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Febrigenic signaling to the brain does not involve nitric oxide

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1   The involvement of peripheral nitric oxide (NO) in febrigenic signaling to the brain has been proposed because peripherally administered NO synthase (NOS) inhibitors attenuate lipopolysaccharide (LPS)-induced fever in rodents. However, how the unstable molecule of NO can reach the brain to trigger fever is unclear. It is also unclear whether NOS inhibitors attenuate fever by blocking febrigenic signaling or, alternatively, by suppressing thermogenesis in brown fat.

2   Male Wistar rats were chronically implanted with jugular catheters; their colonic and tail skin temperatures (T_c and T_{sk}) were monitored.

3   *Study 1* was designed to determine whether the relatively stable, physiologically relevant forms of NO, that is, *S*-nitrosoalbumin (SNA) and *S*-nitrosoglutathione (SNG), are pyrogenic and whether they enhance LPS fever. At a neutral ambient temperature (T_a) of 31°C, afebrile or LPS (1 μg kg^{-1}, i.v.)-treated rats were infused i.v. with SNA (0.34 or 4.1 μmol kg^{-1}; the controls received NaNO$_2$ and albumin) or SNG (10 or 60 μmol kg^{-1}; the controls received glutathione). T_c of SNA- or SNG-treated rats never exceeded that of the controls.

4   In *Study 2*, we tested whether the known fever-attenuating effect of the NOS inhibitor N_ω-nitro-L-arginine methyl ester (L-NAME) at a subneutral T_a (when fever is brought about by thermogenesis) also occurs at a neutral T_a (when fever is brought about by skin vasoconstriction). At a subneutral T_a of 24°C, L-NAME (2.5 mg kg^{-1}, i.v.) attenuated LPS (10 μg kg^{-1}, i.v.) fever, presumably by inhibiting thermogenesis. At 31°C, L-NAME enhanced LPS fever by augmenting skin vasoconstriction (T_{sk} fall).

5   In summary, both SNA and SNG had no pyrogenic effect of their own and failed to enhance LPS fever; peripheral L-NAME attenuated only fever brought about by increased thermogenesis. It is concluded that NO is uninvolved in febrigenic signaling to the brain.
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Introduction

There is little doubt that centrally produced nitric oxide (NO) is involved in fever as an antipyretic agent (Steiner *et al.*, 2002), but the role of peripherally produced NO in the fever response is unclear. It has been hypothesized that peripheral NO is a febrigenic signal to the brain (Steiner & Branco, 2001). The main argument supporting this hypothesis is that peripherally administered inhibitors of NO synthase (NOS) attenuate fevers induced by lipopolysaccharide (LPS) and many other exogenous pyrogens in rats (Scammell *et al.*, 1996; Perotti *et al.*, 1999; Ataoglu *et al.*, 2000; Soszynski, 2001; Kamerman *et al.*, 2002) and guinea-pigs (Roth *et al.*, 1998a; 1999; Kamerman & Fuller, 2000). However, the brief (∼0.1 s) half-life of free NO *in vivo* (Kelm & Schrader, 1990) and, consequently, its curtailed ability to reach tissue targets (Condorelli & George, 2002) severely limit the mechanisms by which peripheral NO might signal the brain to produce fever.

In the plasma, NO exists in a dynamic balance with low-molecular-mass nitrosothiols (such as *S*-nitrosoglutathione (SNG)) and high-molecular-mass nitrosothiols (such as *S*-nitrosoalbumin (SNA)): NO–SNG–SNA (Scharfstein *et al.*, 1994; Gordge *et al.*, 1996; Jourd'heuil *et al.*, 2000b; Tsikas *et al.*, 2001). The predominant, most stable species in this equilibrium is SNA: it has a plasma concentration of ∼10 nM (Rassaf *et al.*, 2003) and a half-life of ∼40 min (Stamler *et al.*, 1992). Since they are relatively stable, the nitrosothiols serve as a high-capacity reservoir of NO (Minamiyama *et al.*, 1996; Jourd'heuil *et al.*, 2000b). The same nitrosothiols also release NO to its distant targets *via* tissue-specific reactions with transition metals, enzymes, and reducing agents (Gaston, 1999; Hogg, 2002). The involvement of SNA and SNG in many biological effects of NO, including vasorelaxation and inhibition of platelet aggregation, has been demonstrated (Stamler *et al.*, 1992; Keaney *et al.*, 1993; De Belder *et al.*, 1994; Gordge *et al.*, 1996; Kashiba *et al.*, 1999). It is, therefore, reasonable to hypothesize that, if peripheral NO signals the brain to produce fever, this signaling involves nitrosothiols. Consistent with this

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hypothesis, the plasma levels of nitrosothiols increase after the peripheral administration of LPS, at least at a high dose (Jourd'heuil *et al*., 2000a). To test whether peripherally administered SNA and SNG are pyrogenic and whether they enhance LPS-induced fever were the first aims of the present work.

Although several studies (Scammell *et al*., 1996; Roth *et al*., 1998a; 1999; Perotti *et al*., 1999; Ataoglu *et al*., 2000; Kamerman & Fuller, 2000; Soszynski, 2001; Kamerman *et al*., 2002) attributed the fever-suppressing effect of NOS inhibitors to their action on NO-mediated mechanisms of immune-to-brain febrigenic signaling, an alternative interpretation was not ruled out. Specifically, these studies did not address the possibility that the observed attenuation of fever might have resulted from altered thermogenesis. All referenced studies were conducted in rats and guinea-pigs at an ambient temperature (T_a) of 22–25°C, which is substantially below the thermoneutral zone for these species in most experimental setups (Pace & Rahlman, 1983; Romanovsky *et al*., 2002). At a subneutral T_a (i.e., in the cold), fever is primarily caused by increased thermogenic activity of the brown adipose tissue (Székely & Szelényi, 1979). As this activity critically depends on the local production of NO (Nagashima *et al*., 1994; Saha & Kuroshima, 2000), NOS inhibitors might attenuate fever by suppressing brown adipose tissue thermogenesis. To either confirm or rule out this possibility was the second aim of the present work.

