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INTRACEREBROVENTRICULAR INJECTION OF NEURONAL AND INDUCIBLE NITRIC OXIDE SYNTHASE INHIBITORS DOES NOT INFLUENCE FEBRILE RESPONSE IN RATS DURING TURPENTINE ABSCESS

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The purpose of this study was to investigate the role of neuronal nitric oxide synthase (nNOS) and inducible NOS (iNOS) in the brain during development of fever in response to localized tissue inflammation caused by injection of turpentine in freely moving biotelemetered rats. To determine the role of both NOSs in turpentine-induced fever, we injected vinyl-L-NIO (N^5 – (1-Imino-3-butenyl) – ornithine (vL-NIO), a selective nNOS inhibitor, and aminoguanidine hydrochloride, a selective iNOS inhibitor, intracerebroventricularly (*i.c.v.*) 5 h after turpentine injection. Rats responded with fever to intramuscular injection of 20 µl of turpentine that commenced about 5 - 6 h after injection and reached peak value between 9 - 11 h post-turpentine. The inhibition of nNOS as well as iNOS in the brain did not affect fever induced by turpentine. Fevers in control rats (treated *i.c.v.* with pyrogen-free water) and iNOS or nNOS inhibitor-*i.c.v.* treated rats injected with turpentine were essentially the same. Furthermore, on the basis of these data, we concluded that iNOS and nNOS inside the brain do not participate in generation of fever to turpentine in rats.

Key words: *body temperature, turpentine, fever, neuronal nitric oxide synthase, inducible nitric oxide synthase, aminoguanidine, vL-NIO, biotelemetry, rat*

INTRODUCTION

Over the past decade, it has been realized that nitric oxide (NO) plays a variety of regulatory functions *in vivo* (1). This free radical gas is generated from

the amino acid L-arginine by three types of nitric oxide synthases (NOS). Two of them, endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutive isoforms and calcium-dependent. eNOS is present in endothelial cells and is involved in regulating of blood vessel tone and – consequently – in organ perfusion (1). The constitutively expressed nNOS is the most abundant isoform in the brain and it can be found in different anatomical and functional regions of the brain, including the hypothalamus (2). The third NOS isoform - a Ca^{2+}-independent inducible enzyme (iNOS) - is preferentially expressed in macrophages (3). This isoenzym is also present in the brain (4, 5). Although astrocytes and microglia have been suggested to be the main source of NO produced by iNOS (6), neurons also have iNOS activity (7).

NO has been well established as an important mediator/modulator of many physiological and pathophysiological phenomena, particularly within cardiovascular, nervous, and immune systems, including thermoregulation and fever (8). Several lines of evidence support hypothesis that NO is an important mediator of lipopolysaccharide (LPS)-induced fever. LPS as well as cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α (well-known endogenous mediators of fever to LPS) are able to increase the expression of NOS's in peripheral tissues and within the brain (1, 9, 10, 11). Moreover, plasma nitrite and nitrate concentrations (two stabile metabolites of NO) have been shown to rise in response to peripheral injection of endotoxin (12). Recently, it has been shown that iNOS and nNOS are responsible for the induction of LPS fever, whereas eNOS is not involved (13). Finally, there is some evidence suggesting the mechanism by which NOS and cyclooxygenase systems within the brain operate together to induce a febrile response (14). The enzymatic activation of cyclooxygenase-2 (COX-2) appears critically important for synthesis of PGE_2, upregulation of COX-2 activity is considered to be necessary for fever production (15, 16).

Whereas NO has been accepted as a potent endogenous mediator of fever due to injection of LPS, conflicting data have been published regarding the induction of NO formation by turpentine. Our previous studies have established that blockade of NO formation by intraperitoneal injection of N^{ω}-nitro-L-arginine methyl ester (L-NAME) enhanced febrile and behavioral responses in rats during turpentine abscess (17). Since L-NAME, a non-selective NOS inhibitor, is capable of crossing the blood-brain barrier (18), the results of studies with systemically administered L-NAME do not allow us to determine the site of the action (*i.e.* inside or outside the central nervous system) of L-NAME as well as to ascertain which of three NOS isoforms is indeed involved in development of turpentine fever. Therefore the purpose of the present study was to assess the effects of two highly selective NOS inhibitors, aminoguanidine (iNOS inhibitor) and vinyl-L-NIO (N^5 – (1-Imino-3-butenyl) – ornithine (nNOS inhibitor) injected into the lateral ventricle on fever response due to turpentine in rats.

