant difference in the mean daytime (Day 0) and nighttime (Night 0) $T_b$ between both groups of rats. The mean daytime temperature of vL-NIO-treated rats was 37.65±0.11°C (versus 37.56±0.15°C in water-injected rats) and nighttime was 38.15±0.21°C (versus 38.20±0.18°C in water injected rats). The following injections of vL-NIO at an i.c.v. dose of 10 µg/rat did not affect daytime and nighttime $T_b$. There

Fig. 2. Seven-hour fever index (mean ± S.E.M.) between 0 and 7 h after LPS injection (50 µg/kg i.p.) of rats treated i.c.v. with vL-NIO (10 µg/animal) or water as a control. On fourth day of experiment, rats from groups "WATER+LPS" and "vL-NIO+LPS" were injected with LPS alone. Sample sizes are indicated in parentheses. * p<0.001 first injection of WATER+LPS versus first injection of vL-NIO+LPS; ** p<0.01 first versus second injection of WATER+LPS; *** p<0.05 first versus second injection of vL-NIO+LPS.

Fig. 3. Body temperature over six consecutive days of rats housed in 12:12-h light-dark cycle. On Days 0, 1 and 2, rats were treated i.c.v. with vL-NIO (10 µg/animal) or water and then injected with LPS (50 µg/kg i.p.). On Day 3, rats from these groups were injected only with LPS. Mean ± S.E.M. of 12-h averages * p<0.01 first injection of WATER+LPS versus first injection of vL-NIO+LPS.
Fig. 4. Effect of aminoguanidine, a selective inhibitor of iNOS, injected intracerebroventricularly at a dose of 10 µg/animal on time course of T<sub>b</sub> during development of tolerance to LPS from <i>E. coli</i> (50 µg/kg i.p.). On fourth day of experiment, rats from groups "WATER+LPS" and "AMINOGUANIDINE+LPS" were injected with LPS alone. Rats were injected at the same time with LPS and iNOS inhibitor or with saline and pyrogen-free water as controls. Values are means ± S.E.M. Arrowhead indicates the time of injection. Sample sizes are indicated in parentheses.
was no significant difference between rats treated i.c.v. with vL-NIO or pyrogen-free water and then injected i.p. with saline in calculated daytime and nighttime T_b (Fig. 3).

Effect of i.c.v. administration of an inducible nitric oxide synthase inhibitor on changes of T_b during the development of tolerance to daily repeated injections of lipopolysaccharide

As can be seen from Fig. 4, the elevation in T_b due to handling and injection in i.c.v. aminoguanidine-pretreated rats was significantly lower compared with that of i.c.v. pyrogen-free-water-pretreated group of rats. This suppression in stress-induced rise in T_b was independent on i.p. injections (LPS or saline). Moreover, there was no significant difference in the mean daytime and nighttime between rats pretreated i.c.v. with aminoguanidine or water and i.p. injected with saline, indicating that aminoguanidine affected neither normal daytime T_b nor normal nighttime T_b (Fig. 6). The mean daytime temperature of aminoguanidine-injected rats was 37.62±0.12°C (versus 37.71±0.04°C in controls) and nighttime was 38.22±0.15°C (versus 38.29±0.18°C in controls).

When LPS-injected control rats (pretreated i.c.v. with water) developed a typical biphasic fever, the rats treated with aminoguanidine responded to LPS with significant lower fever (Fig. 4). The temperature peaks of febrile response for control rats were 38.74±0.23°C (for first peak, 165 min postinjection and 38.69±0.32°C (for second peak, 270 min postinjection), whereas for the aminoguanidine-pretreated and LPS-injected rats the mean T_b at the same time-points were 37.87±0.33°C and 37.84±0.19°C. As a result, fever index calculated for the period of 7.0 hours post-LPS was significantly lower (p<0.01) in rats pretreated with aminoguanidine (2.94±0.58°C × h) compared with that of water-LPS group (7.20±1.06°C × h) (Fig. 5). In contrast to Day 1 of LPS treatment, injection of LPS on Day 2 evoked only a monophasic rise in T_b, indicating that rats developed pyrogenic tolerance to LPS (Fig. 4). A significant
decrease of fever index was observed as early as on the second day of LPS treatment: 7.20±1.06°C × h after the first LPS injection and 3.10±0.75°C × h after the second LPS injection (p<0.01) (Fig. 5). Compared with the first and second LPS-induced fevers, the febrile responses to the third and fourth injection of LPS were abrogated completely.