To address this second aim, the effect of the widely used NOS inhibitor N_ω-nitro-L-arginine methyl ester (L-NAME) on LPS fever was studied both in the cold and at the upper limit of the thermoneutral zone. In the latter case, fever is primarily brought about by tail skin vasoconstriction, and the contribution of thermogenesis for this response is negligible (Székely & Szelényi, 1979). If L-NAME attenuates fever by suppressing thermogenesis, it should fail to affect the febrile response at a neutral T_a. If, however, NO is a febrigenic signal to the brain (as proposed in the earlier studies), L-NAME should attenuate fever regardless of T_a. This approach (i.e., studying an effect on the febrile response at different T_a's to differentiate whether the effect results from altered febrigenic signaling or altered thermoeffector mechanisms) was used successfully in our recent study (Ivanov & Romanovsky, 2002).

Methods

Animals

The studies were conducted in 151 male Wistar rats (B&K Universal, Kent, WA, U.S.A.) weighing 280–320 g at the time of the experiments. Initially, the animals were housed three per standard 'shoe box'; after surgery, they were caged individually. The cages were kept in a rack equipped with a Smart Bio-Pack ventilation system (model SB4100) and Thermo-Pak temperature control system (model TP2000; Allentown Caging Equipment, Allentown, NJ, U.S.A.); the temperature of the incoming air was maintained at 28°C. Standard rat chow (Teklad Rodent Diet 'W' 8604; Harlan Teklad, Madison, WI, U.S.A.) and tap water were available *ad libitum*. The room was on a 12 : 12 h light–dark cycle (lights on at 07:00 h). The cage space was enriched with artificial 'rat holes' (cylindrical confiners made of stainless steel wire). In addition to spending

time in the confiners voluntarily, the rats were systematically habituated to them (seven daily training sessions, 4 h each). The same confiners were used later in the experiments. When well-adapted rats are confined, they exhibit no stress fever (Romanovsky *et al*., 1998). All experiments were started between 08:00 and 09:00 h. Each rat was used in only one experiment and euthanized with an overdose of sodium pentobarbital (100 mg kg^{-1}, i.v.) at the end of the experiment. The protocols were approved by the Institutional Animal Care and Use Committee of St Joseph's Hospital and Medical Center.

Surgery

Each rat was subjected to chronic catheterization of the jugular vein. Under ketamine–xylazine–acepromazine (55.6, 5.5, and 1.1 mg kg^{-1}, i.p., respectively) anesthesia and antibiotic (enrofloxacin, 12 mg kg^{-1}, s.c.) protection, the rat was placed on an operating board. A 1-cm longitudinal incision was made on the ventral surface of the neck, 1 cm left of the trachea. The left jugular vein was exposed, freed from its surrounding connective tissue, and ligated. A silicone catheter (ID 0.5 mm, OD 0.9 mm) filled with heparinized (50 U ml^{-1}) pyrogen-free saline was passed into the superior vena cava through the jugular vein and secured in place with ligatures. The 10-cm free end of the silicone catheter was knotted, tunneled under the skin, and exteriorized at the nape. If the rat was designated for an experiment involving blood pressure measurements (*Experiment* (*Exp*.) *1*), the left carotid artery was also chronically catheterized. The artery was isolated and clamped by a microclip. A PE-50 catheter (ID 0.6 mm, OD 1.0 mm) filled with heparinized saline was inserted into the artery centripetally, the clip was removed, and the catheter was secured in place with ligatures. The free end of the catheter was heat-closed and exteriorized at the nape. The surgical wounds on the ventral and dorsal surfaces of the neck were sutured. The catheters were flushed with heparinized saline every other day.

Instrumentation

All experiments (except for *Exp. 1*) were performed on *Day 5* post-surgery. Each rat was placed in a confiner and equipped with two copper-constantan thermocouples: one for recording the colonic temperature (T_c) and the other for recording the tail skin temperature (T_{sk}). The colonic thermocouple was inserted 10 cm beyond the anal sphincter and fixed to the base of the tail with an adhesive tape. The skin thermocouple was positioned on the lateral surface of the tail (at the boundary of the proximal and middle thirds) and insulated from the environment with tape. The thermocouples were plugged in to a data logger (Dianachart, Rockaway, NJ, U.S.A.), which was connected to a personal computer. The rat was transferred to a climatic chamber (Forma Scientific, Marietta, OH, U.S.A.) set to T_a of 24.0°C (mild cold exposure) or 31.0°C (the upper limit of the thermoneutral zone; see Romanovsky *et al*., 2002). According to the manufacturer, the precision of temperature control in this chamber is ±0.1°C, and the uniformity at 30.0°C is ±0.4°C. The jugular catheter was extended with a length of PE-50 tubing filled with saline, and the extension was passed through a wall port and connected to a syringe filled with the drug of interest. This setup permitted i.v. drug administration without disturbing the animal.

The experiment that involved arterial blood pressure recording (*Exp. 1*) was performed on *Day 1* post-surgery at a T_a of 31.0°C. Each rat was prepared as described above, but no thermocouples were attached. The arterial catheter was extended with a length of PE-50 tubing filled with saline. The extension was passed through a wall port and connected to a blood pressure monitor (World Precision Instruments, Sarasota, FL, U.S.A.) *via* a disposable pressure transducer (Cobe Cardiovascular, Arvada, CO, U.S.A.). The data were registered with the help of a paper chart recorder (Linear Instruments, Delray Beach, FL, U.S.A.).

Drugs

All drugs and reagents were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). A stock suspension of *E. coli* 0111:B4 LPS (2.5 mg ml^{-1}) in saline was stored at −20°C. At the time of the experiment, the stock was diluted to a final concentration of either 1 µg ml^{-1} (*Study 1*) or 10 µg ml^{-1} (*Study 2*). The NOS inhibitor L-NAME (2.5, 5, or 10 mg ml^{-1}), its inactive (Rees *et al.*, 1990) enantiomer D-NAME (2.5 or 10 mg ml^{-1}), SNG (5 or 30 µmol ml^{-1}), and reduced glutathione (5 or 30 µmol ml^{-1}) were all dissolved in saline immediately before the experiment.

SNA was synthesized extemporaneously according to Stamler *et al.* (1992), with minor modifications. Essentially fatty acid-free bovine serum albumin (BSA, 106.4 mg) was dissolved in saline (0.5 ml). NaNO$_2$ (156 µg in 26 µl of saline) was added to achieve a molar NaNO$_2$-to-BSA ratio of 1.4, and the solution was acidified with 0.5 N HCl (1 ml). After a 30-min incubation at room temperature, the solution was neutralized with 0.5 N NaOH (1 ml) and diluted with Tris-buffered saline (10 mM, pH 7.4) to a final volume of 4 ml. If the efficacy of nitrosylation were 100%, the resultant solution would have a final SNA concentration of 400 nmol ml^{-1}. Assuming that the actual yield of SNA in this reaction is 85% (i.e., 85% of BSA molecules are nitrosylated; see Stamler *et al.*, 1992; Seo *et al.*, 1998), the final concentration of SNA was 340 nmol ml^{-1}. Hence, the molar ratio between the added amount of NaNO$_2$ and the synthesized amount of SNA was 1.65, whereas the ratio between the added amount of BSA and the synthesized amount of SNA was 1.18. These molar ratios were used to calculate the amounts of NaNO$_2$ and BSA infused to the control rats in *Study 1*.