MATERIALS AND METHODS

Animals and experimental conditions

Specific-pathogen-free adult male Wistar rats weighing 300-320 g were used throughout the study. Animals were housed individually in plastic cages and maintained in temperature-, humidity-, and light-controlled chamber set at 12:12 h light/dark cycle (lights on at 0600 h), and at 21 ± 1°C. They had free access to food and drinking water. These experiments have been approved by our University Committee on the Use and Care of Laboratory Animals.

Surgery

Body temperature (T_b) of the rats were monitored by precalibrated battery-operated miniature telemetry transmitters (model VM-FH; Mini-Mitter Co., Bend, OR, USA) implanted intraperitoneally. Signals for body temperature were collected at 5 min intervals with a peripheral processor (VitalView 3000; Mini-Mitter Co., Bend, OR, USA) connected to an IBM personal computer. For intracerebroventricular (*i.c.v.*) administration of nNOS and iNOS inhibitors or appropriate control, rats were stereotaxically implanted with 5 mm long 26-gauge steel cannulae (Plastic Products Co., Roanoke, Va., USA) into the lateral ventricle. The coordinates were 1 mm posterior to the bregma and 0.5 mm lateral to the midline (coordinates according to the atlas of Pellegrino *et al.* 19). Before implantations rats were anesthetized with an intramuscular (*i.m.*) injection of a mixture of ketamine (87 mg/kg body weight) and xylazine (13 mg/kg). All surgeries were made at least 1 week before the start of experimental procedure. After the completion of experiments, the animals were sacrificed and then dye was injected through the cannula to mark the ventricular space. The brain section was visually examined to verify that the tip of the stainless steel cannula was located in the right lateral ventricle. Moreover, after experiments, the transmitters were recalibrated to verify calibration values.

Injections

Local inflammation was induced with commercial-grade stem-distilled turpentine (Dorex, Poland). Non-diluted turpentine was injected intramuscularly (*i.m.*) into the left hindlimb of gently restrained rats at a volume of 20 µl/animal with the use a 50-µl Hamilton syringe. Control rats were similarly restrained, and an equal volume of sterile (0.9% sodium chloride), nonpyrogenic saline was administered *i.m.* into the left hindlimb.

Vinyl-L-NIO (N^5 – (1-Imino-3-butenyl) – ornithine; Alexis Biochemicals, USA), a neuronal NOS (nNOS) inhibitor, and aminoguanidine hydrochloride (Sigma, St. Louis, MO, USA), an inducible NOS (iNOS) inhibitor, were dissolved in pyrogen-free water. Both inhibitors were prepared freshly on the day of experiment. For *i.c.v.* injections, rats were restrained in a towel while the dummy cannula was removed and the injection cannula inserted into the guide cannula. Injections were made using a Hamilton syringe. The final volume for *i.c.v.* injection was 5 µl/rat. After injection, the injection cannula was removed and the dummy cannula replaced. Control rats were injected *i.c.v.* with an equal volume of pyrogen-free water. To avoid any effects of the circadian variation in T_b, all injections were made between 8:00 and 9:00 a.m.

