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ORIGINAL PAPER

P.R. Kamerman · D. Mitchell · H.P. Laburn

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Abstract We have administered aminoguanidine, a relatively specific inhibitor of inducible nitric oxide synthase, and N-nitro-L-arginine methyl ester (L-NAME), an unspecific nitric oxide synthase inhibitor, to rats made febrile with the gram-positive pyrogen, muramyl dipeptide and gram-negative pyrogen, lipopolysaccharide. Sprague-Dawley rats, housed individually at ~25 °C with a 12:12 h light:dark cycle (lights on 0700 hours), were injected (at 0900 hours) intraperitoneally with 50 mg/kg aminoguanidine, 25 mg/kg or 50 mg/kg L-NAME, and intramuscularly with 500 μg/kg muramyl dipeptide or 100 μg/kg lipopolysaccharide. Pyrogen injections were spaced at least 14 days apart. Body temperature was measured throughout the study in unrestrained animals using radio-telemetry. Neither muramyl dipeptide nor lipopolysaccharide-induced fevers were affected by aminoguanidine. However, L-NAME administration inhibited muramyl dipeptide and lipopolysaccharide-induced fevers, but only for the 1st 2-4 h of the fevers (two-way ANOVA, $P < 0.05$). After the initial inhibition, lipopolysaccharide fevers developed normally. Therefore, constitutively expressed nitric oxide synthase appears to be involved in the initial phases of fever genesis of gram-negative and gram-positive fevers in rats. On the other hand, inducible nitric oxide synthase appears not to play a role in these fevers.

Keywords Fever · Stress hyperthermia · N-nitro-L-arginine methyl ester · Aminoguanidine

Abbreviation *L-NAME* N-nitro-L-arginine methyl ester

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P.R. Kamerman (✉) · D. Mitchell · H.P. Laburn
School of Physiology,
University of the Witwatersrand,
Medical School, 7 York Road,
Parktown, 2193, South Africa
E-mail: Kamermanpr@physiology.wits.ac.za
Tel.: +27-11-7172261
Fax: +27-11-6432765

Introduction

Systemic administration of unspecific inhibitors of both constitutive and inducible nitric oxide synthase reduce the febrile response to lipopolysaccharide in rats and guinea pigs (Kamerman and Fuller 2000; Perotti et al. 1999; Roth et al. 1998b; Scammell et al. 1996; Soszynski 2001). However, the antipyretic actions of these inhibitors are species-dependent, as inhibition of peripheral nitric oxide synthesis enhanced the febrile response of rabbits (Riedel 1997), and had no significant effects on fevers in pigs (Parrott et al. 1998). Roth and colleagues (1998b) suggested that the interspecies variation in the effects that nitric oxide synthase inhibitors have on lipopolysaccharide-induced fever might result from the different thermal strategies employed by animals to generate a fever; for example, increased metabolic rate in rats and guinea pigs versus decreased heat loss in rabbits.

Although numerous studies have investigated the antipyretic actions of intrathecally injected inhibitors of inducible nitric oxide synthase (Huang et al. 1997; Lin and Lin 1996a, 1996b; Lin et al. 1997), only three studies have investigated the effects on fever of systemically injected inhibitors of the inducible isoenzyme (Kamerman and Fuller 2000; Parrott et al. 1998; Roth et al. 1999). All three of these studies reported that inhibitors of inducible nitric oxide synthase, at doses that do not affect the activity of the constitutively expressed enzymes (Mehta et al. 1998), had no significant effect on lipopolysaccharide-induced fever. We did report, however, that inhibition of inducible nitric oxide synthase reduced the magnitude of fevers induced by the gram-positive pyrogen muramyl dipeptide (Kamerman and Fuller 2000), possibly reflecting previously observed differences in the pyretic pathways employed by gram-positive and gram-negative pyrogens (Goelst and Laburn 1991; Mitchell and Laburn 1997; Roth et al. 1997b).

Therefore, we have investigated the effects of administering aminoguanidine, a relatively selective inhibitor of inducible nitric oxide synthase, and for comparison, the

unspecific nitric oxide synthase inhibitor, N-nitro-L-arginine methyl ester (L-NAME), on the febrile response to muramyl dipeptide and lipopolysaccharide in a species other than the guinea pig, namely the rat. To facilitate correlation between our results in rats and those found previously in guinea pigs, we injected the synthase inhibitors and pyrogens by the same two routes we had used in guinea pigs (Kamerman and Fuller 2000).