To verify the successful synthesis of SNA, UV-visible scanning was performed with a U-3010 spectrophotometer (Hitachi, Tokyo, Japan). The preparation of SNA, but not that of uncomplexed BSA or NaNO$_2$, presented an absorption maximum at ~340 nm, which is characteristic for *S*-nitrosothiols (Stamler *et al.*, 1992; Seo *et al.*, 1998). Assuming a molar absorptivity of 3869 M^{-1} cm^{-1} (as reported by Stamler *et al.* (1992) for a similar preparation of SNA), the value of absorbance measured at 340 nm (1.23, mean of three samples) corresponds to the SNA concentration of 318 nmol ml^{-1}, which differs by only 6% from the concentration calculated based on the product yield in the nitrosylation reaction.

Protocols

Study 1: Thermal effects of SNA and SNG in afebrile and febrile rats. This study consisted of *Exps. 1–3*, which were performed at a neutral T_a (31°C).

Exp. 1: For both SNA and SNG, infusion rates presumed to be biologically active based on previous reports (Stamler *et al.*, 1992; Keaney *et al.*, 1993; Kashiba *et al.*, 1999) were selected. The effectiveness of the selected infusion rates to cause arterial hypotension under the present experimental conditions was then tested. The rats were infused i.v. with SNA (68 nmol kg^{-1} min^{-1}, 200 µl kg^{-1} min^{-1}, 5 min) or SNG (1 µmol kg^{-1} min^{-1}, 33.3 µl kg^{-1} min^{-1}, 1 min), and their arterial blood pressure was recorded. For the SNA infusion, the control treatment was an infusion of the reagents employed in SNA synthesis at the corresponding molar ratios (see *Drugs*): NaNO$_2$ (1.1 µmol kg^{-1} min^{-1}, 200 µl kg^{-1} min^{-1}, 0.5 min), followed by BSA (89.1 nmol kg^{-1} min^{-1}, 200 µl kg^{-1} min^{-1}, 4.5 min). For the SNG infusion, the control treatment was an infusion of reduced glutathione (1 µmol kg^{-1} min^{-1}, 33.3 µl kg^{-1} min^{-1}, 1 min). At the infusion rates used, both SNA and SNG evoked mild hypotensive responses, whereas the control infusions did not affect arterial blood pressure.

Exp. 2 was designed to determine whether SNA and SNG cause fever. In this experiment, the nitrosothiols were infused at the biologically active (mild hypotensive) rates selected in *Exp. 1*. To increase the chance of finding an effect on T_c, the duration of the infusions was extended substantially. SNA was infused over 60 min, so that the total dose administered was 4.1 µmol kg^{-1}; the controls received NaNO$_2$ (6.8 µmol kg^{-1}) over 6 min, followed by BSA (4.8 µmol kg^{-1}) over 54 min. SNG (or glutathione in the controls) was infused over 60 min, so that the total dose administered was 60 µmol kg^{-1}.

Exp. 3 was designed to determine whether SNA and SNG enhance the small, monophasic fever caused by a low dose of LPS (Romanovsky *et al.*, 1997; Simons *et al.*, 1998). While designing this experiment, it was important to notice that both control groups in *Exp. 2* (viz., the group treated with NaNO$_2$ and BSA and that treated with glutathione) exhibited thermal effects (see *Results*). To eliminate the thermal effects of the control solutions and to prevent their potential interference with the fever response to LPS, the infusion rates were decreased in *Exp. 3*. Starting 30 min after the injection of LPS (1 µg kg^{-1}, i.v.), the rats were infused with SNA (5.67 nmol kg^{-1} min^{-1}, 16.8 µl kg^{-1} min^{-1}) or SNG (167 nmol kg^{-1} min^{-1}, 33.4 µl kg^{-1} min^{-1}) for 60 min; the total doses infused were 340 nmol kg^{-1} and 10 µmol kg^{-1}, respectively. The controls were also injected with LPS and then infused with either NaNO$_2$ (92.4 nmol kg^{-1} min^{-1}, 16.8 µl kg^{-1} min^{-1}, 6 min) followed by BSA (7.5 nmol kg^{-1} min^{-1}, 16.8 µl kg^{-1} min^{-1}, 54 min), or with glutathione (167 nmol kg^{-1} min^{-1}, 33.4 µl kg^{-1} min^{-1}, 60 min).

Study 2: Effect of L-NAME on LPS fever at different T_a's. This study consisted of *Exps. 4* and *5*.

Exp. 4: Peripheral administration of L-NAME causes dose-dependent hypothermia in rats at subneutral T_a's (De Luca *et al.*, 1995; Scammell *et al.*, 1996; Branco *et al.*, 1997; Steiner *et al.*, 1998), an effect that would potentially interfere with the fever response to LPS. To avoid such an effect, the highest dose of L-NAME that is thermally ineffective in afebrile rats at both subneutral and neutral T_a's was found in *Exp. 4*. At a subneutral T_a (24°C), the rats were injected with L-NAME (2.5, 5, or 10 mg kg^{-1}, i.v.) or D-NAME (10 mg kg^{-1}) in a volume of 1 ml kg^{-1}. Only the lowest dose caused no hypothermia (see *Results*). The thermal ineffectiveness of this dose was then verified in afebrile rats at a neutral T_a (31°C). Importantly, this dose of L-NAME is adequate

to study the physiological roles of peripherally produced NO: it markedly suppresses NOS activity in peripheral tissues (Rees *et al.*, 1990), but is expected to have little effect on brain NOS (Iadecola *et al.*, 1994). This dose was selected for *Exp. 5*.