Experimental Protocol

The effects of intracerebroventricular injection of nNOS and iNOS inhibitors were tested on thermal response to turpentine in rats. Since the latency of fever onset after turpentine injection was about 5 - 6 h, the 5-h period between *i.m.* injection of turpentine and *i.c.v.* injection of NOS inhibitors

was used in our experiments. In experiment 1, four groups of rats were used to test the role of nNOS in the rat brain in development of febrile response to turpentine: (1) Turpentine 20 µl/rat *i.m.* along with 10 µg/rat vL-NIO *i.c.v.*; (2) Saline 20 µl/rat *i.m.* along with 10µg/rat vL-NIO *i.c.v.*; (3) Turpentine 20 µl/rat *i.m.* along with pyrogen-free water *i.c.v.*; (4) Saline *i.m.* along with pyrogen-free water *i.c.v.*. In experiment 2, four groups of rats were used to test the role of iNOS in the rat brain in pathogenesis of fever due to turpentine injection: (1) Turpentine 20 µl/animal *i.m.* along with 10 µg/rat aminoguanidine *i.c.v.*; (2) Saline 20 µl/animal *i.m.* along with 10 µg/rat aminoguanidine *i.c.v.*; (3) Turpentine 20 µl/rat *i.m.* along with pyrogen-free water *i.c.v.*; (4) Saline *i.p.* along with pyrogen-free water *i.c.v.*. The rationale for using inhibitors at an *i.c.v.* dose of 10 µg/rat is based on our previous experiments that clearly indicated that the dose of 10 µg/rat for both inhibitors was the highest dose that did not affect the normal body temperature in rats (unpublished data).

In both experiments, the T_b was recorded for 2 h before the turpentine or saline (as control treatment) injection and 22 h afterwards. To assess a difference in the magnitude of febrile response during the development of febrile response due to turpentine, changes in T_b from 5 to 15 h after turpentine injection were averaged and the 10-hour fever index (FI; expressed as °C x h) was calculated. The mean values of body temperature 1 h before the turpentine or saline injections were designated as baseline temperatures.

Statistical analysis

All data are reported as means ± S.E. Data collected for T_b at 5-min intervals were pooled into 15-min averages before statistical analysis and graphical presentation. All comparisons between groups were made using one-factor ANOVA followed by pairwise comparisons by Fisher' protected least significant difference. Values of $P < .05$ were considered to be significant different.

RESULTS

Regardless of the treatment all rats responded with a sharp increase in T_b at the time of handling and injection. Intraperitoneal injection of saline as well as intracerebroventricular injection of pyrogen-free water led to a sharp and transient rise in T_b which lasted approximately 1.5 – 2 h (*Fig. 1, 3*). This increase in T_b was followed by a gradual fall of T_b to a normal daytime level. This indicates that either intramuscular administration of saline or intracerebroventricular injection of pyrogen-free water does not affect normal T_b in rats.

Effect of i.c.v. administration of vL-NIO on febrile response due to turpentine injection

Rats injected *i.m.* with saline and then treated with vL-NIO or pyrogen-free water displayed normal circadian rhythm in T_b postinjection: temperature low during day-time and high during night-time (*Fig. 1*). Moreover, there was no significant difference in the mean day-time and night-time T_b between water-treated and vL-NIO-injected rats (data not shown). The mean day-time temperature of v-LNIO-injected rats was 37.71 ± 0.09°C (*vs.* 37.70 ± 0.10°C in controls) and night-time was 38.50 ± 0.08°C (*vs.* 38.47 ± 0.13°C in controls). This indicates that vL-NIO injected into the brain at a dose of 10 µg/animal does not affect normal T_b in rats.

As can be see in *Fig. 1*, rats responded with fever to the sterile turpentine abscess. The fever due to turpentine commenced about 6 h after injection and reached a peak value of 39.60 ± 0.37°C 9 h after injection. By comparison at this time point the T_b of saline controls reached 37.97 ± 0.25°C. There was a significant difference between saline- and turpentine-injected rats in the 10-hour fever index (17.37 ± 1.12°C x h for turpentine-injected rats *vs.* 5.70 ± 1.19°C x h for saline-treated rats; P<0.0001) (*Fig. 2*). The relatively high value of fever index for saline-treated rats is related to the time period chosen for the calculation of the fever index which corresponded to light-on and off conditions during the 12-hour-light/12-hour-dark cycle (5-15 h after turpentine injection). As shown in *Fig. 1*, *i.c.v.* injection of vL-NIO at a dose of 10 µg/animal did not affect turpentine-induced fever. The latency of fever onset was similar in both pyrogen-free water and vL-NIO injected turpentine-treated rats. The pattern of changes in T_b following turpentine injection did not differ markedly between vL-NIO- and pyrogen-free water injected rats. Either vL-NIO injected or pyrogen-free water treated rats responded with a sharp rise in T_b that was especially seen during the period 6 - 9 h after turpentine injection. As a result, there was no statistically significant difference in fever index calculated for 10 h between turpentine-pretreated rats injected with vL-NIO and turpentine-pretreated rats