Materials and methods

Animals

We used female Sprague-Dawley rats with a body mass of 200–250 g. The animals were housed individually in cages at an air temperature of 24.6 ± 1.4 °C, and a light-dark cycle of 12:12 h (lights on at 0700 hours). Food and water were provided ad libitum. The Animal Ethics Screening Committee of the University of the Witwatersrand cleared all procedures (protocol no. 2000/95/4).

Body temperature measurement

Rats anaesthetised with 80 mg/kg ketamine and 20 mg/kg xylazine had sterile, wax-coated, temperature-sensitive radiotelemeters (Mini-Mitter, Sunriver, USA) implanted into their abdomens 7 days before the start of experiments. The telemeters were calibrated over a range of temperatures in a water bath, against a precision quartz-crystal thermometer (Quat 100, Heraeus, Germany), such that abdominal temperature could be measured to an accuracy of 0.1 °C. The output frequency from each telemeter was monitored by a receiver plate (RTA 500, Mini-Mitter, USA) placed under each rat's cage. The frequency received by each plate was fed into a peripheral processor (Datacol-3 Automated Data Acquisition System, Mini-Mitter) connected to a personal computer, and the output expressed in degrees centigrade. Body temperature recordings were made at 10-min intervals.

Pyrogens and drugs

Stock solutions of lipopolysaccharide (Salmonella typhosa, Sigma, USA) and muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine, Sigma, USA) were prepared by dissolving the pyrogens in sterile pyrogen-free saline, such that the appropriate amount of pyrogen could be administered in a 1 ml/kg dose. A suitably pyrogenic dose of muramyl dipeptide was determined in a separate dose-ranging study, and the pyrogenicity of the lipopolysaccharide stock solution was tested by injecting a group of rats with 100 μg/kg lipopolysaccharide.

We used two doses (25 mg/kg and 50 mg/kg) of L-NAME (Sigma, USA) and one dose of aminoguanidine (50 mg/kg, Sigma, USA). The 25-mg/kg dose of L-NAME and 50-mg/kg dose of aminoguanidine have been shown previously to have no significant effect on body temperature of afebrile rats (Kamerman et al. 2001). However, the higher dose of L-NAME (50 mg/kg) has been shown to cause nocturnal, but not diurnal, hypothermia in rats (Kamerman et al. 2001), and was injected to determine whether administration of such a dose of L-NAME may enhance any affects L-NAME may have on fever.

Experimental procedure

Each animal received a single dose of one of the nitric oxide synthase inhibitors in combination with one of the pyrogens. Nitric oxide synthase inhibitors were administered intraperitoneally and the pyrogens intramuscularly. As a control, animals also were injected with saline together with an appropriate dose of synthase inhibitor or pyrogen. Injections were made, in random order, at 0900 hours and pyrogen injections were separated by 14 days, to avoid pyrogenic tolerance.

Data analysis

Data are expressed as mean ± SD. For statistical purposes, the temperature of each animal was averaged over hourly intervals for the 9-h period following each injection. Two-way repeated measures analysis of variance, followed by a Student-Newman-Keuls (SNK) post hoc test, was used to detect for differences within and between groups. In cases where analysis of variance detected significant interaction between main effects, interpretation of the data was based on the results of the post hoc test (Glantz and Slinker 1990).

Results

All injections caused a brief stress-hyperthermia, which lasted for approximately 1 h. The magnitude of the stress-hyperthermia (average magnitude: 38.3 ± 0.3 °C) was not significantly affected by the administration of nitric oxide synthase inhibitors (Fig. 2) nor pyrogen (Figs. 3, 4). The agents also did not alter the time taken for the stress-hyperthermia to reach its peak (average time to peak temperature: 20 ± 10 min).

