Deletion of mu-opioid receptor in mice alters the development of acute neuroinflammation

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Abbreviations: MOP-KO, mu-opioid receptor knockout mice; WT, wild type; Tb, body temperature, LPS, lipopolysaccharide; NO, nitric oxide; IL-1, interleukin-1; IL-6, interleukin-6; TNF, tumor necrosis factor; MIP-1, macrophage inflammatory protein-1; DAMGO, [D-Ala2, N-Me-Phe-4, Gly-ol5]enkephalin; CNS, central nervous system.
Abstract

The realization that the mu opioid system plays a key role in the control of the process of neuroinflammation is a new concept that may lead to identification of novel therapies for this extremely widespread and intractable syndrome. Fever is the hallmark among the defense mechanisms evoked by the entry into the body of pathogens to initiate the innate immune responses. In an attempt to determine the possible involvement of mu opioid receptors in the control of brain inflammation, we examined the effect of their deletion on the fever induced by intracerebroventricular (ICV) injection of lipopolysaccharide (LPS). The first series of experiments examined the thermal consequence of the absence of mu opioid receptors on circadian body temperature rhythm and basal body temperature. Mu-opioid-receptor knockout mice (MOP-KO) showed a normal circadian body temperature rhythm and basal body temperature as compared to the wild type (WT). The second series of experiments investigated ICV administration of LPS on body temperature in WT and MOP-KO. In the WT, ICV injection of 100 ng LPS induced fever, but there was no increase in body temperature in the MOP-KO mice. Saline, given ICV, did not alter the body temperature, either in WT or MOP-KO. These results show that the mu opioid system participates in the control of acute neuroinflammation, further reinforcing our earlier finding that the opioid system is involved in the pathogenesis of fever induced by bacterial LPS, and that mu opioid receptors are the target for morphine-induced hyperthermia.
Introduction

Because it is now known that opioids have many diverse effects on the immune system, there is continuing interest in elucidating their actions. Morphine, given in vivo either parenterally or centrally, has been shown to suppress a variety of immune responses including Natural Killer (NK) cell activity (Carr et al, 1994, Lysle et al, 1993), antibody responses (Eisenstein and Hilburger, 1998), spleen and peripheral blood cell responses to mitogens (Bayer et al, 1990) and macrophage function (Bussiere et al, 1993, Rojavin et al, 1993). In addition, exogenous opioids have been shown to modify release of the cytokines interleukin-β and tumor necrosis factor-1α (IL-1β and TNF-α) (Pacifici et al, 2000). Studies examining the involvement of opioids in LPS-induced fever have reported an elevation in plasma, cerebrospinal fluid, and hypothalamic levels of β-endorphin during lipopolysaccharide (LPS) and endogenous pyrogen-induced fever (Carr et al, 1982, Leshin and Malven, 1984), and the mu opioid receptors within the preoptic anterior hypothalamus mediate, at least in the early phases, LPS-induced fever (Benamar et al, 2000).

Since the discovery that the opioid system has many diverse effects on the immune system and is involved in the pathogenesis of fever, targeting this system has represented a promising therapeutic approach. A prominent component of the acute-phase reaction to immune and inflammatory stimuli is the development of fever. Although fever is an important indicator for the severity of the inflammation, no one has investigated and/or linked this parameter with the opioid system during neuroinflammation. Using a model of acute neuroinflammation, we sought to determine
the effects of the genetic deletion of the μ opioid receptor on the fever by ICV injection of LPS.

Methods

Animals. Mu-opioid receptor knockout mice (MOP-KO) mice were developed by disruption of exon-1 of the MOP-1 gene through homologous recombination as described previously (Schuller et al., 1999). The 129S6 × C57BL/6J chimeras were directly crossed with 129S6 mice to produce the inbred 129S6 MOP-1 mutant strain, while the 129S6 × C57BL/6J F1 mutants were produced by directly crossing F10 C57BL/6J MOP-1 KOs with the 129S6 MOP-1-deficient strain. Mice (WT C57BL/6J mice as well as MOP-KO) weighing 20-30 g were used in this study. They were housed five per cage for at least 1 week before surgery and were fed laboratory chow and water ad libitum. Mice were housed on a light-dark cycle (12 h on/off; lights on at 06:00 AM) at an ambient temperature of 22 ± 0.3°C. All experiments were started between 09:00 and 10:00 h to minimize the effect of circadian variation in body temperature. 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.