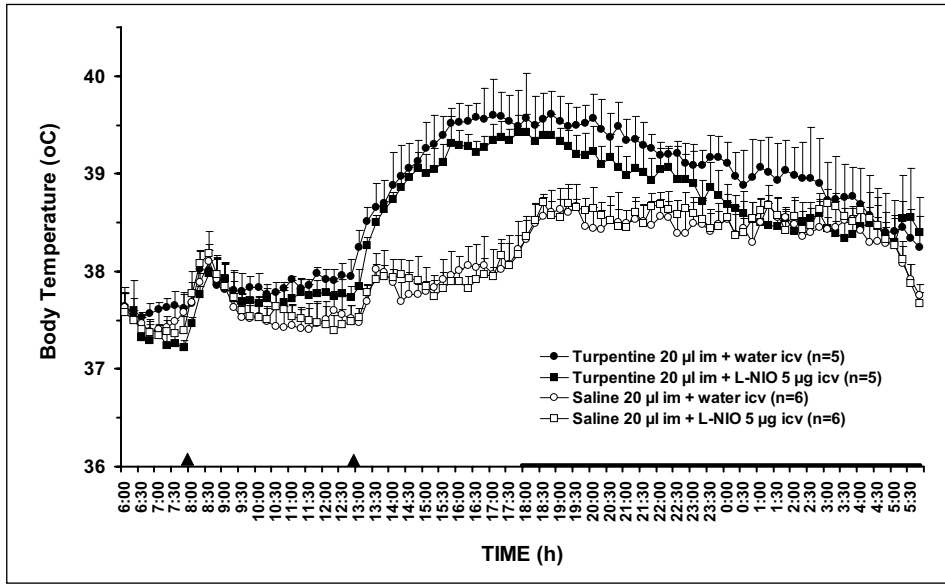

Fig. 1. Change in body temperature over time of rats injected intramuscularly with turpentine at a dose of 20 µl/rat and intracerebroventricularly with vL-NIO at a dose of 10 µg/animal or pyrogen-free water as a control 5 h after turpentine. Arrowheads indicate time of injection. Black horizontal bar indicates dark period in 12-hour-light/12-hour-dark cycle. Values are means ± SE. Sample sizes are indicated in parentheses.