Exp. 5 was designed to determine the effect of L-NAME on LPS-induced fever at both subneutral and neutral T_a's. To this end, L-NAME or D-NAME (2.5 mg kg^{-1}, i.v.) was injected 30 min before LPS (10 μg kg^{-1}, i.v.) at either 24 or 31°C. The 10 μg kg^{-1} dose of LPS causes a long-lasting, polyphasic fever of a relatively high magnitude (Romanovsky *et al.*, 1997; 1998). This dose of LPS was chosen because the expected effect of L-NAME was a reduction in the fever height. The duration of the febrile response to this dose of LPS was not a problem because the NOS-inhibiting effect of a single injection of L-NAME lasts a few hours to a few days (Rees *et al.*, 1990; Iadecola *et al.*, 1994).

Data processing and analysis

The absolute value of T_c, rather than the change in T_c, was used to evaluate deep body temperature responses (for justification, see Romanovsky *et al.*, 2003). The heat loss index (HLI) was used to evaluate the thermoeffector responses of tail skin vasculature. As justified elsewhere (Romanovsky *et al.*, 2002), the HLI was calculated according to the formula: $HLI = (T_{sk} - T_a)/(T_c - T_a)$. The theoretical limits of the HLI are 0 (maximal skin vasoconstriction) and 1 (maximal vasodilation). In practice, however, the upper limit depends on the position of the tail skin thermocouple. When T_{sk} is measured at the boundary of the proximal and middle thirds of the tail, as in the present work, the HLI rarely exceeds 0.6 (Romanovsky *et al.*, 2002). The mean arterial pressure (MAP) was calculated according to the formula: $MAP = AP_{min} + (AP_{max} - AP_{min})/3$ (Sindrup *et al.*, 1991), where AP_{min} and AP_{max} are the minimal and maximal values of arterial pressure, respectively. The T_c, HLI, and MAP responses were compared across treatments and time points by two-way ANOVA for repeated measures using Statistica AX'99 (StatSoft, Tulsa, OK, U.S.A.). The data are reported as means ± s.e.

Results

Study 1: Thermal effects of SNA and SNG in afebrile and febrile rats

Exp. 1: When infused i.v. at the rate of 68 nmol kg^{-1} min^{-1}, SNA produced mild arterial hypotension (MAP decrease of ~20 mmHg; $P = 3.4 \times 10^{-2}$), whereas the control infusion (NaNO$_2$ + BSA) had no effect on the MAP (Figure 1a). The i.v. infusion of SNG (but not of glutathione) at the rate of 1 μmol kg^{-1} min^{-1} caused a similar decrease in the MAP ($P = 3.9 \times 10^{-2}$; Figure 2a). The biologically active (mild hypotensive) infusion rates of SNA and SNG used in this experiment and the corresponding rates of the control solutions were used in *Exp. 2*.

Exp. 2: When NaNO$_2$ and BSA were administered sequentially (as a control to the SNA infusion), they evoked a biphasic rise in T_c (Figure 1b) accompanied by tail skin vasoconstriction (biphasic decrease in the HLI; Figure 1c). Experiments with an i.v. infusion of BSA alone (data not
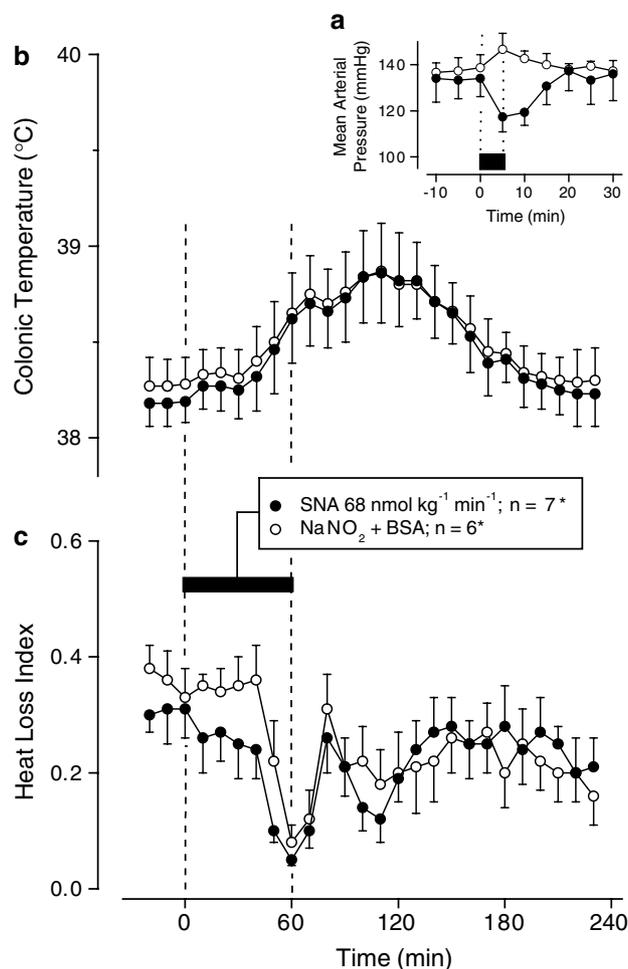
Figure 1 Effects of SNA (infusion rate indicated) on the MAP (a), colonic temperature (b), and HLI (c) of afebrile rats at a neutral ambient temperature of 31°C. The controls received the corresponding amounts of NaNO$_2$ and BSA; see *Methods* for details. The black bars indicate the duration of infusions. *In panel (a), $n = 3$ for each group.