Surgery procedures. Mice were anesthetized with an intraperitoneal (IP) injection of a mixture of ketamine hydrochloride (100-150 mg/kg) and acepromazine maleate (0.2 mg/kg). An incision 0.5 cm in length was made along the linea alba, and the underlying tissue was dissected and retracted. A transmitter (model E-4000, Mini-Mitter Co. Inc., OR, U.S.A.) was then implanted in the IP space. After the transmitter was
passed through the incision, the abdominal musculature and dermis were sutured independently (Benamar et al., 2005). On the same day as the surgery, each animal was placed into a stereotaxic instrument (Cunningham Mouse and Neonatal Rat Adaptor, Stoelting, IL, USA). The position of the head was adjusted so that the height of skull surface at bregma and lambda was the same. A sterilized stainless steel C315-GS-4 cannula guide (26 gauge, Plastic One, Roanoke) was implanted ICV. Stereotaxic coordinates were as follows: -0.5 mm anterior to bregma, 1 mm from midline and 3 mm ventral to the dura mater (Paxinos G, Franklin, 2001). A C315DCS cannula dummy (Plastic One) of identical length was inserted into the guide tube to prevent its occlusion. The animals were returned to individual cages in the environmental room.

**Microinjection and measurement of body temperature.** At 1 week post-surgery, the mice were tested in an environmental room (22 ± 0.3°C ambient temperature and 52% ± 2 relative humidity). After 1 h of adaptation, two readings were averaged to determine the baseline. During the recording period (pre- and post-injection), the body temperature was measured at 15-min intervals. The body temperature and circadian body temperature were measured by a biotelemetry system using calibrated transmitters. Signals from the transmitter were delivered through a computer-linked receiver. This method minimized stress to animals during the body temperature reading. Thus, the body temperature was monitored continuously and recorded without restraint or any disturbance to the animal. Either saline or drug was microinjected ICV in a volume of 3 µl. With aseptic procedures, the C315IS-4 internal cannula (33 gauge, Plastics One) was connected by polyethylene tubing to a 10-µl Hamilton syringe. The mice were placed into
individual plastic cages in an environmental room kept at 21 ± 0.3 °C with 52 ± 2% relative humidity.

**Statistical and histology analysis.** All results were expressed as mean ± S.E.M. Statistical analysis of differences between groups was determined by analysis of variance (ANOVA) followed by Dunnett’s test. A value of $P$ less than 0.05 was considered statistically significant. Cannula placement was confirmed by checking the location of the tip by 1% Evan’s Blue injection after the experiment according to standard procedures in our laboratory (Xin et al, 1997a).

**Drugs.** Morphine sulfate and the selective mu-opioid receptor agonist [D-Ala2, N-Me-Phe-4, Gly-ol5]enkephalin (DAMGO) (supplied by the National Institute on Drug Abuse) were dissolved in sterile pyrogen-free saline. LPS was a phenol-extracted preparation of *E. coli* (0111: B4) and was obtained from Sigma-Aldrich (St Louis, MO) and dissolved in pyrogen-free saline.

**Results**

**Baseline body temperature and circadian changes in body temperature on MOP-KO and WT.** To examine whether the absence of mu opioid receptors influenced the normal circadian body temperature rhythm, we compared the daily change in body temperature in the MOP-KO with WT. The recordings of the diurnal body temperature changes displayed no significant differences between the two groups (Fig.1, $P>0.05$). The basal body temperature measured before any treatment was comparable in the MOP-KO and WT, indicating that basal body temperature is the same in both groups (Fig.1, $P>0.05$).
To prove that these MOP-KO and WT responded properly to the action of morphine on body temperature and to define the role of the mu opioid receptors in morphine-induced hyperthermia, we injected a hyperthermic dose of morphine (1 mg/kg, IP) and examined the thermal response in WT and MOP-KO. In WT, IP injection of morphine (1 mg/kg) produced an elevation in body temperature that peaked at 60 min (1.41 ± 0.19 °C) and remained approximately 1 °C above baseline for 120 min. The administration of morphine (1 mg/kg, IP) to MOP-KO did not evoke any elevation in body temperature during the 210-min recording period (Fig. 2, $F_{3, 28} = 2.95$ $P<0.005$). Mean body temperature before injection was 36.68 ± 0.13 °C for the WT group, 36.79 ± 0.12 °C for MOP-KO. A similar effect was found using the selective mu-opioid receptor agonist [D-Ala², N-Me-Phe⁴, Gly-ol⁵]enkephalin. (DAMGO) given ICV (Fig. 3).