The effects of intramuscular injections of lipopolysaccharide and muramyl dipeptide on the body temperature of the rats are shown in Fig. 1. Injection of lipopolysaccharide induced a robust, biphasic fever lasting approximately 8 h (Fig. 1A). Both doses of muramyl dipeptide induced fevers that were similar in magnitude, latency and duration (~ 5 h) to each other (Fig. 1B). The fevers induced by muramyl dipeptide, however, differed from those induced by lipopolysaccharide, though their

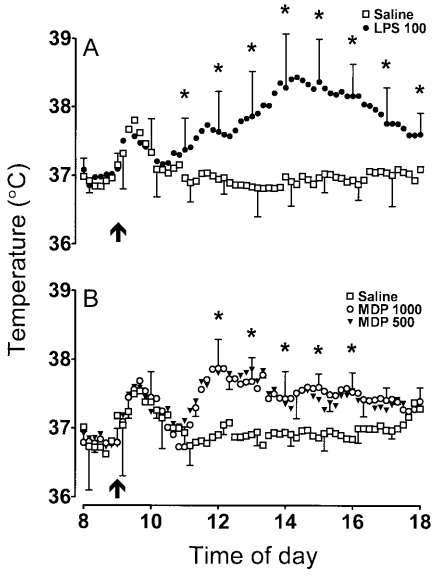

Fig. 1. Mean (SD) body temperature as a function of time of day of rats ($n = 5$) injected intramuscularly with (**A**) 100 μg/kg lipopolysaccharide (*LPS 100*) or saline and (**B**) 1000 μg/kg or 500 μg/kg muramyl dipeptide (*MDP 1000* and *MDP 500*, respectively) or saline. $^*P < 0.05$

latencies were similar. Whereas fevers induced by lipopolysaccharide administration took up to 6 h to reach peak temperature, muramyl dipeptide fevers took only 3 h on average. Also, peak febrile temperature was significantly lower in the muramyl dipeptide than lipopolysaccharide injected animals (one-way ANOVA, $F_{(2,11)} = 6$, $P = 0.018$). As the fevers induced by intramuscular injection of 1000 μg/kg and 500 μg/kg muramyl dipeptide were similar (Fig. 1B), we decided to use the lower dose for the remainder of the study.

Fig. 2 shows the effects on body temperature of an intramuscular injection of saline, rather than a pyrogen, together with an intraperitoneal injection of saline, L-NAME (Fig. 2A, B), and aminoguanidine (Fig. 2C). No statistically significant differences were found between the temperatures of the animals given saline or synthase inhibitors, confirming that the inhibitors, at the doses employed, do not affect daytime body temperature in afebrile rats.

The effects of inhibiting the synthesis of nitric oxide on fevers induced by muramyl dipeptide are shown in Fig. 3. Following the stress-hyperthermia, the 25-mg/kg doses (Fig. 3A; SNK, $P < 0.05$) and the 50-mg/kg doses (Fig. 3B; SNK, $P < 0.05$) of L-NAME caused a 3–4 h attenuation of the febrile response to muramyl dipeptide. On average, the 50-mg/kg dose of L-NAME inhibited the febrile response for approximately 1 h longer

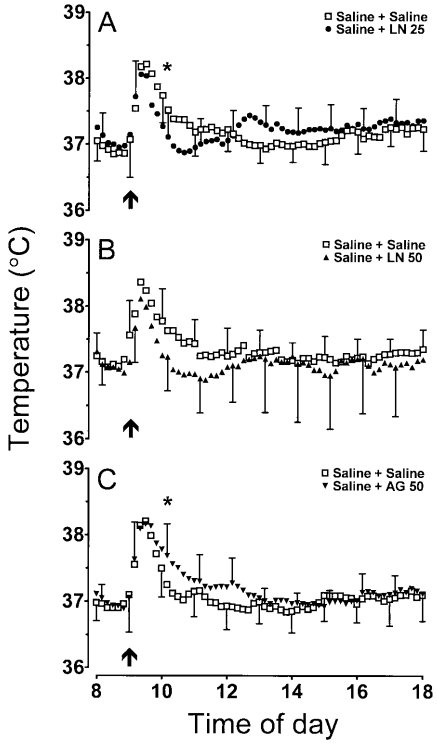

Fig. 2. Mean (SD) body temperature as a function of time of day of rats injected intramuscularly with saline and intraperitoneally with (**A**) 25 mg/kg L-NAME (*LN 25*, $n = 12$) or saline, (**B**) 50 mg/kg L-NAME (*LN 50* $n = 10$) or saline, and (**C**) 50 mg/kg aminoguanidine (*AG 50*, $n = 13$) or saline