shown) suggest that these changes are likely caused by the BSA preparation. When SNA was infused for 60 min, the T_c (Figure 1b) and HLI (Figure 1c) responses were identical to those of the controls. The infusion of glutathione (control to SNG) tended to produce a small monophasic increase in T_c (Figure 2b) and a monophasic decrease in the HLI (Figure 2c), whereas SNG tended to reduce T_c (Figure 2b) and did not cause skin vasoconstriction (Figure 2c). Although not significant by themselves, the changes in T_c of the SNG- and glutathione-treated rats differed significantly from each other ($P = 3.4 \times 10^{-2}$).
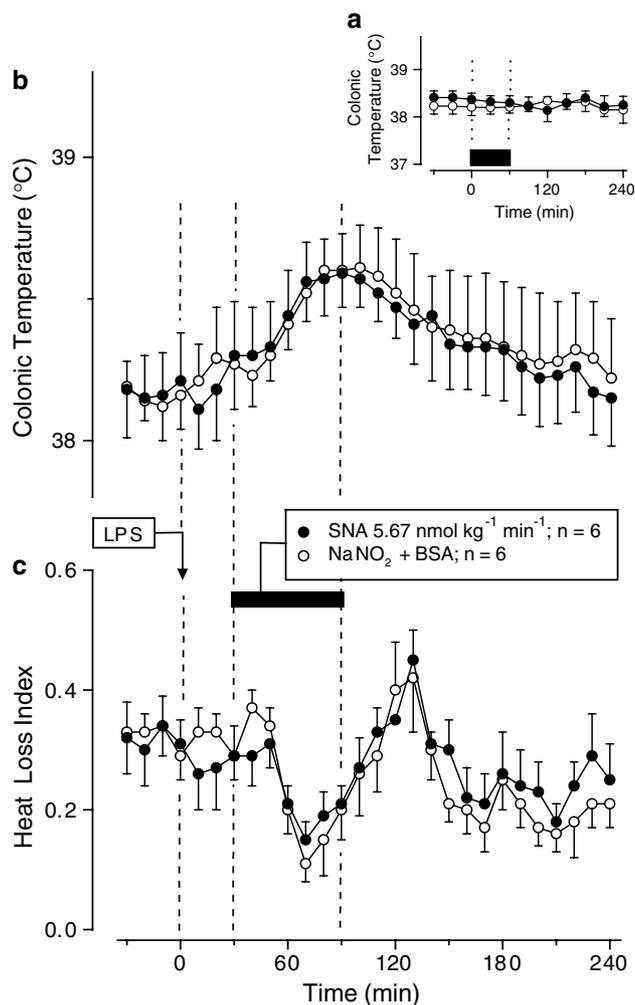
Exp. 3: To avoid potential interference of the thermal effects of the control solutions with LPS fever (subject of study in *Exp. 3*), SNA, SNG, and their control solutions were infused at lower rates: 5.67 nmol kg^{-1} min^{-1} for SNA and 167 nmol kg^{-1} min^{-1} for SNG. At these infusion rates, the nitrosothiols and their respective control solutions produced no change in T_c of the afebrile rats (Figures 3a and 4a). When SNA or SNG was infused during the rising phase of LPS (1 μg kg^{-1}, i.v.)-induced monophasic fever, they affected neither the febrile T_c rise (Figures 3b and 4b) nor the associated decrease in the HLI (Figures 3c and 4c).
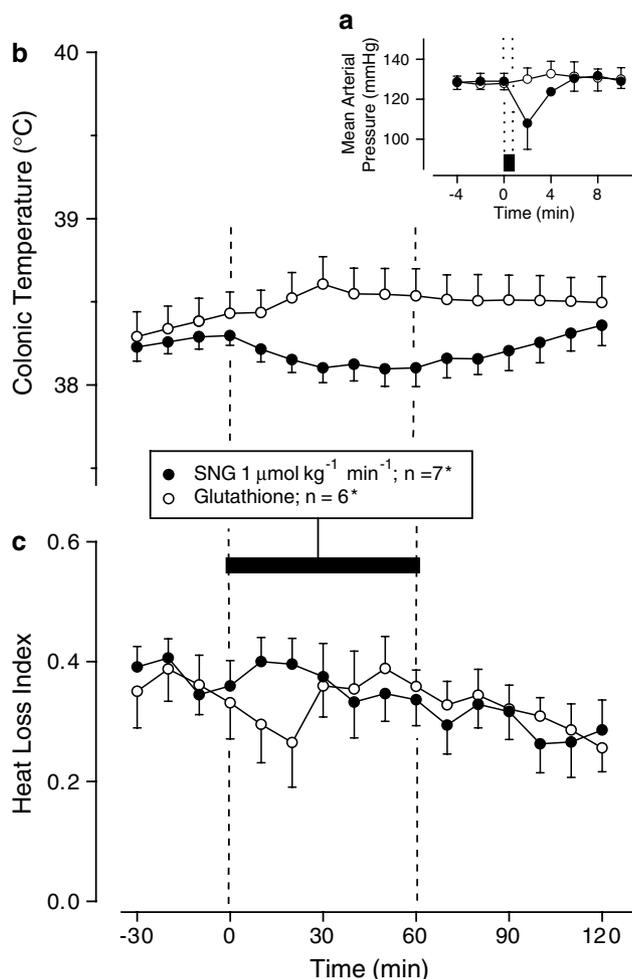
Figure 2 Effects of SNG (infusion rate indicated) on the MAP (a), colonic temperature (b), and HLI (c) of afebrile rats at 31°C. The controls received reduced glutathione. The black bars indicate the duration of infusions. *In panel (a), $n = 3$ for each group.

Figure 3 Effects of SNA (infusion rate indicated) on the colonic temperature of afebrile rats (a) and on the colonic temperature (b) and HLI (c) of LPS ($1 \mu g \, kg^{-1}$, i.v.)-treated rats at 31°C. The controls were infused with the corresponding amounts of $NaNO_2$ and BSA. The black bars indicate the duration of infusions.

Study 2: Effect of L-NAME on LPS fever at different T_a's

Exp. 4: At a subneutral T_a of 24°C, the D-NAME-treated rats presented a slight (~ 0.5°C) decrease in T_c over the course of the experiment (Figure 5a). Such a decrease often occurs in untreated or saline-treated rats during the light phase of the day at a subneutral and even neutral T_a (Honma & Hiroshige, 1978; Romanovsky *et al.*, 1996; Simons *et al.*, 1998; Gautier & Murariu, 1999; Ivanov & Romanovsky, 2002; Ivanov *et al.*, 2003a), presumably reflecting the circadian rhythm of T_c (Honma & Hiroshige, 1978; Scales & Kluger, 1987). Compared to D-NAME, L-NAME evoked a larger decrease in T_c (Figure 5a). This decrease was dose-dependent: whereas T_c of the D-NAME-treated controls was 37.2 ± 0.1°C at 90 min postinjection, T_c of the L-NAME-treated rats was 37.1 ± 0.2°C ($2.5 \, mg \, kg^{-1}$), 36.9 ± 0.2°C ($5 \, mg \, kg^{-1}$), or 36.5 ± 0.3°C ($10 \, mg \, kg^{-1}$). The thermal responses to the two higher doses of L-NAME were significantly different from that of the control group ($P = 3.7 \times 10^{-2}$ and $P = 1.3 \times 10^{-2}$, respectively), but the response to the lowest dose ($2.5 \, mg \, kg^{-1}$) was not. This lowest dose was also thermally ineffective at a neutral T_a of 31°C (Figure 5b). At neither 24°C nor 31°C did this dose

interfere with the basal functioning of skin vessels, as evidenced by the absence of any changes in the HLI (data not shown). This thermally ineffective dose of L-NAME was used in *Exp. 5*.