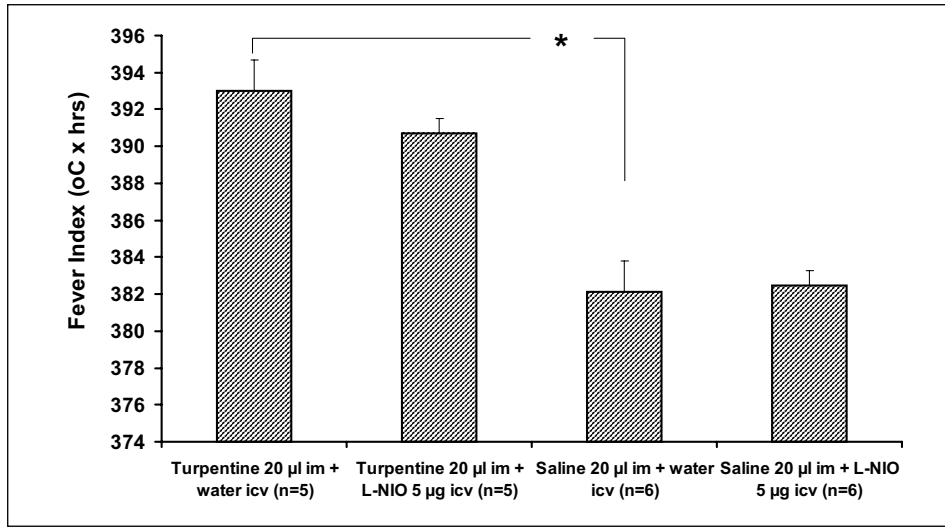

Fig. 2. Effect of vL-NIO, a selective nNOS inhibitor, injected *i.c.v.* at a dose of 10 µg/rat on fever index for 10 h during turpentine (20 µl/rat *i.m.*)-induced fever. Rats were injected with turpentine and vL-NIO or pyroge-free water as a control 5 h after turpentine treatment. Values are means ± S.E. Sample size is indicated in parentheses.

injected with pyrogen-free water (17.98 ± 0.64°C x h *vs.* 17.37 ± 1.12°C x h) (P = 0.415; *Fig. 2*).

Effect of i.c.v. administration of aminoguanidine on febrile response due to turpentine injection

Fig. 3 shows the mean abdominal temperature of four group of rats pretreated intramuscularly with turpentine at a dose of 20 µl/animal or saline and then injected intracerebroventricularly with pyrogen-free water or aminoguanidine. Rats pretreated *i.m.* with saline and injected 5 h later with aminoguanidine or pyrogen-free water displayed normal circadian rhythm in T_b postinjection: temperature low during day-time and high during night-time (*Fig. 3*). There was no significant difference in the mean day-time and night-time T_b between water-treated and aminoguanidine-injected rats (data not shown). The mean day-time temperature of aminoguanidine-injected rats was 37.81 ± 0.06°C (vs. 37.82 ± 0.10°C in controls) and night-time was 38.33 ± 0.09°C (vs. 38.55 ± 0.15°C in controls). This indicates that aminoguanidine at an *i.c.v.* dose of 10 µg/animal does not affect normal T_b in rats. Intramuscular administration of turpentine into rats later injected *i.c.v.* with pyrogen-free water induced fever, which started within 6 hour from the injection, lasted about 10-11 h, and reached a peak value of 39.55 ± 0.37°C 11 h after turpentine injection (*Fig. 3*). By comparison at the same time point the abdominal temperature of saline controls was 38.62 ± 0.23°C. Moreover,

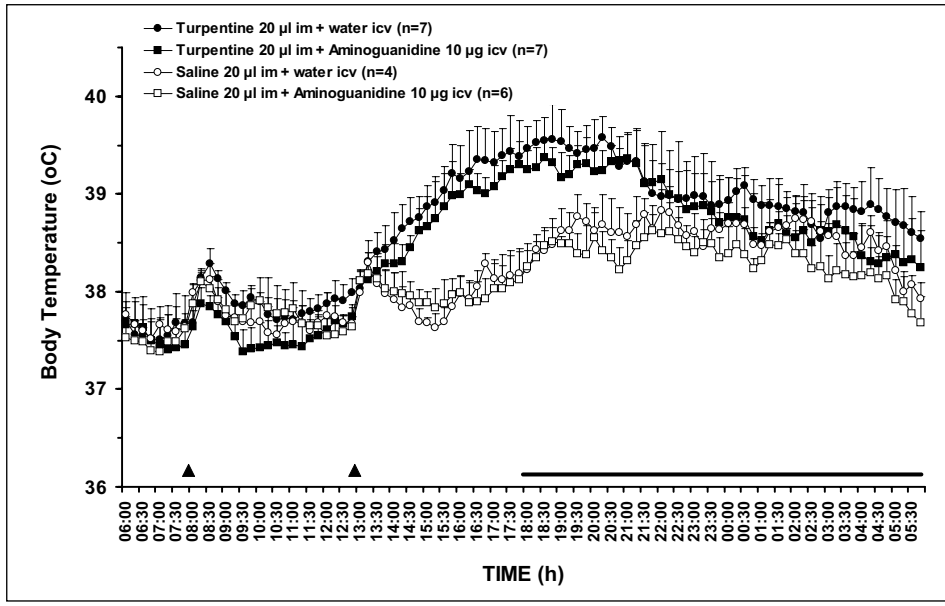

Fig. 3. Effect of aminoguanidine, a selective inhibitor of iNOS, injected intracerebroventricularly at a dose of 10 µg/animal on time course of T_b during turpentine (20 µl/rat *i.m.*)-induced fever. Rats were injected with LPS and iNOS inhibitor or pyrogen-free water as a control 5 h after turpentine treatment. Values are means ± S.E. Arrowhead indicates time of injection. Sample size is indicated in parentheses. Black horizontal bar indicates dark period in 12:12-h light-dark cycle.

