FEVER IS A REGULATED INCREASE of body temperature triggered by infectious or inflammatory agents referred to as exogenous pyrogens and is mediated by a number of endogenous factors (i.e., endogenous pyrogens). Cytokines such as interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor-α (TNF-α), among other cytokines stimulated by exogenous stressors, are regarded as the most important endogenous mediators and regulators of fever. Endogenous pyrogens subsequently induce a cyclooxygenase-2 (COX-2)-dependent metabolism of arachidonic acid, leading to the synthesis of prostaglandin (PG). Because inhibitors of COX-2 block fever triggered by injections of exogenous and endogenous pyrogens into laboratory animals, PG, PGE₂ in particular, is thought to act as a proximal mediator of fever (for review, see Refs. 8, 12, 25, 27).

Exogenous pyrogens and proinflammatory, profebrile cytokines are also known to stimulate the generation of nitric oxide (NO), a diffusible gaseous messenger molecule synthesized from L-arginine (15, 20, 57). NO has been shown to participate in a large variety of homeostatic control mechanisms (35). Numerous studies have also indicated the involvement of NO in temperature regulation (16), which, together with the ability of cytokines to stimulate NO synthesis, implies a role for NO in fever. However, results of studies on the role of NO in fever are conflicting, as both pyretic and antipyretic functions of NO have been suggested. For example, in a substantial number of studies, inhibitors of NO synthesis have been shown to reduce fever in laboratory animals, indicating that NO is a mediator of fever (2, 4, 38, 46, 47, 51, 55). In contrast, there are also reports indicating that NO is not important in the development of fever or that it plays an antipyretic role, since administration of inhibitors of NO synthesis had either no effect on fever (41) or they augmented fever (1, 18, 43, 54, 55). These discrepancies may have resulted from, among others, differences in the relative affinity of the inhibitors to various nitric oxide synthase (NOS) isoforms as well as from a tissue-specific distribution of various NOS enzymes. Thus NOS occurs as three isoforms (19): neuronal NOS (nNOS; encoded by Nos1 gene), inducible NOS (iNOS; encoded by Nos2 gene), and endothelial NOS (eNOS; encoded by Nos3 gene). nNOS and eNOS isoforms are expressed constitutively. nNOS is encountered in spinal cord, brain, and peripheral blood vessels, whereas iNOS is inducible in response to inflammatory stimuli and is present in a variety of cell types, including macrophages, monocytes, and some epithelial cells.

Edited by W. Kozak, Wieslaw, and Anna Kozak.
brain, kidney, and sympathetic ganglia, whereas eNOS is largely found in endothelial cells and plays a substantial role in blood pressure control (19). An inducible isozyme (iNOS) becomes expressed in leukocytes and in parenchymal cells of liver, muscles, kidney, and brain in response to cytokines and exogenous pyrogens (15, 57). Therefore, one can hypothesize that among the three known NOS isomers, iNOS may principally be involved in fever.

The synthesis of NO can be inhibited experimentally by analogs of arginine, including nitro-L-arginine-methyl ester (L-NAME), \( N^G \)-monomethyl-L-arginine (L-NMMA), and \( N^G, N^C \)-dimethyl arginine (ADMA), which inhibit all three NOS isoforms (35). Methylated arginines such as ADMA and L-NMMA are naturally occurring NOS inhibitors (35); however, the majority of studies on the role of NO in fever have been performed with L-NAME. In rats, pigs, and guinea pigs, L-NAME reduced fevers in response to IL-1β (42, 47) and lipo-polysaccharide (LPS; a standard laboratory pyrogen derived from gram-negative bacteria) (38, 48, 51, 55). L-NAME, however, induced hypothermia when administered at higher doses (46, 51). Administration of S-methylisothiourea, which are relatively selective inhibitors of the iNOS, resulted in suppression of the early phase of fever in guinea pigs challenged with LPS (46). However, both agents exhibited these inhibitory effects only when given at relatively high doses (46). In other studies, aminoguanidine failed to reduce fever in rats after injections of IL-1β, LPS, and muramyl dipeptide (a pyrogen derived from gram-positive bacteria) (24, 42). In guinea pigs challenged with muramyl dipeptide, on the other hand, fever was reduced by administration of aminoguanidine (23). Results of these studies suggest that iNOS may account for a part of fever, but its involvement may depend on the type of pyrogen and animal species. Moreover, these data also imply a role for the constitutive NOSs in generation of fever. In support, a systemic injection of 7-nitroindazole, a selective inhibitor of nNOS, reduced the LPS-provoked fever in rats (39). However, 7-nitroindazole impaired fever when administered at a dose that alone produced a drop in body temperature in control rats (39). On the basis of these pharmacological studies, due to a significant impact on normal body temperature of the fever-preventing doses of specific NOS inhibitors, it is difficult to ascertain which of these NOS isoforms is indeed involved in fever. To our knowledge, there have been no studies to address the functions of constitutive eNOS in fever.