**Fever evoked by intracerebroventricular injection of LPS.** Animals were injected ICV with either LPS or vehicle (sterile, pyrogen-free saline), and body temperature monitored 360 min after injection. The ICV injection of LPS 50 ng did not significantly affect the body temperature relative to vehicle (Fig. 4, $P>0.05$). However, LPS in doses of 75 or 100 ng caused a fever which reached a peak of 1.15 ± 0.2°C and 1.47 ± 0.23 °C at 240 min, respectively (Fig. 4, $F_{3, 40} = 2.84$ $P<0.05$). Mean body temperature before injection was 36.67 ± 0.15°C for the saline group, 36.76 ±0.12 for LPS (50 ng) group, 36.84 ± 0.19 for LPS (75 ng) group and 36.75 ± 0.07 LPS (100 ng) group. There was no significant difference in baseline body temperature among these groups.
The effect of LPS (100 ng, ICV) on body temperature in MOP-KO and WT.
The administration of LPS (100 ng, ICV) to WT caused an increase in body temperature of approximately 1.56 ± 0.3 °C, which remained elevated throughout the 360-min recording period. However, LPS (100 ng, ICV) administration to MOP-KO did not show any increase in body temperature compared to WT (Fig. 5, $F_{3, 40} = 2.84$, $P < 0.05$).

The effect of LPS (0.1-4 µg, IP) on body temperature. To verify that the transfer of LPS from the CNS into the periphery is not responsible for the fever after ICV administration, we administered LPS IP at doses ranging from 0.1 to 4 µg/kg to WT. As can be seen in table 1, these doses did not alter WT body temperature as compared to saline controls.

Discussion

In the first series of experiments, we determined the thermoregulatory characteristics of these MOP-KO mice. We examined the consequence of the absence of mu opioid receptors on the basal body temperature and circadian body temperature rhythm. These receptors do not seem to be critical for the normal body temperature and circadian body temperature rhythm, as both the WT and MOP-KO showed similar baseline body temperature and diurnal/nocturnal fluctuations. Interestingly, although mu opioid receptors play an important physiologic role, the knockout mice did not demonstrate any apparent developmental or physiological abnormalities during the time of observation. Other studies have also reported that size, development, fertility and locomotor activity of knockout animals did not differ significantly from WT (Schuller et al, 1999, Tian et al, 1997). Previous study has been shown that naloxone blocks
morphine-induced hyperthermia (Geller et al., 1983). In using a genetic approach to correlate functional activity of the MOP gene with the known pharmacology of morphine on body temperature, and to show that MOP-KO respond properly to the effect of mu-opioid agonist, we administered a hyperthermic dose of morphine to MOP-KO and WT. Morphine (1 mg/kg) produced hyperthermia in WT mice but did not evoke the same response in MOP-KO during the recording period. Because morphine is not a selective mu opioid agonist, it could be argued that the effect of morphine on body temperature may involve actions on receptors other than mu. In the present study we tested this possibility. The selective mu opioid agonist DAMGO was administered to WT and MOP-KO and body temperature was monitored. As expected, a normally hyperthermic dose of DAMGO produced an increase in body temperature in WT mice, but did not evoke the same response in MOP-KO. These findings clearly indicate that the mu opioid receptor is required for the action of morphine on body temperature in mice, confirming the pharmacological effects of the selective mu-opioid-receptor antagonists (Adler and Geller, 1993, Handler et al, 1992, Spencer et al, 1988).

