Role of the Nitric-Oxide Synthase Isoforms during Morphine-Induced Hyperthermia in Rats

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ABSTRACT
Recently, we demonstrated that the diffusible messenger molecule nitric oxide (NO) is involved in the hyperthermic response induced by morphine by using a nonselective nitric-oxide synthase inhibitor, N-nitro-L-arginine methyl ester. The present work extended these studies to include 7-nitroindazole (7-NI), an inhibitor specific for neuronal nitric-oxide synthase (nNOS), N(S)-(5-iminoethyl)-L-ornithine (L-NIO), an inhibitor of endothelial NOS (eNOS), and aminoguanidine (AG), a potent inhibitor of inducible NOS (iNOS). A biotelemetry system was used in this study to measure the body temperature (Tb). A dose of 7-NI (5 or 10 mg/kg), which did not affect Tb by itself, blocked the hyperthermia induced by morphine in a dose-dependent manner (15 mg/kg i.p.). However, pretreatment with L-NIO (10–20 mg/kg) or AG (50 mg/kg) failed to alter the hyperthermia induced by morphine. L-NIO (10–20 mg/kg) or AG (50 mg/kg) had no effect on Tb. These results suggest the involvement of nNOS in morphine-induced hyperthermia.

Morphine has effects on a number of physiological functions, including antinociception, respiration, gastrointestinal function, blood pressure, immunoregulation, and thermoregulation. With respect to the last, previous results from this and other laboratories demonstrated that intracerebroventricular (i.c.v.) administration of selective μ receptor agonists produced hyperthermia ( Handler et al., 1992; Adler and Geller, 1993; Xin et al., 1997) that could be blocked or antagonized by selective μ receptor antagonists, whereas κ receptor agonists produced hypothermia (Xin et al., 1997) that could be blocked or antagonized by selective κ receptor antagonists ( Handler et al., 1992). These findings indicate that the hyperthermic response to opioids in rats is mediated by the μ receptor and the hypothermic response is mediated by the κ receptor (Xin et al., 1997). The thermoregulatory effects of δ opioid receptors are not as clear. Although some studies observed hypothermia after the application of δ receptor agonists (Spencer et al., 1988), others did not find a significant change in body temperature (Tb) ( Handler et al., 1992), or noted a biphasic effect (Salmi et al., 2003). In addition, several studies strongly support the involvement of the opioid system in the fever process. Thus, μ opioid receptor-selective antagonists given into the preoptic anterior hypothalamus block the fever induced by lipopolysaccharide (Benamar et al., 2000) and interleukin-6 (Benamar et al., 2002).

Nitric oxide (NO), recently recognized as a prominent second messenger (Breder and Saper, 1996), is produced by the enzyme nitric-oxide synthase (NOS) that utilizes L-arginine to make L-citrulline and the radical gas NO. NOS has been found in peripheral and central neurons (Bredt and Snyder, 1992). Three different isoforms of NOS have been described ( Lopez-Figueroa et al., 1998). Two are constitutive forms, endothelial and neuronal (Moncada et al., 1991), and the third is inducible (Lowenstein et al., 1992). NOS was first described in the neurons of the central and peripheral nervous systems. eNOS is generally found in the endothelium of blood vessels responsible for vasodilatation. iNOS is located in the cytosol of cells in the immune system, in smooth muscles, and hepatocytes. It was originally described as inducible and is almost undetectable under basal conditions but is induced at the transcriptional level by LPS and cytokines (Xie et al., 1992).

Within the last few years, a number of studies have been conducted to investigate whether NO plays a role in temperature regulation, fever, and hypothermia. Some authors have suggested that NO has an antipyretic function (Moncada et al., 1991; Gourine, 1995), and some have shown that NO is involved in hypothermia (Branco et al., 1997; Steiner et al., 1998; Almeida and Branco, 2001). However, other reports provide evidence that the formation of NO participates in the

ABBREVIATIONS: Abbreviation: Tb, body temperature; NO, nitric oxide; NOS, NO synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS; 7-NI, 7-nitroindazole; L-NIO, N(S)-(5-iminoethyl)-L-ornithine; AG, aminoguanidine; L-NNAME, N⁴-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; DMSO, dimethyl sulfoxide.
development of a febrile response (Lin and Lin, 1996; Scammell et al., 1996; Roth et al., 1998; Benamar et al., 2000). It should be noted that these studies differed in strategies to assess the role of NO, species of experimental animal, pyrogen administered, and route of administration, as well as in inhibitors used.