To further examine the role of the three specific NOS isomers in the febrile response, we have used genetically engineered mice deficient in the respective Nos gene [Nos1, Nos2, and Nos3 knockout (KO) mice: nNOS KO, iNOS KO, and eNOS KO, respectively]. Mice have been extensively used in studies on the role of NO in various aspects of physiological and pathological regulation. There are no data, however, focused on the involvement of NO in fever in this laboratory species. The use of genetically engineered mice deficient in genes for the particular NOS may circumvent the problems inherent in the injection of pharmacological agents mentioned above. However, this experimental paradigm may also have limitations because KO mice may generate a functional redundancy for the deficiency in specific genes, particularly those encoding signaling molecules functioning within a wide array of physiological and pathological processes. Therefore, in addition to the studies with mice that used a single Nos gene deletion, we have also applied a pharmacological approach using L-NMMA to inhibit all three NOS isoforms in wild-type and KO mice. Intravenous injection of LPS was used to trigger systemic inflammation, whereas subcutaneous administration of turpentine oil was used to induce localized inflammation (sterile tissue abscess). We found that L-NMMA differentially influenced fever, depending on the pyrogen used; L-NMMA reduced the LPS-induced fever, whereas it augmented fever in mice challenged with turpentine oil. We demonstrate that lack of iNOS and nNOS resulted in partial reduction of fever in mice challenged with LPS and had no effect on fever induced by injection of turpentine. Lack of eNOS, on the other hand, led to exaggeration of fever to turpentine oil and had no effect on the febrile response to LPS. We conclude that iNOS and nNOS are involved in generation of fever in mice injected with LPS, whereas none of the NOs contributed in triggering fever after the injection of turpentine. In contrast, eNOS appears to contribute to down-regulation of fever induced by turpentine.

**METHODS**

Genetically engineered mice. The protocol for this study was approved by the Animal Care and Use Committee of the Medical College of Georgia. Experiments were performed on ~10- to 11-wk-old male mice with the C57BL/6J genetic background. All mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Homozygous Nos1, Nos2, and Nos3 gene KO mice were from stock number 002986, 002609, and 002684, respectively. Age-matched control male mice (wild type for all KO mice) were from colony C57BL/6J 000664. At arrival, the mice were 8 wk old. Mice were kept in a specific pathogen-free facility and housed in individual plastic cages. All mice were maintained in a temperature-humidity-light-controlled chamber set at 30 ± 1°C, 12:12-h light-dark cycle, with light on at 0600. Rodent laboratory chow (Teklad rodent diet, W8604) and drinking water were provided ad libitum.

Body temperature measurement. We measured deep body temperature (Tb) of the mice with an accuracy of ±0.1°C using battery-operated miniature biotelemetry (model VMFH MiniMitter, Sunriver, OR). One week after their arrival, at 9 wk of age, the mice were anesthetized (isoflurane; Abbott Laboratories) and implanted intra-abdominally with telemetry devices (animals were not treated with antibiotics postsurgery; for details, see Ref. 26). Experiments were started after 7 days of postsurgery recovery. Injections and treatments were preceded by a 3-day monitoring of the regular rhythm of Tb in undisturbed freely moving mice. We made recordings at 5-min intervals using a peripheral processor (Dataquest III System) connected to an IBM personal computer.

LPS-induced systemic inflammation. LPS derived from *Escherichia coli* (0111:B4, Sigma Chemical, St. Louis, MO)
was dissolved in sterile 0.9% sodium chloride (saline) at a stock concentration of 2 mg/ml and kept frozen (−20°C). Before use and dilution to desired concentration, a portion of the stock was preheated to 37°C, vortexed, and briefly sonicated. LPS was injected intravenously into the tail vein at a dose of 50 µg/kg in an injection volume of 0.05 ml/mouse. Pyrogen-free saline was used for control injections. Mice were restrained and not anesthetized during the LPS and/or saline intravenous injections. After injections, the mice were placed in their home cages to monitor changes in Tb.

Turpentine sterile abscess. Sterile tissue damage (local inflammation) was induced with commercial-grade steam-distilled turpentine (Sunneyside, Wheeling, IL). Pure nondiluted turpentine oil (100%) was injected subcutaneously into the left hindlimb of a lightly anesthetized (inhaled isoflurane) mouse. Injections (30 µl/mouse) were made with a 100-µl Hamilton syringe connected to PE50 tubing filled with turpentine and equipped with a 30-gauge needle. Pyrogen-free saline was used for control injections. Injected mice were returned to their home cages and not further disturbed.