In the second series of experiments, we used an experimental model of fever associated with brain inflammation, in which mice received an ICV injection of LPS, to investigate the role of mu opioid receptors in LPS-induced fever. Using pharmacological approaches, it has been shown that mu-opioid antagonists (naloxone or CTAP) block the LPS-induced fever (Blatteis et al., 1991; Benamar et al., 2000), indicating that opioid system is involved in the pathogenesis of fever. And using gene deletion of mu opioid receptors, recently we have confirmed that the mu opioid receptor mediated the fever
induced by systemic administration of LPS (Benamar et al, 2005). The purpose of the present study was to determine whether such an effect occurs when LPS is given centrally. The injection of LPS, an endotoxin derived from the cell wall of Gram-negative bacteria, directly into the brain has been used as an animal model for the study of neuroinflammation. The present studies show that the ICV injection of LPS (50-100 ng) produced a dose-dependent, significant elevation in body temperature in WT during the 360-min recording period. However, the administration of LPS (100 ng, ICV) to MOP-KO did not evoke any increase in body temperature during the same recording period, indicating that the mu opioid receptors are critical for the development of fever induced by central administration of LPS in mice. One study has suggested that transfer of LPS from the CNS into the periphery in significant amounts is what accounts for the observed effects of ICV LPS (Cunningham et al, 2005). In our study, it is highly unlikely that the small amount of LPS (100 ng) injected via the ICV route evoked fever through its leakage into the system compartment, because the same amount of LPS, when injected peripherally, did not evoke fever, even at a dose 10 times higher. Our results suggest that an inflammatory response occurs in the brain following the administration of LPS ICV, manifested by increases in body temperature.

It has been shown that ICV administration of LPS caused a rapid and prolonged elevation of IL-1 throughout the brain (Quan et al, 1994). Microglial cells readily produce detectable IL-1 (Cunningham et al, 2005) and intense immunoreactivity to IL-1β in hypothalamic microglial cells (Gonzalez et al, 2004) after ICV stimulation. The in vivo stereotaxic injection of LPS into the brain has been reported to lead to a rapid production
by microglial cells of pro-inflammatory factors such as TNFα (Kalehua et al, 2000). Previous experimental data strongly suggest the important roles of IL-1β, IL-6 TNF-α and MIP-1β in fever induced by LPS (Blatteis, 2006), and recent results showed that microinjection of a selective mu-opioid-receptor antagonist centrally prevents the fever produced by interleukin-1β, TNF-α, MIP-1β, IL-6 and LPS (Benamar et al, 2000, Benamar et al, 2002, Handler et al, 1998, Xin et al, 1997b), indicating that mu opioid receptors are involved in the pathogenesis of fever induced by these endogenous and exogenous pyrogens. Mu opioids have also been shown to alter the release of cytokines important for both host defense and the inflammatory response (Chao et al, 1993, Lysle et al, 1993). Cells involved in neuro inflammation, astrocytes and microglia, as well as neurons, express mu opioid receptors (Ruzicka et al, 1995). Furthermore, mu-opioid-receptor mRNA has been observed in various regions in the brain, including the preoptic anterior hypothalamus (Mansour et al, 1995), the main area involved in fever and thermoregulation. In view of these findings, an interaction between the cytokine/chemokine and the mu opioid systems could take place under neuroinflammatory conditions.

Although a large part of the response to LPS-induced fever has been attributed to the action of cytokines, nitric oxide (NO), a proinflammatory mediator in the immune system with both antiviral (Lowenstein et al, 1996) and antibacterial (Nathan and Hibbs, 1991) actions, is one of the mediators produced following brain inflammation (Zamora et al, 2000). It is also considered to be an important mediator of LPS-induced fever (Roth et al, 1998). In addition, we have shown that NO produced by neuronal nitric oxide
synthase (nNOS) mediates morphine-induced hyperthermia (Benamar et al, 2003, Benamar et al, 2001). Another explanation of our data is that by deleting the mu opioid receptor, it is possible that the NO release decreases, leading to a decline in cumulative NO levels and therefore absence of LPS-induced fever mediated by endogenous NO.