Aminoguanidine pretreatment not only significantly suppressed febrile response due to LPS in rats on Day 0 but also markedly altered the pattern of changes in Tb, due to daily repeated injections of LPS. As can be seen on Fig. 4, on the second day of experiment, aminoguanidine-pretreated rats did not develop febrile response, whereas rats pretreated i.e.v. with water developed monophasic fever due to second injection of LPS. Compared to rats injected for the second time with LPS and water, the fever index calculated for 7-h postinjection was significantly lower in rats treated secondly with LPS along with aminoguanidine (0.44±1.27°C × h for aminoguanidine-pretreated rats versus 3.10±0.75°C × h for water-pretreated rats; p<0.001) (Fig. 5). No further significant reduction of fever index was seen after the third injection of LPS in aminoguanidine-pretreated rats. When rats injected three times with aminoguanidine and LPS had received LPS alone on the fourth day of experiment, they responded with changes of Tb similar to that group of rats that were given three injections of water and LPS (Fig. 4).

As can be seen from Fig. 6, during the day of injection (Day 0), rats injected with LPS along with aminoguanidine had significantly lower 12-h average of Tb than rats injected with LPS along with water (37.76±0.10°C versus 38.09±0.13°C; p<0.01), indicating that aminoguanidine suppressed rise in Tb due to the first LPS injection. Furthermore, there was significant difference between both groups of rats in calculated 12-h average of Tb on Day 1 (37.54±0.07 and 37.74±0.11°C, respectively), for rats pretreated with aminoguanidine and water; p<0.05). Starting with Night 1, there was no significant difference between both group of rats in calculated daytime and nighttime Tb (Fig. 6).

DISCUSSION

The results of the present study show that vinyl-L-NIO (Figs. 1 and 2) and aminoguanidine (Figs. 4 and 5), the specific inhibitors for nNOS and iNOS respectively, injected intracerebroventricularly at a dose of 10 μg/rat suppress the LPS-induced fever in rats. The same dose of both inhibitors, however, had no effect on normal body temperature. There were no significant differences between both phases of fever and normal body temperature of the rats. It seems to be more important in pathogenesis of fever than activation of nNOS. These results further support the hypothesis developed by us and others that NO formation in the brain is required for the febrile response to LPS in rats and therefore has pyretic properties (14, 15, 24, 25). Interestingly, it has been reported in guinea pigs that systemic administration of L-NAME, nonselective NOS inhibitor (capable of crossing blood-brain barrier), attenuates of LPS-fever without concomitant alterations of the circulating LPS-induced tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (26). Thus, it is tempting to speculate, that propyreic activity of nitric oxide inside the brain is independent of any influence on circulating proinflammatory cytokines. Relevant to this, are data showing the positive correlation between NO formation and cyclooxygenase-2 (COX-2) gene expression (27, 28). The enzymatic activation of COX-2 appears critically important for synthesis of PGE2, which is considered to be a proximal centrally acting mediator of fever (29, 30). Recently, it has been documented that LPS-induced fever is also PGD2-dependent phenomena (31).

The present results show that daily repeated intraperitoneal injections of LPS results in the development of tolerance to the thermal effect of LPS (Figs. 1-4). The pyrogenic tolerance to LPS was already seen after second injection of endotoxin and was accompanied by the disappearance of the second phase of febrile response to LPS in rats (29, 30). A significant decrease of fever index was observed as early as on the second day of LPS treatment. Febrile response to the third and fourth injection of LPS were abrogated almost completely. The results of this experiment confirm the data obtained previously, where alteration of an endotoxin fever to monophasic with disappearance on the second peak of fever, was also seen within 24 hours after first LPS injection (2, 32, 33).