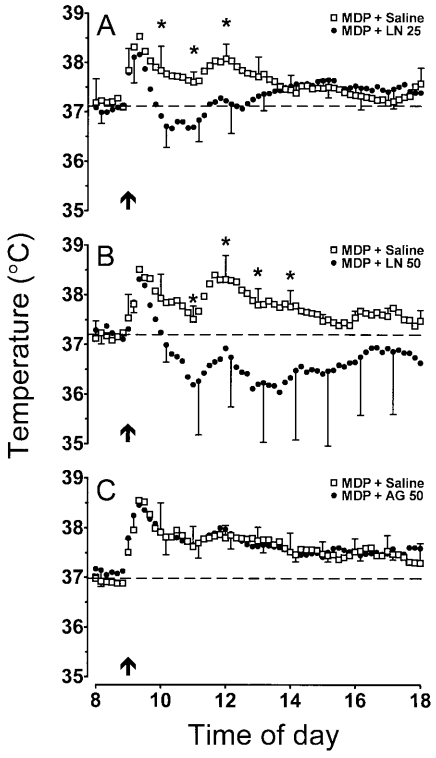

Fig. 3. Mean (SD) body temperature as a function of time of day of rats injected intramuscularly with 500 μg/kg MDP and intraperitoneally with (**A**) 25 mg/kg L-NAME (*LN 25*, $n = 6$) or saline, (**B**) 50 mg/kg L-NAME (*LN 50* $n = 5$) or saline, and (**C**) 50 mg/kg aminoguanidine (*AG 50*, $n = 7$) or saline. The *dashed horizontal line* denotes the average pre-injection temperature. $^*P < 0.05$

than did the 25-mg/kg dose (Fig. 3A, B). Injection of aminoguanidine had no significant effect on the febrile response elicited by muramyl dipeptide administration (Fig. 3C).

The effects of administrating an inhibitor of nitric oxide synthase on the febrile response to lipopolysaccharide are shown in Fig. 4. Similar to their effects on muramyl dipeptide-induced fevers, 25 mg/kg (Fig. 4A; SNK, $P < 0.05$) and 50 mg/kg (Fig. 4B; SNK, $P < 0.05$) L-NAME inhibited the 1st 3–4 h of lipopolysaccharide-induced fevers. However, subsequent to the initial antipyresis, the body temperature of animals injected with L-NAME and lipopolysaccharide rose, such that there was no significant difference between the peak febrile temperature when the animals, injected with lipopolysaccharide, were given saline or L-NAME (Fig. 4A, B). Aminoguanidine had no antipyretic actions on lipopolysaccharide-induced fever (Fig. 4C).

Discussion

We have investigated the effects of administering aminoguanidine, a relatively selective inhibitor of inducible nitric oxide synthase, and L-NAME, an unspecific nitric oxide synthase inhibitor, on the febrile response of rats

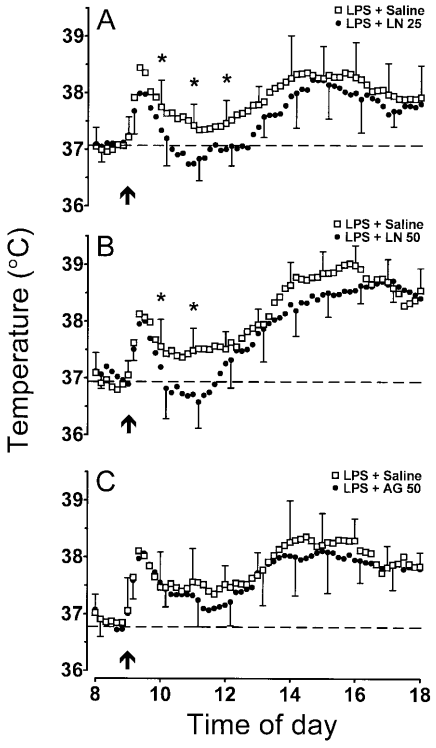

Fig. 4. Mean (SD) body temperature as a function of time of day of rats injected intramuscularly with 100 μg/kg LPS and intraperitoneally with (**A**) 25 mg/kg L-NAME (*LN 25*, $n=6$) or saline, (**B**) 50 mg/kg L-NAME (*LN 50* $n=5$) or saline, and (**C**) 50 mg/kg aminoguanidine (*AG 50*, $n=7$) or saline. The *dashed horizontal line* denotes the average pre-injection temperature. $*P<0.05$

to a gram-positive pyrogen, muramyl dipeptide, and a gram-negative pyrogen, lipopolysaccharide. We found that at the doses we employed, L-NAME inhibited the 1st 2–4 h of fevers induced by muramyl dipeptide and lipopolysaccharide, but aminoguanidine had no significant antipyretic actions. In addition, neither nitric oxide synthase inhibitor affected the magnitude or timing of the stress-hyperthermia induced by handling and injection procedures. Therefore, our results support the view that, in the rat, the full expression of fevers induced by gram-positive and gram-negative pyrogens requires the synthesis of nitric oxide by the constitutive, but not the inducible, synthase.