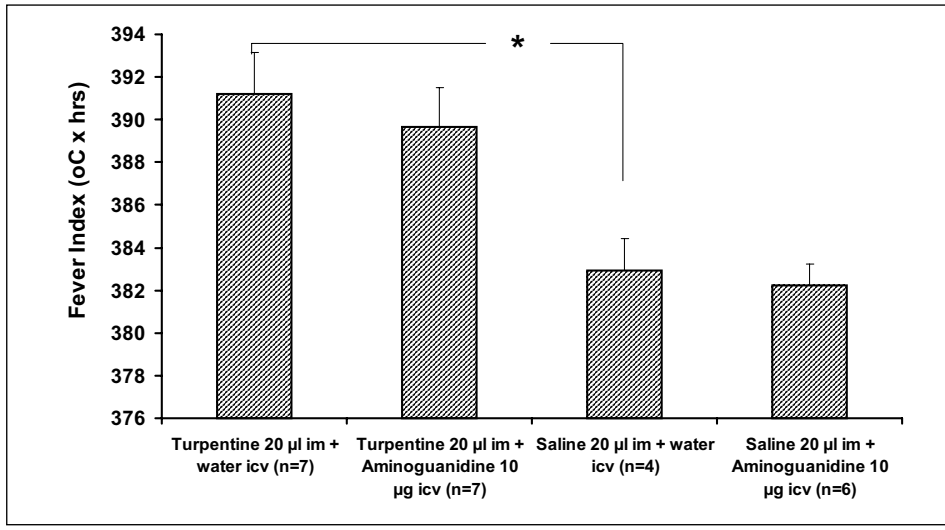

Fig. 4. Effect of aminoguanidine, a selective iNOS inhibitor, injected *i.c.v.* at a dose of 10 µg on fever index for 10 h during turpentine (20 µl/rat)-induced fever. Rats were injected with turpentine and aminoguanidine or pyroge-free water as a control 5 h post-turpentine. Values are means ± S.E. Sample size is indicated in parentheses.

there was a significant difference between saline- and turpentine-injected rats in the 10-hour fever index (14.92 ± 3.36°C x h for turpentine-injected rats *vs.* 5.42 ± 2.16°C x h for saline-treated rats; P<0.001) (*Fig. 4*). Inhibition of iNOS in the rat brain did not affect fever induced by turpentine. *Fig. 3* demonstrates that fevers due to turpentine in pyrogen-free water and aminoguanidine injected rats were essentially the same in terms of latency and time course of fever, indicating that iNOS cannot be responsible for turpentine-induced fever. Moreover, both fevers reached a maximal values at the same time point – 11 h post-turpentine (39.55 ± 0.37°C for pyrogen-free water/turpentine group of rats *vs.* 39.42 ± 0.19°C for aminoguanidine/turpentine group of animals). Therefore, there was no significant difference in fever index calculated for both group of rats (14.92 ± 3.36°C x h *vs.* 15.10 ± 1.24°C x h for turpentine pretreated rats injected with pyrogen-free water or aminoguanidine, respectively; P = 0.912) (*Fig. 4*).