The current report demonstrates a role of mu opioid receptors in an animal model of acute neuroinflammation, pointing out their critical role in the fever induced by central administration of LPS. The realization that the mu opioid system plays a key role in the control of the process of neuroinflammation is a new concept and may well lead to a fruitful approach to identify novel therapies for neuroinflammatory conditions. For example, it may be possible to use a mu opioid antagonist as a therapeutic strategy to prevent and treat brain diseases associated with neuroinflammation (e.g., Multiple Sclerosis, Alzheimer’s Disease). Also, these studies confirm that the mu-opioid system is involved in bacterial LPS-induced fever. In uncontrolled conditions, fever can threaten cellular homeostasis and survival. Treating such dysregulation of body temperature would be aided by an understanding of the role of mu opioid system in the pathogenesis of fever. In addition, these results provide direct genetic evidence that mu opioid receptors play a predominant role in morphine-induced hyperthermia and reinforce our earlier pharmacological findings.

Acknowledgments

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References


Footnotes

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Legends for figures

Fig. 1. Diurnal temperature changes in WT and MOP-KO. WT and KO mice displayed similar diurnal temperature variations. Data are expressed as the mean ± S.E.M. of body temperature. N, number of mice. Horizontal bar indicates dark phase.

Fig. 2. Effect of morphine on body temperature response in WT or MOP-KO knockout mice. Morphine (1 mg/kg, IP) injected at time zero. Data are expressed as the mean ± S.E.M. of body temperature. N, number of mice. * * P < 0.001

Fig. 3. Effect of DAMGO on body temperature response in WT or MOP-KO knockout mice. Morphine (1 µg, ICV) injected at time zero. Data are expressed as the mean ± S.E.M. of body temperature. N, number of mice. * P < 0.05, * * P < 0.01

Fig. 4. Effect of ICV injection of LPS (50-100 ng) on body temperature. LPS was injected at time 0. Data are expressed as the mean ± S.E.M. from baseline. N, number of rats. * P < 0.05.

Fig. 5. Effects of LPS on body temperature response in MOP-KO mice. LPS (100 ng) injected at time zero. Data are expressed as the mean ± S.E.M. of body temperature. N, number of mice. * P < 0.05
Table 1. Maximal change (mean ± S.E.M.) in body temperature induced by 0.1 to 4 µg of LPS in WT. Tb, variation in body temperature. N, number of mice.

<table>
<thead>
<tr>
<th>Dose (µg/kg, IP)</th>
<th>N</th>
<th>Baseline Tb (degree C)</th>
<th>Maximum increase Tb (degree C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6</td>
<td>36.56 ± 0.14</td>
<td>0.24 ± 0.09</td>
</tr>
<tr>
<td>0.1</td>
<td>6</td>
<td>36.51 ± 0.14</td>
<td>0.20 ± 0.10</td>
</tr>
<tr>
<td>0.5</td>
<td>6</td>
<td>36.61 ± 0.18</td>
<td>0.32 ± 0.10</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>36.68 ± 0.20</td>
<td>0.37 ± 0.15</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>36.81 ± 0.24</td>
<td>0.41 ± 0.16</td>
</tr>
</tbody>
</table>
Figure 1

WT N=6
MOR-KO N=6

Mean Tb (degree C) ± S.E.M.

6 AM 6 PM 6 AM 6 PM 6 AM 6 PM
Figure 2

- WT, morphine 1 mg/kg N=6
- MOP-KO, morphine 1 mg/kg N=6
- WT, saline N=6
- MOP-KO, saline N=6
Figure 3

WT, DAMGO (1 µg) ICV N=8
MOP-KO, DAMGO (1 µg) ICV N=6
WT, saline N=6
MOP-KO, saline N=6
Figure 4

Mean change in Tb (degree C) ± S.E.M.

- LPS (50 ng) ICV N=6
- LPS (100 ng) ICV N=6
- LPS (75 ng) ICV N=6
- Saline ICV N=6
WT, LPS (100 ng) ICV N=6
MOP-KO, LPS (100 ng) ICV N=10
WT, saline ICV N=6
MOP-KO, saline ICV N=6

Mean change in Tb (degree C) ± S.E.M.

Time (min)

Figure 5