Exp. 5: The effect of L-NAME on LPS ($10 \, \mu g \, kg^{-1}$, i.v.)-induced fever was studied at both subneutral (24°C) and neutral (31°C) T_a's. At 24°C, the D-NAME-treated controls responded to LPS with complex changes in T_c: the initial drop (nadir at ~ 80 min post-LPS) was followed by two T_c rises (peaks at ~ 140 and 370 min) (Figure 6a). During the second rise, T_c reached a febrile level (~ 0.5°C above the baseline). Pretreatment with L-NAME had little effect on the initial hypothermia, but it markedly attenuated the first ($P = 2.9 \times 10^{-2}$) and second ($P = 1.0 \times 10^{-2}$) T_c rises (Figure 6a). In both the D-NAME- and L-NAME-treated rats, the HLI remained at a near-zero level throughout the experiment, indicating maximal tail skin vasoconstriction (Figure 6b).

At 31°C, the D-NAME-treated controls responded to LPS with a typical polyphasic fever: three febrile phases peaked at ~ 60, 150, and 280 min post-LPS (Figure 7a), and they
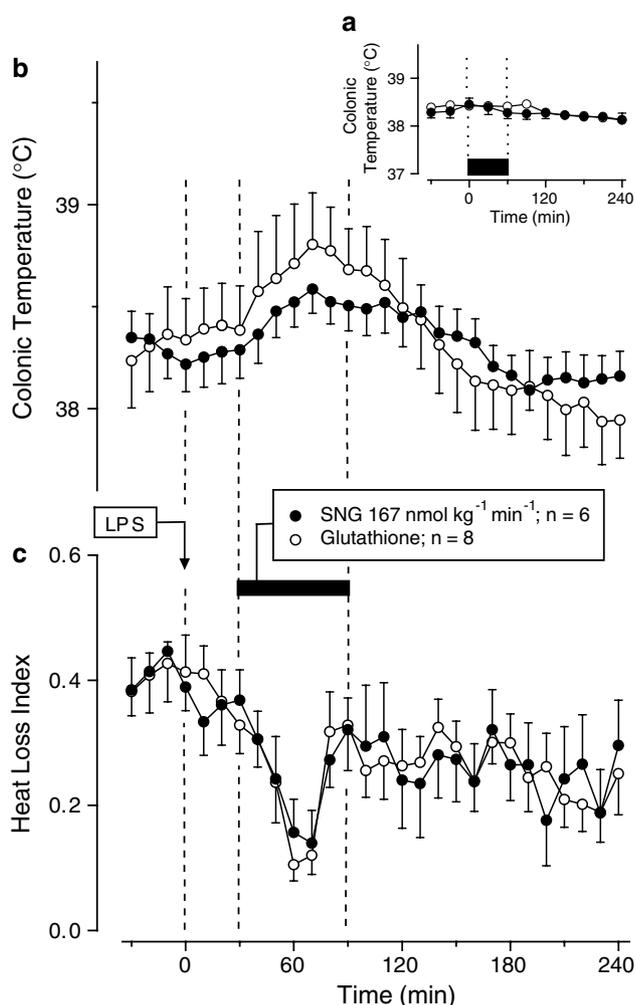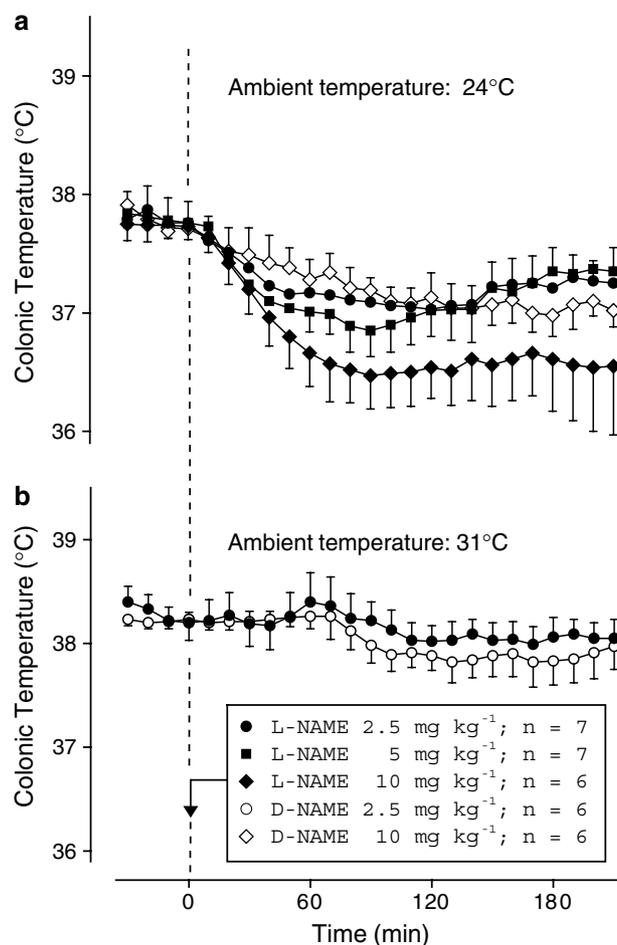
Figure 4 Effects of SNG (infusion rate indicated) on the colonic temperature of afebrile rats (a) and on the colonic temperature (b) and HLI (c) of LPS ($1\,\mu g\,kg^{-1}$, i.v.)-treated rats at 31°C. The controls received reduced glutathione. The black bars indicate the duration of infusions.

Figure 5 Effect of intravenous injection of the NOS inhibitor L-NAME (doses indicated) or its inactive enantiomer D-NAME (doses indicated) on the colonic temperature of rats at an ambient temperature of 24°C (a) or 31°C (b).

were preceded by three waves of skin vasoconstriction (decrease in the HLI; Figure 7b). In contrast to its fever-reducing effect at 24°C, L-NAME significantly enhanced the second ($P = 2.8 \times 10^{-2}$) and third ($P = 1.2 \times 10^{-2}$) febrile phases (Figure 7a). In contrast to its lack of effect on the HLI at 24°C, L-NAME tended to enhance the LPS-induced skin vasoconstriction at the first and second febrile phases and significantly ($P = 1.4 \times 10^{-2}$) enhanced it at the third phase (Figure 7b).