DISCUSSION

The present results are the first to demonstrate that inhibition of neuronal NOS as well as inducible NOS inside the brain do not influence fever due to intramuscular injection of turpentine in rat (*Fig, 1, 3*). Of note, vL-NIO and aminoguanidine, nNOS and iNOS selective inhibitor, respectively, injected at a dose of 10 μg/animal *i.c.v.* did not affect normal T_b. The fever indices calculated for 10-h period (5–15 h after turpentine treatment) did not significantly differ compared to that calculated for vehicle-injected and turpentine-treated rats (*Fig. 2, 4*). Analyses of changes in body temperature as a function of time indicate that fevers to turpentine in vehicle-injected and vL-NIO or aminoguanidine injected rats were essentially the same in terms of latency and time course of fever. Our results on rats are consistent with a results obtained recently on mice, which demonstrated that deficiency in constitutive nNOS (nNOS KO mice) as well as in inducible NOS (iNOS KO mice) did not influence fever induced by injection of turpentine (13). However, they also found that the lack of endothelial NOS (eNOS KO mice) led to exaggeration of turpentine fever (13). Based of these results they concluded that eNOS is responsible for down-regulation of turpentine fever in mice. Moreover, we (17) and others (13) have also shown that after injection of turpentine, fevers were significantly higher in rats or mice treated with L-NAME, a nonspecific inhibitor of all three NOS isoenzymes. These data indicate indirectly that augmentation of febrile response due to turpentine is predominantly related to an inhibition of endothelial NOS. Taken together, our results along with results published by others (13) strongly suggest that both iNOS and nNOS inside the brain do not participate in generation of fever due to turpentine injection in rats. It indicates, furthermore, that neither of NOS studied in the present paper contributes to generation of fever during localized

inflammation, and from three NOS isoforms only eNOS is able to modulate febrile response to turpentine.

Data obtained with gene knockout mice support a serial pathway of cytokine production in the regulation of turpentine fever: (i) interleukin (IL)-1β knockout mice do not develop fever after turpentine injection (20), (ii) IL-1 type I receptor (IL-1rtI) knockout mice and interleukin-6 (IL-6) knockout mice are resistant to turpentine fever (21, 22). It has been found that NO may downregulate IL-6 as well as IL-1 production in macrophages activated by exogenous pyrogens (23, 24). Therefore, it is possible that the augmentation of fever following turpentine treatment by NOS inhibitors is due to the lack of a negative feedback of NO on the production of both cytokines. This conclusion still needs further verification by the measurement of IL-1β and IL-6 plasma levels during febrile response to turpentine with or without inhibition of NOS activity. The current model of fever assumes that the pyrogenic activity of proinflammatory cytokine requires the initiation of the cyclooxygenase pathway of arachidonic acid, and consequently production of prostaglandin E_2 where PGE_2 is considered to be a proximal centrally acting mediator of fever (25). Administration of cyclooxygenase inhibitor suppressed febrile rise in body temperature during localized inflammation, indicating that this fever response is PGE_2-dependent phenomena (26). As mentioned in Introduction, there are some evidence, suggesting the mechanism in which NOS and cycloxygenase systems within the brain operate together to induce a febrile response (14). Therefore, it was interesting to investigate whether PGE_2 synthesis is NO-dependent. It has been reported, however, that the elevation of plasma levels of PGE_2 measured 24 h after injection of turpentine did not differ between control and L-NAME-treated mice (13).

In view of the data pointing NO as an antipyretic molecule within the brain, one could suggest that inhibition of NO synthesis could be responsible for the enhancement of activity of hypothalamic-pituitary-adrenal axis in rats injected with turpentine (27). It is well documented that glucocorticoids are able to inhibit both biosynthesis and biological activity of proinflammatory cytokines (28, 29). Since glucocorticoids are potent inhibitors of the induction of NOS (30), it is possible that glucocorticoids and nitric oxide act in negative feedback.

Since turpentine did not change concentration of NO in plasma (12), the role of NO, as mediator/modulator, in development of turpentine fever appears to be controversial and needs further experimental verification.

Recently, it has been shown that intracerebroventricular injection of nNOS and iNOS inhibitors attenuates febrile response due to LPS in rat, suggesting that upregulation of iNOS as well as nNOS activity in the brain is critically important for LPS fever production (31).

The presented data provide strong evidence that enzymatic activity of iNOS and nNOS within the brain is not involved in turpentine-induced fever and suggest that molecular scenario of fever induction on the level of the central

nervous system during localized inflammation is differ from that during systemic inflammation due to LPS injection.

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