Treatment of mice with L-NMMA and D-NMMA. To test the effect of a nonspecific NOS inhibitor on fever, groups of mice (wild-type and NOS-deficient) were treated once per day intragastrically (gavage) with 80 mg/kg of Nω-monomethyl-l-arginine acetate salt (L-NMMA; M7033, Sigma-Aldrich, St. Louis, MO) and/or Nω-monomethyl-d-arginine acetate salt (D-NMMA; M7034, Sigma-Aldrich) for 3 consecutive days before injection of pyrogens (last administration at 24 h before the pyrogen injection). Both agents were suspended in corn oil (10 mg/ml; C8267, Sigma-Aldrich), warmed to 37°C, and briefly sonicated before administration. Mice were lightly sedated (inhaled isoflurane) during intragastric administration. Suspension was administered by oral gavage with 20-gauge barrel-tip feeding needle (Fine Science Tools, Belmont, CA) in a volume of 0.2 ml/mouse. Data from L-NMMA-treated mice were compared with mice treated with corn oil alone or corn oil supplemented with 80 mg/kg of D-NMMA (D-NMMA is not an inhibitor of NOS and is used as a negative control for the activity of L-NMMA). In preliminary studies, we determined that L-NMMA and D-NMMA, at doses ranging from 60 to 120 mg·kg⁻¹·day⁻¹ given by oral gavage, did not affect a daytime and nighttime variation of Tb and motor activity in wild-type and KO mice. In contrast, a bolus intraperitoneal injection of a water solution of L-NMMA at a dose of 50 mg/kg and higher (doses of L-NMMA usually applied for the acute inhibition of NO synthesis in mice and rats) elicited a significant drop of Tb in mice. The hypothermia-like effect was dose dependent and lasted ~110 min in mice injected with 50 mg/kg L-NMMA. Intragastric administration of L-NMMA for 3 consecutive days at a dose of 80 mg·kg⁻¹·day⁻¹ has been shown to inhibit the NO-dependent choroidal neovascularization in mice (3). Therefore, we have applied this regimen to our study and empirically determined that this dose of L-NMMA was sufficient in influencing fevers triggered by LPS or turpentine in C57BL/6J mice.

Data analysis. Values are reported as means ± SE. Five-minute temperature recordings were collapsed into 0.5-h and 1-h averages for presentation. Fever index (FI; expressed as °C × h) for each animal was computed for statistical analyses of the results. In experiments with LPS, the FI was calculated for 9.5 h as mean hourly Tb of 5 h (5-min change in Tb, from the baseline, converted into average AT/h) for the period of 0830 (the time of LPS and/or saline injection) to 1800 post-LPS (the time of lights off) multiplied by 9.5 h (average Tb, between 0800 and 0830 was taken as reference for the calculation of change in Tb). Similar analysis was performed in experiments with turpentine; however, FI was computed for 33 h between 0900 (the time of turpentine injection) and 1800 the next day, with “zero” reference temperature monitored for 30 min before turpentine injection. Data were analyzed with the use of Statview SE+ Graphics (Abacus Concepts, Berkeley, CA). ANOVA with repeated measures was used to determine differences among groups in posttreatment temperature changes. Occasionally, ANOVA followed by Scheffe’s pairwise comparisons was used to test for statistical differences among groups at individual time points. Differences were considered significant at P < 0.05.

RESULTS

L-NMMA inhibits fever in normal (wild-type) mice challenged with LPS and augments fever in mice during turpentine abscess. Three separate groups of C57BL/6J mice (16 mice in each group) were treated for 3 days with L-NMMA, D-NMMA, and corn oil. One day after the third gavage, the mice were challenged with LPS or saline. Figure 1 shows results of this experiment. For the clarity of presentation, the changes in Tb of mice treated with corn oil (vehicle control group) are not shown. They were not different, however, from that shown for mice treated with D-NMMA (control for L-NMMA) and injected with LPS and/or saline. Mice treated with L-NMMA, D-NMMA, and corn oil and then injected with sterile saline (vehicle for LPS) displayed normal circadian rhythm in Tb postinjection: temperature low during daytime and high during nighttime. This indicates that intragastric L-NMMA at a dose of 80 mg·kg⁻¹·day⁻¹ does not affect normal Tb in mice. Regardless of the treatment, however, all mice responded with a sharp increase in Tb at the time of handling and injections (Fig. 1), followed by a decrease in Tb to a normal daytime level in mice injected with control vehicle.
Intravenous injection of LPS into mice treated with d-NMMA-induced fever, which started within 90 min from the injection, lasted the entire postinjection daytime period (Fig. 1). Two peaks of fever can be distinguished in these mice: the first peak at 3 h and the second at 5 h post-LPS. d-NMMA reduced fever by ~60% (calculated FIs for d-NMMA-LPS and L-NMMA-LPS groups were 12.3 ± 0.4 and 5.4 ± 0.3, respectively). As can be seen, mice treated with t-NMMA also displayed two peaks of T_b elevation after injection of LPS. They were, however, significantly lower and phase shifted compared with that of the d-NMMA-LPS group. The first peak started within ~3 h post-LPS and occurred at the time of T_b subsidence between the two peaks seen in the d-NMMA-LPS group, whereas the second peak in the L-NMMA-LPS group corresponded to the descending part of fever in mice treated with d-NMMA-LPS. Interestingly, T_b during post-LPS nighttime (especially during the second half of the night) of d-NMMA-treated mice was lower than that of L-NMMA-treated and control mice (those injected with saline) (Fig. 1).