To facilitate correlation between our results in rats and those found previously in guinea pigs, we injected the nitric oxide synthase inhibitors and pyrogens by the same two routes we had used in guinea pigs (Kamerman and Fuller 2000). However, because of the relative insensitivity of rats to pyrogens (Kluger 1991), we had to use higher doses of muramyl dipeptide and lipopolysaccharide in our rats to approximate the magnitude of the fevers induced previously in guinea pigs, by the same pyrogens (Kamerman and Fuller 2000; Roth et al. 1998b, 1999). Also, we did not measure any markers of nitric oxide synthase activity, and therefore we have had to rely on previous research to infer that the agents we used, at the doses we employed, inhibited nitric oxide synthesis (Alden et al. 1998; Iadecola et al. 1994, 1995; Mehta et al. 1998; Rees et al. 1990). Finally, we injected only a single dose of aminoguanidine because, unlike L-NAME, higher doses of aminoguanidine do not affect nocturnal body temperature independently of daytime body temperature (Kamerman et al. 2001), and higher doses of aminoguanidine affect the activity of inducible and constitutive nitric oxide synthase (Mehta et al. 1998; Misko et al. 1993).

Previous studies on rabbits injected intrathecally with gram-positive (Huang et al. 1997) or gram-negative (Lin and Lin 1996a, 1996b; Lin et al. 1997) pyrogens, and inhibitors of the inducible nitric oxide synthase, indicated that the inducible isoenzyme may play a role in transducing pyrogenic signals over the blood brain barrier. However, constant with our current findings, when lipopolysaccharide and moderate doses of inhibitors of inducible nitric oxide synthase were administered systemically in guinea pigs (Kamerman and Fuller 2000; Roth et al. 1999) and pigs (Parrott et al. 1998), the fevers were not suppressed. When Roth and colleagues (1999) observed aminoguanidine to inhibit fevers induced by lipopolysaccharide, they attributed the antipyresis to spillover inhibition of constitutive nitric oxide synthases by high doses of aminoguanidine. Nevertheless, aminoguanidine, at a dose that does not affect constitutive nitric oxide synthase activity (Mehta et al. 1998), did inhibit muramyl dipeptide fevers in guinea pigs (Kamerman and Fuller 2000). The antipyretic effects of aminoguanidine in our guinea pigs, but not our rats, may reflect species differences in the pyrogenic pathways induced by muramyl dipeptide. For example, repeated administration of muramyl dipeptide does not readily lead to the development of pyrogenic tolerance in guinea pigs (Roth et al. 1997a, 1997b), but does so in rats (Ferreira et al. 2001). Moreover, Gath et al. (1999) found that inducible nitric oxide synthase is strongly induced in the brain of rats only during sepsis, and even then the latency to induction is several hours. So we do not think that the inducible isoenzyme plays an important role in fever genesis in rats. The ability to inhibit fevers in rabbits, with inhibitors of inducible nitric oxide synthase applied intrathecally (Huang et al. 1997; Lin and Lin 1996a, 1996b; Lin et al. 1997), probably reflects a species difference between rats and rabbits. Alternatively, inducible nitric oxide synthase may have a special role in the signal transduction mechanisms of pyrogens, when pyrogens are injected directly into the central nervous system.

Intraperitoneal or intravenous administration of inhibitors of constitutive nitric oxide synthase previously have been shown to reduce the magnitude of the entire febrile response to muramyl dipeptide (Kamerman and Fuller 2000) and lipopolysaccharide (Kamerman and Fuller 2000; Perotti et al. 1999; Roth et al. 1998b; Soszynski 2001). While we have shown that the unspecific nitric oxide synthase inhibitor, L-NAME, attenuates the entire febrile response to muramyl dipeptide, L-NAME reduced only the first few hours of lipopoly-

saccharide fevers. However, the antipyresis induced by L-NAME did occur in the same 3–4 h post-injection period following pyrogen administration, in both lipopolysaccharide and muramyl dipeptide fevers. Therefore, the discrepancy in the duration of antipyresis induced by L-NAME probably reflects differences in the duration of muramyl dipeptide and lipopolysaccharide fevers, rather than pyrogen-dependent differences in the role of nitric oxide synthase in fever genesis. That is, given that muramyl dipeptide fevers lasted only 5 h on average in our rats (Fig. 1B) and lipopolysaccharide fevers lasted approximately 8 h (Fig. 1A), it is not surprising that L-NAME inhibited the entire gram-positive fever, but only the first half of the gram-negative fever, if L-NAME was active for only 3–4 h.