Although NO has been implicated in the hyperthermia induced by morphine (Benamar et al., 2001), the NOS isoform involved in the induction of NO remains unknown. Therefore, the present study was undertaken to identify which type of NOS isoform is involved in morphine-induced hyperthermia, using selective inhibitors of the three types of NOS isoforms.

Materials and Methods

Animals. All animal use procedures were conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Zivic-Miller Laboratories, Zelienople, PA) weighing 250 to 300 g were used in this study. They were housed two per cage for at least 1 week before surgery and were fed laboratory chow and water ad libitum. The ambient temperature was 22 ± 2°C, and a 12 h light/dark cycle was used. All experiments were started between 9:00 and 10:00 AM to minimize the effect of circadian variation in Tb.

Surgery Procedures. Rats were anesthetized with an intraperitoneal injection of a mixture of ketamine hydrochloride (100–150 mg/kg) and acepromazine maleate (0.2 mg/kg). An incision 2 cm in length was made along the linea alba, and the underlying tissue was dissected and retracted. A transmitter (Mini-Mitter, Sunriver, OR) was implanted in the intraperitoneal space. After the transmitter was passed through the incision, the abdominal musculature and dermis were sutured independently. The animals were returned to individual cages in an environmental room.

Measurement of Body Temperature. One week after surgery, the rats were tested in an environmental room (21 ± 0.3°C ambient temperature and 52 ± 2% relative humidity). Tb was measured by a biotelemetry system (Mini-Mitter) using calibrated transmitters implanted i.p. Signals from the transmitter were delivered through a computer-linked receiver. This method minimizes stress to animals during the Tb reading. Thus, the Tb could be monitored continuously and recorded without restraint or any disturbance to the animal.

Drugs. Morphine sulfate (National Institute on Drug Abuse) was dissolved in sterile pyrogen-free saline. AG was purchased from Sigma-Aldrich (St Louis, MO) and was dissolved in saline. L-NIO and 7-NI were obtained from Sigma-Aldrich and dissolved in DMSO.

Statistical Analysis. All results were expressed as mean ± S.E.M. Statistical analysis of differences between groups was determined by analysis of variance followed by Tukey's post hoc test. A value of P less than 0.05 was considered statistically significant.

Results

Effect of Intraperitoneal Injection of 7-NI on Body Temperature. During the 180-min recording period, no significant change in Tb was observed after injection of 7-NI at doses of 5 or 10 mg/kg i.p., compared with the effect of an injection of an equivalent volume of vehicle (DMSO) (Fig. 1, P > 0.05). However, 7-NI at a dose of 20 mg/kg i.p. altered Tb significantly compared with the effect of an injection of an equivalent volume of vehicle (DMSO) (Fig. 1, P < 0.05). Accordingly, a dose of 5 or 10 mg/kg was used in these experiments to investigate the role of nNOS during morphine hyperthermia. Mean Tb before injection was 37.56 ± 0.14 for the 5 mg/kg 7-NI group, 37.63 ± 0.17 for the 10 mg/kg group, and 38.10 ± 0.18 for 20 mg/kg group.

Effect of Intraperitoneal Injection of 7-NI on Morphine-Induced Hyperthermia. 7-NI injected 30 min before morphine blocked the morphine-induced hyperthermia (Fig. 2). The effect of 7-NI was dose-dependent. Doses of 5 or 10 significantly suppressed the morphine-induced hyperthermia (Fig. 2). The effect of 7-NI was dose-dependent. Doses of 5 or 10 significantly suppressed the morphine-induced hyperthermia (Fig. 2). The effect of 7-NI was dose-dependent. Doses of 5 or 10 significantly suppressed the morphine-induced hyperthermia (Fig. 2).