Discussion

Several short-lived and/or poorly water-soluble mediators of fever (e.g., prostaglandin (PG) E_2 and platelet-activating factor (PAF)) are solubilized, stabilized, and transported in the bloodstream by carrier molecules, primarily serum albumin (Clay *et al.*, 1990; Peters, 1996). Consequently, their predominant, albumin-bound form is physiologically the most relevant (Peters, 1996). Experiments in which these mediators are administered in the albumin-bound form are more likely to

reveal their intrinsic physiological actions. The use of the albumin-bound form was crucial to show that i.v. PGE_2 causes fever, thus providing an important argument in favor of the febrigenic role of blood-borne PGE_2 (Romanovsky *et al.*, 1999). The albumin-bound form was also important for the discovery of the pyrogenic action of PAF (Ivanov *et al.*, 2003b). Since NO is also a short-lived molecule that circulates primarily in its more stable, bound form (either to albumin (SNA) or to low-molecular-mass molecules such as glutathione (SNG)), we investigated whether SNA and SNG could have pyrogenic effects of their own and/or enhance LPS-induced fever.

Even at biologically active (mild hypotensive) doses, neither SNA nor SNG increased T_c of afebrile rats at a neutral T_a to above the levels seen in the controls. SNG even tended to decrease T_c, an action that agrees with the ability of NO donors to elicit hypothermia in rabbits in a thermoneutral environment (Mathai *et al.*, 1997). When SNA and SNG were infused during LPS-induced fever, they also failed to exaggerate this response. (The infusion rates for the fever experiment were selected as the maximal rates at which the control solutions were thermally ineffective in afebrile rats.) SNA and SNG also failed to affect the HLI of either afebrile or febrile rats. Although the latter observation suggests that
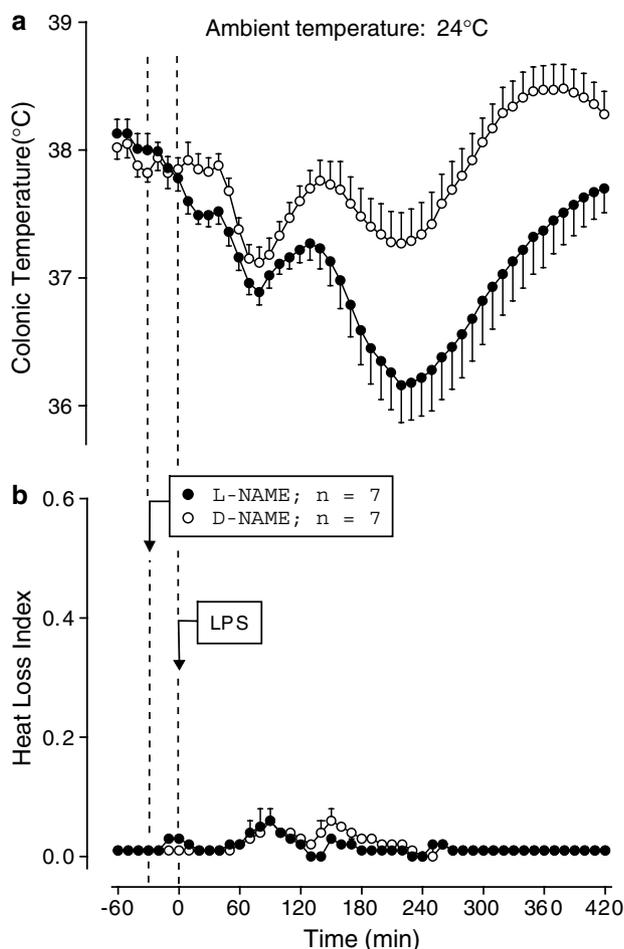
Figure 6 Effect of pretreatment with L-NAME ($2.5\,\mathrm{mg\,kg^{-1}}$, i.v.) or D-NAME on the colonic temperature (a) and HLI (b) of LPS ($10\,\mu\mathrm{g\,kg^{-1}}$, i.v.)-treated rats exposed to an ambient temperature of 24°C.

Figure 7 Effect of L-NAME ($2.5\,\mathrm{mg\,kg^{-1}}$, i.v.) or D-NAME on the colonic temperature (a) and HLI (b) of LPS ($10\,\mathrm{mg\,kg^{-1}}$, i.v.)-treated rats exposed to an ambient temperature of 31°C.

the tail skin vasculature in the rat is insensitive to circulating nitrosothiols, it does not rule out the possibility that locally produced, free NO may still play a role in thermoregulatory cutaneous vasodilation. Indeed, such vasodilation in the human forearm can be blocked by dialysis of a NOS inhibitor (Minson *et al.*, 2001).

Although the low responsiveness of skin blood vessels to nitrosothiols *in vivo* (De Belder *et al.*, 1994; present results) seemingly contradicts the high responsiveness of other vessels (e.g. the aorta) to nitrosothiols *in vitro* (Stamler *et al.*, 1992; Seo *et al.*, 1998; Jia & Stamler, 1999), the sensitivity to the vasorelaxant action of nitrosothiols is known to vary widely among vascular beds (Jia & Stamler, 1999; Gluckman *et al.*, 2002). In contrast to the spontaneous release of NO by many synthetic donors, the release of NO from nitrosothiols depends on the presence of transition metals, enzymes, and reducing agents, many of which are distributed throughout the body in a tissue-specific manner (Gaston, 1999; Hogg, 2002). For example, SNG readily releases NO in platelets due to the presence of a copper-dependent enzyme in these post-cellular structures (Gordge *et al.*, 1996). The effects of nitrosothiols are also likely to be less tissue-specific *in vitro* than *in vivo*. *In vitro*, nitrosothiols promptly release NO by reacting with transition metal ions that exist as unavoidable

contaminants of all buffers. *In vivo*, transition metal ions are actively sequestered; they are present at much lower concentrations and distributed throughout the body in a tissue-specific manner (Hogg, 2002).

Since both SNA and SNG lacked a febrigenic effect of their own and failed to enhance fever in the present work, they are likely uninvolved in febrigenic signaling to the brain. However, a few speculative scenarios are still compatible with an involvement of free NO, produced outside or at the blood-brain barrier, in febrigenic signaling. One scenario is that NO may be produced in the liver, in the vicinity of sensory endings of the nodose ganglion neurons. These neurons, which are stimulated by NO (Lawrence *et al.*, 1997), convey a centripetal febrigenic message (Simons *et al.*, 1998), which is likely to be important for triggering monophasic fever and, possibly, the first, early phase of polyphasic fever (Romanovsky *et al.*, 2000). Another unstable mediator, PGE_2, which is synthesized in the liver already at the onset of the first febrile phase (Ivanov *et al.*, 2002), can also activate hepatic vagal sensory fibers (Niijima, 1996; Ek *et al.*, 1998).