Our results demonstrate for the first time that the activation of nNOS and iNOS inside the brain is not important for the development of tolerant state to repeated injections of LPS. As mentioned above, both, the highly selective nNOS inhibitor (Figs. 1 and 2) and iNOS inhibitor injected i.c.v. at the selected doses, significantly inhibit febrile rise in Tb due to the first LPS injection (Figs. 4 and 5). As can be seen on Fig. 1, on the second day of experiment, the vL-NIO-pretreated rats developed markedly lower monophasic fever that water-pretreated and LPS-injected rats. However, there was no significant difference in fever index between both groups of rats on the second and third day of experiment (Fig. 2). In case of experiment with iNOS inhibitor, febrile response due to the second and third injection of LPS was completely abrogated by i.c.v. injection of aminoguanidine (Figs. 4 and 5). When rats, which had treated three times with nNOS or iNOS inhibitor along with LPS, were then treated the fourth time with endotoxin alone, they all responded virtually identical to that of the group of rats that were given three injections of water and LPS (Figs. 1 and 4), indicating that rats repeatedly injected i.c.v. with nNOS or iNOS inhibitors along with LPS became tolerant to the pyrogenic effect of LPS. We cannot, however, rule out the possibility that nitric oxide is involved in some processes leading to the tolerance development. Our study on the influence of inhibition of NOS activity in the brain provides further insight into the possible role of NO in development of endotoxin tolerance. As mentioned in the Introduction, the intraperitoneal injection of L-NAME (a nonselective NOS inhibitor) delayed the development of tolerance to LPS in rats (17), and iNOS-KO mice did not become tolerant to the repeated injection of LPS (16). Moreover, these observations along with results presented in this paper, leading us to believe that formation of NO in peripheral tissues may account for the stepwise attenuation of febrile response during the development of tolerant state to repeated LPS administration.

It is widely accepted that development of endotoxin tolerance is related to the down-regulation of proinflammatory cytokines network (32, 34). Although the role of particular proinflammatory cytokines in pathophysiology of tolerance formation remain incompletely understood, studies suggest a prominent role of tumor necrosis factor-α (TNF-α) (7, 32, 35, 36).

It has been demonstrated that endotoxin tolerance is characterized by reduced nuclear factor B (NF-kB), which is...
one of the transcription factors activated by LPS and responsible for the transcription of many cytokine genes (37). It has been suggested that the decrease TNF-α mRNA transcription is mediated via a postreceptor mechanism involving NF-kB during the development of endotoxin tolerance (38). However, the causal relationship between nitric oxide and the suppression of TNF-α synthesis during LPS tolerance formation is not fully recognized. The NF-kB transcription factor family may mediate expression of the cytokines encoding genes as well as inducible form of nitric oxide synthase (iNOS) (39). It has been shown that NO produced by newy expressed iNOS might then in turn affects activity of NF-kB (40). Indeed, NO-generating compounds such as sodium nitroprusside (SNP) and S-nitrosyl-N-acetylpenicillamine (SNAP) were able to inhibit the DNA binding activity of NF-kB and decrease expression of cytokine encoding genes, including TNF-α encoding gene (40). Moreover, it has been reported that NO down-regulates LPS-induced synthesis of TNF-α in the murine macrophage line RAW 264.7 (41). However, the role of NO in modulating the activation of NF-kB remains confused. Others, studying models of LPS mediated signaling, documented that the observed inhibition of NF-kB in LPS tolerant cells comes rather from the elevation of the level of inhibitory protein IκB than from the overproduction of nitric oxide (42, 43).

In summary, the presented data along with previous results demonstrated by us and others (16, 19) provide evidence that NO formation in peripheral tissues rather than inside the brain is involved in the development of tolerance to the repeated injections of endotoxin. Additional studies are needed to explore the precise role of NO in mechanisms responsible for development of endotoxin tolerance. Recently, it has been reported that N-acetylcysteine possesses antipyretic activity, probably due to interaction with NF-kB and NOS activity (44). These data indirectly show that N-acetylcysteine may be a potentially useful tool in studies on pathogenesis of endotoxin tolerance.

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REFERENCES


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