In contrast to the inhibitory effect on LPS-induced fever, L-NMMA significantly augmented fever in mice injected with turpentine (Fig. 2), an exogenous pyrogen that provokes localized tissue abscess rather than systemic inflammation. Changes in T_b of mice treated with corn oil and injected with turpentine are not shown in Fig. 2 for the clarity of presentation. These changes, however, were essentially the same as those of mice treated with d-NMMA and injected with turpentine (note that mice were lightly anesthetized for injections; therefore, in contrast to those presented in Fig. 1, they did not display a stress-induced transient elevation of T_b at the time of handling and injection). FI computed for 33 h postinjection of mice treated with d-NMMA increased from 10.8 ± 2.1 (for mice injected with saline) to 32.3 ± 2.6 for those injected with turpentine (n = 8 in each group: ANOVA post hoc comparison between groups showed significant difference in FI at P < 0.05 between saline and turpentine injections). Mice treated with L-NMMA before administration of turpentine showed a further increase of FI to 54.1 ± 4.9 (n = 8; P < 0.01 between d-NMMA-turpentine and L-NMMA-turpentine groups), i.e., increasing by ~65%.

To investigate what NOS isozymes are responsible for these effects of L-NMMA, i.e., reduction of fever to LPS and augmentation of fever to turpentine, we used respective NOS KO mice for further study.

iNOS deficiency results in reduction of fever induced by LPS but not fever induced by turpentine. Stress-induced (handling and injection of LPS or saline) elevation of T_b was similar in all animals regardless of iNOS deficiency. Mice deficient in iNOS (iNOS KO mice) responded with partially reduced fever to LPS (Fig. 3). The computed FI for iNOS KO mice injected with LPS was 8.1 ± 0.3, whereas for wild-type mice injected with LPS it was 12.9 ± 0.4 (n = 8 per group; P < 0.05 between the two groups treated with LPS) (inhibition by ~35%). However, the time course of fever (as shown in Fig. 3) reveals that the response to LPS was initiated but not sustained in the iNOS KO mice. These data suggest that inhibition of iNOS was, at least in part, responsible for the later phase reduction of fever in mice treated with L-NMMA shown in Fig. 1. In addition, it can be seen from Fig. 3 that iNOS KO mice injected with LPS, similar to the L-NMMA-LPS-treated mice shown in Fig. 1, maintained elevated nighttime T_b similar to all saline (control)-injected groups. In contrast, wild-type mice injected with LPS revealed lower nighttime T_b (Fig. 3), similar to that of the d-NMMA-LPS group shown in Fig. 1.

In contrast to the response to LPS, lack of iNOS in mice did not affect fever induced by turpentine. Figure
4 demonstrates that fevers in wild-type and iNOS KO mice injected with turpentine were essentially the same, indicating that iNOS cannot be responsible for the exaggeration of fever to turpentine seen in the experiment with a nonspecific inhibitor of NOS (shown in Fig. 2). These data suggest that inhibition of constitutive NOSs may account for the augmentation of fever to turpentine in mice pretreated with L-NMMA.

Constitutive nNOS deficiency results in partial reduction of fever in mice injected with LPS. Studies on the effect of nNOS and eNOS deficiency on LPS-induced fever in mice were conducted simultaneously, using the respective NOS KO mice and one wild-type control group (Fig. 5). There was no difference in normal circadian rhythm in $T_b$ among eNOS and nNOS KO mice and wild-type mice. There was also no difference in their response to injection of saline (vehicle for LPS). Saline-injected nNOS and eNOS KO mice displayed a stress-induced increase in $T_b$ and then a return to normal temperature identical to that of wild-type mice injected with saline (data not shown). Therefore, only data from wild-type mice injected with saline are presented in Fig. 5.

As can be seen from Fig. 5, deficiency in constitutive eNOS in mice did not influence fever induced by injection of LPS. In contrast, lack of constitutive nNOS resulted in reduced fever, similar to the reduction of fever in iNOS KO mice challenged with LPS. The calculated FI for wild-type mice injected with LPS in this experiment was $13.1 \pm 0.4$ ($n = 8$), whereas for nNOS KO mice it was $8.0 \pm 0.2$ ($n = 6; P < 0.05$). It can be noticed, however, that, in contrast to the late phase of fever depleted in mice deficient in iNOS (shown in Fig. 3), lack of nNOS affected predominantly the early phase of post-LPS $T_b$ elevation. These data suggest that inhibition of nNOS in concert with the inhibition of iNOS accounts for the reduction of fever to LPS following treatment of mice with L-NMMA, a nonspecific NOS inhibitor (shown in Fig. 1). Furthermore, on the basis of these data, we conclude that eNOS does not participate in generation of fever to LPS in mice.

Interestingly, both eNOS KO and nNOS KO mice injected with LPS exhibited lower nighttime $T_b$ than that of saline (control)-treated mice. Comparing these data (Fig. 5) with those presented in Fig. 3, we hypothesize that the delayed suppressive effect of LPS on nighttime $T_b$ of mice is mediated predominantly by an iNOS.