We are not the first to show that inhibition of nitric oxide synthase attenuates only the first hours of a lipopolysaccharide-induced fever. Roth and colleagues (1999) showed that inducible nitric oxide synthase inhibitors, at doses that probably inhibited the constitutively expressed enzyme, affected only the first phase of lipopolysaccharide fever in guinea pigs. Since cytokine release is not affected by L-NAME administration (Roth et al. 1998b, 1998c), we do not believe that alteration by L-NAME of cytokine release, or cytokine signalling, was responsible for the suppression of the early part of the fever in our rats. Moreover, whether altered secretion of cytokines that rise in the early part of a fever, such as tumour necrosis factor, causes antipyresis is equivocal (Long et al. 1990; Mabika and Laburn 1999; Roth et al. 1994, 1998a). Although we do not believe that cytokines were affected, inhibition by L-NAME of a cytokine-independent pathway, for example, the putative pyrogen-activated toll-like receptor pathway (Dinarello et al. 1999), may have been responsible for suppressing the early phase, but not the cytokine-dependent late phase of the fever. It remains possible, though, that species differences, or the high pyrogen doses we used relative to the L-NAME dosages, may explain the incomplete inhibition of lipopolysaccharide fever in our rats.

The contribution of nitric oxide to fever genesis usually is considered to reside in the pathways that lead to an elevation in set-point temperature. An alternative hypothesis is that nitric oxide is required in effector pathways, particularly those responsible for elevating metabolism during a fever. So the antipyretic action of L-NAME, for example, could result from reduced sympathetic output to brown adipose tissue (De Luca et al. 1995) or decreased brown adipose tissue flood flow (Nagashima et al. 1994). However, nitric oxide also acts as an inhibitor of cytochrome oxidase (Brown 2000), so that blocking nitric oxide synthesis removes this inhibition, and elevates metabolic rate (Koivisto et al. 1997; Shen et al. 1994). Whether metabolism is increased or decreased by inhibitors of the constitutive isoenzymes probably depends on the relative contribution of constitutive nitric oxide synthase to each of the opposing pathways. That body temperature usually is depressed by high doses of nitric oxide synthase inhibitors may reflect a predominance of the depressing effects of the inhibitors on metabolism. There is no evidence, though, against the hypothesis that nitric oxide is involved in set-point elevation during fever.

In addition to the effects that L-NAME has on fever, the drug recently has been reported to inhibit open-field stress hyperthermia in rats (Soszynski 2001). The parallels between the actions of L-NAME and inhibitors of prostaglandin synthesis on fever and open-field stress hyperthermia (Kluger et al. 1987; Soszynski et al. 1998) support the concept that open-field stress hyperthermia is a true fever. However, both ourselves and Soszynski (2001) found that the hyperthermia induced by handling is not inhibited by L-NAME administration in rats. It may be that the pathways that mediate the elevation in temperature induced by handling are not the same as those induced by pyrogens or open-field stress. Alternatively, the pathways may be the same, but in the case of handling, the pathway may be induced too rapidly for the simultaneous intraperitoneal injection of nitric oxide synthase inhibitors to have an effect on them.

In conclusion, we have shown that intraperitoneal administration of aminoguanidine, an inhibitor of inducible nitric oxide synthase, does not inhibit fevers induced by intramuscular administration of a gram-positive or a gram-negative pyrogen in rats. On the other hand, L-NAME, an unspecific inhibitor of all nitric oxide synthase isoforms, inhibited the entire febrile response to muramyl dipeptide, and the 1st 2–4 h of longer-lasting lipopolysaccharide fevers. These findings imply that the constitutive nitric oxide synthases, rather than the inducible isoenzyme, have a role in the development of short-duration gram-positive fevers and the early stages of long-duration gram-negative fevers in rats. Our study also provides evidence of species differences in the antipyretic actions of L-NAME and aminoguanidine on gram-positive and gram-negative fevers, and evidence against stress hyperthermia, induced by handling, being a fever.

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