Effect of Intraperitoneal Injection of AG on Morphine-Induced Hyperthermia. Pretreatment with AG (20 mg/kg) failed to alter the hyperthermic response during the 180-min recording period (Fig. 3, P > 0.05). No significant change in Tb was observed after AG injection compared with the effect of an injection of an equivalent volume of vehicle.
Altered morphine hyperthermia, L-NIO was ineffective and led to a clear involvement of NO during morphine-induced hyperthermia. Although L-NAME caused changes in the hyperthermic response induced by morphine, it is a nonselective NOS inhibitor and acts on both constitutive and inducible isoforms of the enzyme. To identify the NOS isoforms involved in morphine hyperthermia, we used nNOS, iNOS, and eNOS inhibitors. Pharmacokinetic studies show that 7-NI inhibits rat cerebellar NOS activity in vivo and in vitro in different brain regions with maximum inhibition occurring 30 min after i.p. administration and slowly returning to normal in 24 h (MacKenzie et al., 1994). Under the present experimental conditions, i.p. injection of 7-NI at doses of 5 or 10 mg/kg itself caused no modification in Tb. However, 7-NI blocked the hyperthermia induced by morphine (15 mg/kg) in a dose-dependent manner with maximum inhibition occurring at 30 min coinciding with the pharmacokinetics of 7-NI (MacKenzie et al., 1994). Our results demonstrate that nNOS participates in the hyperthermic effect of morphine, and they are compatible with the hypothesis that nNOS is involved in the mediation of other types of hyperthermia. Indeed, the hyperthermia caused by heat stress is associated with marked up-regulation of nNOS in many parts of the brain (Sharma et al., 1999). In addition, Western blotting identified nNOS in homogenates of whole brain, and specific immunofluorescence staining showed a high nNOS signal in the preoptic anterior hypothalamus during LPS-induced fever in rats (Gath et al., 1999). In addition, i.p. administration of 7-NI at a dose of 25 mg/kg significantly attenuated the rise in Tb elicited by restraint stress, indicating that the nNOS isoform plays an important role in the development of restraint stress-induced fever (Sanches et al., 2002). Similarly, pretreatment with 7-NI blocked the fever induced by LPS (Perroni et al., 1999).

Morphine is routinely used for pain management in the critically ill; thus, it is of clinical relevance to determine the role of NO as a mediator of the physiological and pathological process in the temperature responses induced by morphine. Recently we have demonstrated that L-NAME blocks the hyperthermia induced by morphine (Benamar et al., 2001). However, L-NAME produces an increase in blood pressure. In the present study we could prevent the morphine-induced hyperthermia by using 7-NI, which did not increase blood pressure (MacKenzie et al., 1994).

To investigate the role of iNOS in hyperthermia induced by morphine, we used AG. Pretreatment with a dose of AG (50 mg/kg) that did not affect the Tb did not alter the hyperthermia induced by morphine. Interestingly, a similar effect of AG (50 mg/kg) has been found in restraint stress-induced fever in rats.
(Sanches et al., 2002) and in LPS-induced fever in pigs (Roth et al., 1999). Also, it has been demonstrated that iNOS could neither be detected by immunofluorescence staining nor on Western blots in preparations from rats treated with a pyrogenic dose of LPS (Gath et al., 1999). Moreover, a very small population of iNOS-positive microglial cells appeared several hours after administration of LPS in rats (VanDam et al., 1995), and no significant elevation of NO activity is detected in brain a short time after LPS administration (Sehic et al., 1997). The iNOS is almost undetectable under basal conditions but is induced at the transcriptional level by LPS and cytokines (Xie et al., 1992) until the enzyme is synthesized. This is a process that requires several hours (Mustafa and Olson, 1998). The administration of morphine induced an increase in Tb starting 10 to 20 min after injection, before iNOS was produced. That AG failed to alter the hyperthermia induced by morphine seems reasonable. However, some authors have shown that iNOS is detected in hypothalamic cells (Miñano et al., 1997) after LPS administration, and intra-OVL (organum vasculosum of the lamina terminalis) injection of AG inhibited the fever induced by LPS (Lin and Lin, 1996). It should be noted that these studies varied in conditions used to assess the role of NO.

The implication of eNOS as the source of NO whose production was stimulated by LPS has been reported recently (Yang and Krukoff, 2000). Also, glial gene expression of eNOS was shown to increase in response to viral infection (Barna et al., 1996) suggesting that, under special circumstances, glia can be induced to produce greater amounts of eNOS. In this study, the possible participation of eNOS has been investigated by using L-NIO, an inhibitor that acts predominantly on eNOS but also has effects on nNOS. Neither 10 nor 20 mg/kg of L-NIO by itself altered Tb. The pretreatment with L-NIO did not affect morphine-induced hyperthermia, suggesting that eNOS does not play a role in mediating the hyperthermic response to morphine.

In conclusion, the present studies have demonstrated that morphine injected at a dose of 15 mg/kg i.p. in rats produced hyperthermia, and the effect could be blocked by an nNOS inhibitor but not by an iNOS or nNOS inhibitor, underscoring the differing roles of NOS isoforms in the hyperthermic processes. This finding indicates clearly that NO produced by nNOS mediates morphine-induced hyperthermia. However, eNOS and iNOS seem not to be involved in the morphine-induced hyperthermia.

References


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