Lack of eNOS in mice results in exaggeration of fever induced by turpentine. Figure 6 demonstrates changes in $T_b$ of wild-type and nNOS KO mice injected with turpentine. As can be seen, fever induced by turpentine in mice deficient in nNOS was not different from that of wild-type mice. In contrast, when eNOS KO mice were injected subcutaneously with the same dose of LPS KO / LPS (n=6) nNOS KO / LPS (n=6) Wild-type / LPS (n=8) Wild-type / saline (n=8)

Fig. 5. Changes in body temperature of mice deficient in constitutive endothelial NOS (eNOS KO) and neuronal NOS (nNOS KO) and control wild-type mice to intravenous injection of LPS (50 $\mu$g/kg; arrowhead at 0830). Values are means ± SE at 30-min averages; $n$, number of mice in each group. Black horizontal bar indicates dark period in 12:12-h light-dark cycle.

Fig. 6. Fever in mice deficient in constitutive nNOS (nNOS KO) and wild-type control in response to subcutaneous injection of turpentine (30 $\mu$l/mouse; arrowhead at 0930). Values are means ± SE at 60-min averages; $n$, number of mice in each group. Black horizontal bars indicate dark period in 12:12-h light-dark cycle.
Effect of L-NMMA on fevers induced by LPS and turpentine in Nos gene-KO mice. To elaborate and confirm conclusions from experiments with NOS KO mice injected with LPS or turpentine, we performed an additional set of experiments in which separate groups of mice deficient in the respective NOS (5 mice per group per experiment) and wild-type mice (8 per experiment) were treated with L-NMMA and D-NMMA (gavage) and then injected with LPS (experiment 1) or turpentine (experiment 2). Results of these experiments are presented in Figs. 8 and 9, respectively. Injection of saline into L-NMMA- and/or D-NMMA-treated animals was used as a control for each experiment.

When treated with D-NMMA, wild-type mice and mice deficient in eNOS responded with similar fevers to LPS (FI of 12.6 ± 1.1 and 13.4 ± 0.9, respectively), whereas iNOS and nNOS KO mice treated with D-NMMA responded to LPS with significantly lower fever (FI of 8.1 ± 0.4 and 7.9 ± 0.7, respectively). Treatment with L-NMMA reduced LPS-induced fever in all groups of mice. The fevers were significantly lower, however, in nNOS KO (FI = 3.4 ± 0.2) and iNOS KO (FI = 3.6 ± 0.1) mice than in wild-type (FI = 5.9 ± 0.2) and eNOS KO mice (FI = 6.1 ± 0.2).

Injection of turpentine into D-NMMA-treated mice induced fever, which was significantly higher in eNOS KO (FI = 61.1 ± 7.3) mice than in wild-type (33.5 ± 5.3), iNOS KO (FI = 29.8 ± 6.1), and nNOS KO (FI = 32.4 ± 6.3) mice (Fig. 9). These values indicate that mice deficient in iNOS and nNOS and treated with D-NMMA responded with similar fever to turpentine as wild-type mice treated with D-NMMA. Pretreatment with L-NMMA did not affect the already high fever in eNOS KO mice (FI = 53.4 ± 8.2). However, it significantly augmented fever induced by turpentine in wild-type, iNOS KO, and nNOS KO mice.

In general, experiments with the use of a nonspecific inhibitor of NOS in iNOS, nNOS, and eNOS KO mice supported the conclusions from the experiments, in which fever was induced in mice deficient in the respective NOS. Pretreatment with L-NMMA resulted in reduction of LPS-induced fever in all three gene KO mice studied (Fig. 8). Inhibition, however, in nNOS and iNOS KO mice was greater than in eNOS KO and wild-type mice, confirming the conclusion that both nNOS and iNOS participate in the generation of fever in mice challenged with LPS. L-NMMA potentiated fevers in iNOS- and nNOS-deficient mice injected with turpentine (Fig. 9). It did not, however, exacerbate a turpentine-induced fever in eNOS KO mice, confirming the conclusion from the experiments shown in Figs. 2 and 7 that inhibition of eNOS accounts for higher fever induced by turpentine in mice.

DISCUSSION

The main result of the present study is that fever in mice can be regulated by NO, similarly to that reported...
injection with turpentine was not different regardless of D-NMMA or turpentine, except for the group designated as eNOS KO, in which FI after Fig. 1. Significant difference of FI of the group designated as eNOS KO d-NMMA/turpentine from that of all other groups treated with d-NMMA and injected with turpentine (P < 0.05). #Significant difference in FI of nNOS KO, iNOS KO, and wild-type groups treated with l-NMMA/turpentine from that treated with d-NMMA/turpentine, except for the group designated as eNOS KO, in which FI after injection with turpentine was not different regardless of d-NMMA or l-NMMA treatment (P < 0.05) (see text for details).