A second scenario emphasizes the hypothalamic microvasculature, which produces febrigenic PGE_2 (Matsumura *et al.*, 1998; Yamagata *et al.*, 2001) during the second and third febrile phases (Ivanov *et al.*, 2002). By analogy, it is

conceivable that circulating LPS and cytokines can induce NO synthesis in hypothalamic capillaries. After being released from their antiluminal surface, NO (or NO-derived species) may trigger fever by increasing the permeability of the blood–brain barrier (Mayhan, 1999) to circulating pyrogenic molecules or by activating an important PGE_2-synthesizing enzyme, cyclooxygenase-2 (Salvemini *et al.*, 1993; Goodwin *et al.*, 1999).

Yet, a completely different scenario is possible: NO may be uninvolved in febrigenic signaling. Although this less intriguing scenario has received little attention in the literature, it is consistent with the following data. First, when L-NAME attenuates the febrile response to peripheral pyrogens in a cool environment, it decreases neither the blood levels of propyretic cytokines interleukin (IL)-6 and tumor necrosis factor-α (Roth *et al.*, 1998a) nor the brain level of PGE_2 (Redford *et al.*, 1995). Second, fevers caused by central administration of IL-1β (Monroy *et al.*, 2001) or by psychological stressors (De Paula *et al.*, 2000; Soszynski, 2001) in a cool environment are also sensitive to peripheral administration of NOS inhibitors – even though they are initiated within the brain and do not require transduction of a febrigenic signal from the periphery. Third, systemic production of NO may not even be enhanced during fever. Only high doses of LPS (those that cause shock and hypothermia) induce overexpression of the inducible NOS isoform (Titheradge, 1999), leading to a massive production of NO and to a surge of plasma SNA (Jourd'heuil *et al.*, 2000a). Lower, pyrogenic doses suppress NO synthesis, possibly by inhibiting constitutive NOS isoforms (Riedel, 1997; Steiner *et al.*, 2002).

In the present work, L-NAME attenuated LPS fever in a cool environment but did not attenuate (and rather enhanced) it at a neutral T_a. These results are consistent with multiple studies showing that NOS inhibitors suppress fever in rats (Reimers *et al.*, 1994; Scammell *et al.*, 1996; Roth *et al.*, 1998b; Perotti *et al.*, 1999; Ataoglu *et al.*, 2000; Soszynski, 2001; Kamerman *et al.*, 2002) and guinea-pigs (Roth *et al.*, 1998a; 1999; Kamerman & Fuller, 2000) at 22–25°C, that is, at their subneutral T_a's (Pace & Rahlman, 1983; Romanovsky *et al.*, 2002). Our finding also agrees with studies showing that NOS inhibitors do not suppress fever in rabbits (Kapás *et al.*, 1994; Gagalo *et al.*, 1996; Riedel, 1997), cats (Redford *et al.*, 1995), and pigs (Parrott *et al.*, 1998) at 20–26°, that is, at their neutral T_a's (Gonzalez *et al.*, 1971; Gautier *et al.*, 1989; Noblet *et al.*, 1997). Together with our data, these studies suggest that NO affects fever by acting on a process or mechanism that strongly depends on T_a. Febrigenic signaling is unlikely to be a subject of such dependence.

What strongly depends on T_a is the set of thermoeffectors recruited for the development of fever. In the cold, fever is brought about largely by thermogenesis; at thermoneutrality, its major effector mechanism is skin vasoconstriction (Crawshaw & Stitt, 1975; Székely & Szelényi, 1979). Therefore, it can be inferred that NOS inhibitors attenuate fever by affecting thermoregulatory effector mechanisms rather than by blocking febrigenic signaling to the brain. Indeed, both activation of thermogenesis in the brown adipose tissue (Nagashima *et al.*, 1994; Saha & Kuroshima, 2000; Kikuchi-Utsumi *et al.*, 2002) and dilation of skin vasculature (Minson *et al.*, 2001) are thought to depend critically on locally produced NO. In the present work, the fever-attenuating effect of L-NAME at a subneutral T_a was not associated with changes in the HLI (the

tail skin vessels remained constricted); the effect of L-NAME likely resulted from inhibition of thermogenesis. In contrast, the fever-enhancing effect of L-NAME at a neutral T_a probably reflects enhanced vasoconstriction in the tail because L-NAME augmented the LPS-induced decreases in the HLI. These results are in agreement with a recent report (Kamerman *et al.*, 2003) showing that the thermal effect of peripheral L-NAME in afebrile rats also depends on T_a: L-NAME inhibits thermogenesis and causes hypothermia at a subneutral, but not at a neutral T_a.

Conclusions

The major findings of the present work can be summarized as follows. Neither SNA nor SNG (physiologically relevant forms of NO) have a febrigenic action of their own or enhance LPS fever. Intravenous L-NAME (a NOS inhibitor) attenuates LPS-induced fever in rats only at a subneutral T_a, that is, when this response is predominantly brought about by nonshivering thermogenesis. Intravenous L-NAME does not attenuate LPS fever when it is brought about by skin vasoconstriction (at or above thermoneutrality). Rather, L-NAME enhances the febrile response to LPS at thermoneutrality by enhancing skin vasoconstriction. Together, these results suggest that peripheral NO is uninvolved in febrigenic signaling to the brain.

Perspectives

While this report was in preparation, Kozak & Kozak (2003) used selective genetic deletion of individual isoforms of NOS and chronic oral administration of a NOS inhibitor to study LPS- and turpentine oil-induced fevers in mice. The authors' conclusion that the inducible and neuronal isoforms of NOS (but not the endothelial isoform) are involved in febrigenic signaling contradicts the main conclusion of the present work. Such a contradiction can be attributed to at least two methodological factors. First, due to compensatory mechanisms of genetic ablation in knockout and mutant animals, experiments in such animals (Ivanov *et al.*, 2003a), and specifically in NOS knockout mice (Szabó, 1998), often lead to conclusions later proved erroneous by further pharmacological studies. Second, when administered chronically (as done by Kozak & Kozak, 2003), NOS inhibitors often produce qualitatively different results than when administered acutely (Bryant *et al.*, 1995). Although the main conclusion of our present work differs from that of Kozak & Kozak (2003), we agree with their final remark that the complex relationships between the NOS system and the febrile response are far from being understood and that the role of NO in fevers caused by various pyrogenic insults merits further investigation.

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