Fig. 9. FI of mice deficient in respective NOS (nNOS KO, eNOS KO, and iNOS KO) and wild-type mice injected subcutaneously with turpentine (30 μl/mouse) or saline. Before injections, the mice were treated with l-NMMA and d-NMMA as described in METHODS and in other species. We demonstrate that involvement of NO in fever in mice is complex and depends on the pyrogenic factor used to trigger the febrile response and NOSs. The mouse has not been a traditional laboratory species used to study the mechanisms of fever. For many decades, mice were thought to be not capable of generating fever due to a low body mass-to-body surface ratio. Introduction of a biotelemetry technique that is able to monitor changes of Tb in freely moving, unstressed animals revealed that mice generate fever similarly to other laboratory species (see discussion in Ref. 26). It is worth noticing, however, that laboratory animals with a larger body mass, such as rats, guinea pigs, and rabbits, usually respond with biphasic fever to the injection of LPS, the most common used exogenous pyrogen (44). In our present report, we observed a biphasic-like fever in C57BL/6J mice in the experiment shown in Fig. 1. Although the experimental environment did not change during our study, the biphasic characteristics of LPS-induced fever in mice was not consistent between experiments, indicating that the mouse model may have some limitations in investigating particular aspects of fever, e.g., the dynamics of the febrile response to LPS. One of the possible explanations is that conscious mice always respond with a significant increase in Tb during stress due to handling and injection. This profound stressful response may mask the immediate early stages of the experimental fever in this species. It is also noteworthy that lightly anesthetized mice for the turpentine injection also did not display a biphasic fever to this inflammatory challenge. Nevertheless, genetically engineered mice have already proven to be a valuable model in demonstrating the role of cytokines (27), cyclooxygenases (30), PG receptors (59), and complement (31) in fever. Interpretation of the pathophysiological data obtained with gene KO mice, especially data that contradict results obtained via “traditional” pharmacological means, may also have limitations, since mutant mice bred for generations may develop compensatory mechanisms for the lack of a certain gene. Therefore, in the present report, focusing on a role of NO in fever in mice, we contrast data obtained by using specific Nos gene KO mice with data obtained by using a nonspecific NOS inhibitor. This approach allows us to compare and confirm pharmacological data reported earlier that used different animal species and conclude more accurately regarding the complex role of NO in fever in mice. As already mentioned in the introduction, antipyretic as well as pyretic functions of NO have been suggested. Several investigators (23, 24, 38, 48) have concluded that different results of research on the role of NO in fever may be due to different types of pyrogens used (e.g., originating from gram-negative and gram-positive bacteria or viruses), different routes of administration of different NO inhibitors, and the use of different animal species that normally use distinct thermoregulatory strategies to elevate Tb . Our data on mice indicate that the complexity of the NOs is an additional factor that needs to be taken into consideration.

It has been repeatedly demonstrated that inflammation is accompanied by an increase in synthesis of NO (57). Mediators and modulators of fever and inflammation, such as bacterial toxins and cytokines, are well-known inducers of iNOS in a large variety of immune and nonimmune cells (16, 20, 35, 57). However, different types of inflammation, e.g., systemic infectious bacterial-origin type or localized sterile abscess and tissue damage type, may result in activation of different NOS isoforms. In support, Geller et al. (15) compared the induction of iNOS in hepatocytes of rats injected with LPS to that of rats treated with turpentine and showed that iNOS expression was induced only by injection of LPS. In addition to the induction of iNOS, cytokines can also dynamically regulate expression of constitutive NOS isoforms. This latter effect, however, is complex, and it is becoming evident that mediators of inflammation and fever may regulate distinct NOS isoform expression in different ways depending on the cytokine combination associated with a particular type of inflammation, the dose and concentration of the inflammatory factor used in vivo and in vitro studies, the animal species, and the cell type analyzed (see Refs. 13 and 37 for review). For example, it has been shown that TNF-α downregulated eNOS mRNA, protein, and activity in human and bovine endothelial cells, whereas interferon-α/β (IFN-α/β) and LPS, also known to induce TNF-α, resulted in activa-
Fever in NOS Knockout Mice

2541

J Appl Physiol • VOL 94 • JUNE 2003 • www.jap.org

...tion of eNOS expression in these cells (13). Rats injected with LPS exhibited an upregulation of eNOS in the liver (10), downregulation of eNOS in aorta, heart, lung, and gastric mucosa (13), and upregulation of eNOS and nNOS in the hypothalamus (14). Administration of IL-1β increased (29), whereas IFN-γ decreased (6), the expression of nNOS in the rat hypothalamus. Interestingly, mice injected systemically with IL-12 exhibited enhanced eNOS immunoreactivity in astrocytes (7). Thus these diverse interactions between cytokines and various NOS isoforms may greatly affect the results of studies focused on the role of NO in fever.

NO can affect fever development either systemically or centrally (16, 28). Brain structures such as preoptic-anterior hypothalamus and organum vasculosum laminae terminalis (OVLT) of the third ventricle are extremely important in thermoregulation and fever (8, 12, 25). Initial in vitro work showed that both exogenous and endogenous pyrogens increased the expression of NO within cultured neuronal (9) and glial cells (45). Therefore, several in vivo studies were designated to assess changes in the expression of NOS isoforms in these brain structures during fever. It has been shown that, in guinea pigs challenged intravenously with a pyrogenic dose of LPS, there was no elevation in NOS activity in the OVLT within 5 min from the pyrogen administration (52). Analyses of the rat brain several hours after the pyrogen injection revealed rather moderate increases in iNOS expression in the hypothalamus and OVLT (34, 60). Recent studies by Gath et al. (14) showed that a pyrogenic dose of LPS triggered an upregulation of nNOS and eNOS in the rat brain, whereas expression of iNOS occurred only in response to high (septic) doses of LPS. Together, these data do not confirm an assumption that there might be a distinct correlation between fever and activation of NOS isoforms in the brain, particularly the correlation between brain iNOS and fever. However, injection of the minute amounts of nonspecific NOS inhibitors into the brain has been shown to affect fever provoked by central or peripheral injection of exogenous pyrogens and endogenous mediators of fever. In the majority of studies, intrahypothalamic or intracerebroventricular administration of NO inhibitors led to the augmentation of fever magnitude in the rat (1, 17, 36, 40, 56), suggesting that NO, within the central nervous system, is functioning as an antipyretic agent in this species. In contrast, in rabbits, intrav-OVLT administration of iNOS inhibitors prevented fever induced by LPS, IL-1β, and PGE2 (32) as well as staphylococcal enterotoxin A (21). In cats, however, intracerebroventricular administration of NO inhibitor did not influence fever induced by LPS and IL-1β (41), suggesting no role for NO in fever in this species. Thus the action of brain NO on fever may be site specific and species specific. Whether systemic administration of NO inhibitors affect fever via acting on the OVLT and preoptic-anterior hypothalamus is open to question. In contrast to the effect of central administration of the inhibitors, most of the studies with systemic injection of NO inhibitors into rats, pigs, and guinea pigs showed attenuated fever irrespective of whether the pyrogen used was yeast cell wall mannans, LPS, or IL-1β (4, 38, 42, 47, 51, 55), indicating a pyretic role of peripheral NO in these species. In rabbits, however, some studies suggested antipyretic functioning of the peripheral NO (18, 43), whereas others indicated a pyretic function, demonstrating that systemic NO inhibitors impaired heat generation (33). Our data revealed that oral administration of a nonspecific inhibitor of NO attenuated the LPS-induced fever in mice. Whether this effect of L-NMMA was central or peripheral cannot be assumed on the basis of our results. Also, our studies with NOS KO mice cannot add to this debate, since mice depleted in specific Nos genes do not express the gene on both sides of the blood-brain barrier. However, the availability of genetically engineered mice lacking the different NOSs allowed us to explore the role of three known Nos isoforms in fevers associated with two different inflammatory insults: LPS generating a systemic inflammation and turpentine inducing a localized sterile abscess.

The effect of L-NMMA on the LPS-induced fever in normal mice, i.e., resulting in a reduced fever exhibiting two phase-shifted peaks compared with the d-NMMA-LPS group (Fig. 1), may be interpreted in terms of the significance of various NOS isoforms in different phases of fever. Indeed, experiments with the use of KO mice supported this assumption and revealed that iNOS and constitutive nNOS share the capacity of upregulation of fever to LPS in mice. Data presented in Figs. 3 and 5 suggest, however, that iNOS may be involved in the later phase, whereas nNOS seems to participate mostly in the early phase of fever. Another constitutive form of NO, eNOS, does not seem to be involved in any phase of LPS-induced fever in mice.

As can be seen from Fig. 1, mice treated with d-NMMA and injected with LPS exhibited reduced Tb during the first night following the injection of pyrogen. The same behavior can be seen in wild-type mice injected with LPS (Figs. 3 and 5), as well as in nNOS KO and eNOS KO mice treated with LPS (Fig. 5). In contrast, mice treated with L-NMMA-LPS (Fig. 1) and iNOS KO-LPS (Fig. 3) revealed no nighttime reduction of Tb. Additional simultaneous monitoring of the motor activity in these groups of mice demonstrated a similar pattern, i.e., LPS provoked a significant nighttime lethargy in d-NMMA, eNOS KO, and nNOS KO mice compared with that of L-NMMA and iNOS KO (data not shown). These data indicate that iNOS, in addition to its role in maintaining the later part of fever, may also be responsible for the late sickness effects shown in LPS-treated mice.

Surprisingly, none of the NOSs contributes to generation of fever during localized tissue abscess in mice. Fever was evident regardless of the deficiency in Nos genes in mice challenged with turpentine oil. The most striking observation, however, was that, after injection of turpentine, fever was significantly higher in mice treated with L-NMMA, a nonspecific inhibitor of all
three NOS isoenzymes. Our results on mice are consistent with a recent report by Soszynski (54), who demonstrated that \( L-\text{NAME} \), another nonspecific inhibitor of NOS, augmented the turpentine-induced fever in rats. Our results also complement results reported by Turnbull and Rivier (58), who showed that \( L-\text{NAME} \) exacerbated the activation of the hypothalamic-pituitary-adrenal (HPA) axis in rats during acute local inflammation induced by injection of turpentine oil. Together, these data indicate that, during fever triggered by a turpentine-induced abscess, NO contributes to endogenous antipyresis, a physiological mechanism that counteracts the action of pyrogens (25). Our studies with gene KO mice indicate that, among the NOSs investigated, the constitutive eNOS is responsible for the regulatory action of NO on fever during localized abscess. Whether eNOS expression is also responsible for the limitation of the HPA axis activation on localized inflammation requires further investigation. It is possible, however, that eNOS may form a functional regulatory link between acuteness of the inflammatory process, fever, and stimulation of the HPA axis.

Because all three NOS isoforms generate the same product (NO), it is unclear why fever induced by LPS was affected by deficiencies in iNOS and nNOS and not eNOS and, furthermore, why fever induced by turpentine was influenced by deficiency in eNOS and not iNOS and nNOS. One possible explanation is that diversity in the distribution of the various NOSs in tissues, in combination with the role of these tissues in fever and the autocrine nature of the action of NO, may together account for the different effects. iNOS has been shown to be widespread throughout the body and is present in peripheral cells and tissues directly engaged in generation of metabolic heat (11, 33, 49), release of the mediators of fever (15, 16), and brain centers responsible for fever (see, e.g., Ref. 34). Expression of constitutive nNOS, although demonstrated also in the kidney, occurs mostly in nervous tissues, including hypothalamic preoptic nuclei (5, 14, 22). Thus the expression of iNOS and nNOS in tissues crucial for fever may have a role in the LPS-induced fever in mice. Expression of eNOS, on the other hand, has been demonstrated predominantly in endothelial cells (35). Numerous studies have demonstrated that autocrine action of NO generated in these cells contributed mostly to vasorelaxation processes (19). Assuming that NO use activation of the generation of metabolic heat and vasoconstriction for heat conservation as strategies for the induction of fever by LPS and other inflammatory stimuli, one can conclude that the role of eNOS in fever of mice may indeed appear to be insignificant.

A striking difference in sensitivity of fever to NOS isoforms of mice in response to injections of LPS and turpentine oil substantiates the notion that involvement of NO in fever is complex. It indicates, furthermore, that the mechanisms of these two febrile responses may be different. In our previous studies with IL-18 KO and IL-6 KO mice, we have shown that fevers to LPS and turpentine differ in their profile of cytokines (27). For example, we have not been able to detect any elevation of plasma TNF\( \alpha \) in mice after injection of turpentine (27). In contrast, response to the injection of LPS is accompanied by a transient increase of TNF\( \alpha \) during early phases of fever in mice (27). However, in studies by Roth et al. (48) on guinea pigs challenged with LPS, the antipyretic effect of \( L-\text{NAME} \) occurred without influencing the LPS-induced elevations in plasma IL-6 and TNF\( \alpha \), indicating that NO acts on fever downstream to cytokines, presumably via enhancing a cytokine-induced synthesis of PG. In accordance with this possibility, Salvemini et al. (50) showed that NO directly stimulates production of PGE\( _2 \). This might imply, therefore, that differences in a cytokine profile may not account for the contrasting action of \( L-\text{NMMA} \) on fever in response to turpentine and LPS. It has been shown, however, that induction of NOS after injection of LPS in rats is acutely regulated by increases in TNF\( \alpha \) (57). It has also been reported that NO downregulates LPS-induced synthesis of TNF\( \alpha \) in murine macrophages (53), suggesting a regulatory feedback between TNF\( \alpha \) and NO on stimulation with LPS. Such a mechanism may be absent during stimulation with turpentine, which is supported by studies of Geller et al. (15), who demonstrated lack of iNOS expression in remote tissues during localized inflammation. Because febrile responses to both LPS and turpentine are PG sensitive, i.e., they can be blocked by inhibitors of cyclooxygenases (see, e.g., Refs. 8 and 27), one can speculate that NO is involved in the generation of PG during response to LPS, whereas, during the response to turpentine, synthesis of PG is NO independent. In support, our unpublished data indicate that there is no difference in elevation of plasma levels of PGE\( _2 \) measured 24 h after injection of turpentine in control and \( L-\text{NMMA} \)-treated mice. In contrast, \( L-\text{NMMA} \) suppressed a significant portion of the increase of plasma PGE\( _2 \) measured 5 h after injection of LPS in mice. Together, data presented in this report as well as those reported by other investigators support the notion that the impact of the complexity of the NOS system on fever is far from being sufficiently understood. Because there is a strong indication of the therapeutic application of this system (35), the exact role of NO in responses to various pyrogenic insults merits further investigation.

This study was supported by Intramural Research Funds from the Medical College of Georgia and was conducted in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

REFERENCES


44. Roth J, Storr B, Goldbach JM, Voigt K, and Zeisberger E. Dose-dependent attenuation of lipopolysaccharide-fever by in-


