Enhanced thermal antinociceptive potency and anti-allodynic effects of morphine following spinal administration of endotoxin

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Abstract

Recently, an animal model of central inflammation characterized by widespread cutaneous hyperalgesia and allodynia following intracerebroventricular (i.c.v.) administration of lipopolysaccharide (LPS) was described. In the present study, we demonstrate that central administration of LPS via intrathecal (i.t.) injection produces bilateral tactile allodynia and thermal hyperalgesia in the rat. Also, the effects of morphine-induced antinociception were determined in this model. Here we demonstrate enhanced thermal antinociceptive potency of i.t. morphine in LPS-treated rats compared to controls. Intrathecal morphine was also effective in alleviating the tactile allodynia induced by LPS. Both the antinociceptive and anti-allodynic effects produced by i.t. morphine were completely antagonized by pretreatment with subcutaneous naloxone (1 mg kg $^{-1}$). This study demonstrates the presence of both heat hyperalgesia and mechanical allodynia following central administration of LPS, and an increased antinociceptive potency of i.t. morphine in this model.

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1. Introduction

Prolonged tissue damage or nerve injury often leads to chronic pain characterized by the genesis of hyperalgesia and/or allodynia, which persists after healing. Many animal models have been developed in an attempt to understand the etiologies of various pain syndromes following an inflammatory insult. These models include application of various chemical irritants to induce peripheral inflammation (complete Freund’s adjuvant, carrageenan, mustard oil and formalin), all of which elicit spontaneous pain and/or thermal hyperalgesia and tactile allodynia [15]. The models described above have broadened our knowledge of the mechanisms underlying pain following the induction of peripheral inflammation, but few studies have examined pain mechanisms following the induction of central inflammation.

It is now well established that peripheral administration of lipopolysaccharide (LPS; endotoxin) interacts with various cell types to release cytokines IL-1, IL-6, and TNFα in the periphery and central nervous system, which can in turn precipitate hyperalgesia [51–54]. Central administration of endotoxin can also initiate the production of cytokines [17,20,43] known to modulate nociception. For example, intracerebroventricular (i.c.v.) administration of IL-1β, IL-6 or TNFα elicited thermal hyperalgesia and enhanced the response to wide dynamic range neurons in the trigeminal nucleus caudalis to noxious stimulation in rats [22,34,35]. We and others have also reported cutaneous thermal hyperalgesia and tactile allodynia following i.c.v. administration of LPS [4,50]. In addition, spinal administration of LPS produced an increase in the concentration of TNFα in the cerebrospinal fluid [48] as well as precipi-
tating thermal hyperalgesia [30] and enhancing activity of dorsal horn neurons [41].

It has been well established that the antinociceptive potency of systemically administered opioids [26,27,32] and spinal opioids [24,46] is enhanced in peripheral inflammatory states. In the spinal cord, the complexity of changes induced by inflammation on opioid receptor number, changes in neurochemical release and neuronal firing patterns makes it difficult to ascertain the exact mechanism of enhanced opioid sensitivity. It has been demonstrated that all three opioid receptors are expressed in dorsal root ganglion neurons and are differentially regulated by inflammation [1,2,12]. Moreover, in situ hybridization has shown that inflammation increases the percentage of dorsal root ganglion cells expressing mu-opioid receptors [25].

In the current study, we characterize the nociceptive behaviors following spinal administration of LPS, by examining the occurrence of heat hyperalgesia and tactile allodynia using the hot plate test and von Frey hair mechanical stimulation. We have also examined the effect of priming with LPS to enhance the behavioral nociceptive response to intrathecal (i.t.) administration of endotoxin, as priming was shown in an earlier study to augment the nociceptive response [4]. Finally, we evaluate the efficacy and potency of morphine in this model by comparing nociceptive thresholds in LPS-treated and saline-treated control animals.

2. Materials and methods

2.1. Animals

Experiments were performed on male Long Evans hooded rats (200–250 g; Charles River, Quebec, Canada) housed in groups of three per cage. Rats were maintained on a 12/12-h light/dark cycle and were allowed free access to food and water. Each rat was only used once and separate groups were used for mechanical versus thermal testing. Experiments were carried out according to a protocol approved by the animal care committee at the Clinical Research Institute of Montreal and McGill University, and were in accordance with guidelines from the Canadian Council on Animal Care and I.A.S.P. Committee for Research and Ethical Issues.

2.2. Behavioral testing

2.2.1. Thermal hyperalgesia testing

Thermal nociceptive thresholds were determined using a constant temperature hot plate (48°C). The response latency to a hind paw lick or vigorous shaking was recorded (baseline 25–35 s; cutoff 50 s). Animals were habituated to the testing apparatus for at least 15 min on the day prior to testing.

2.2.2. Mechanical alldynia testing

Mechanical response thresholds were quantified by determining the hind paw withdrawal response to von Frey filament stimulation according to the method described by Chaplan et al. [9]. In brief, animals were placed in a Plexiglas® box (21×16×27 cm) with a wire grid bottom through which the von Frey filaments were applied to the plantar surface of both hind paws. Filaments were applied in either ascending or descending strength as necessary to determine the filament closest to the threshold of response. The minimum stimulus intensity was 0.25 g and the maximum was 15 g. Based on the response pattern and the force of the final filament, the 50% response threshold (g) was calculated. Animals were habituated to the testing apparatus for at least 15 min on the day prior to testing and for 10 min prior to testing.

2.3. Drug administration schedule

Spinal administration of either vehicle or LPS (E. coli lipopolysaccharide (Sigma stereotype 0111:B4) in 0.9% sterile saline) was accomplished by injecting a 30-μl volume into the subarachnoid space via a lumbar puncture (between vertebrae L4 and L5), while rats were briefly anesthetized with halothane. Behavioral testing was performed in two treatment groups: primed and unprimed. Primed rats received an i.t. injection of LPS (2.0 μg) 24 h prior to the experiment when a second LPS (0.02, 0.2, 2.0 or 20 μg) injection was given, the ‘challenge dose’. The priming dose of LPS was chosen based on experiments described by Meller et al. [30]. Control (un-primed) animals received saline rather than endotoxin 24 h prior to the LPS challenge. To determine a time course for the effects of LPS on our behavioral test, we used 2.0 μg LPS for priming and 20 μg LPS for challenge injections.

Morphine was administered by either intrathecal (i.t.), subcutaneous (s.c.) or i.c.v. injection. I.c.v. administration was accomplished by injection into the lateral ventricle via a 30-gauge stainless steel tubing through the previously implanted guide cannula (23-gauge needle) to a depth of 4 mm below the surface of the skull. Implantation of i.c.v. cannula was performed under halothane anesthesia 6–7 days prior to behavioral testing to allow the animals to recover from surgery. I.t. administration of morphine was performed by acute lumbar puncture as described above.

Morphine antinociception was evaluated in both primed (2.0 μg followed by 20 μg) and unprimed (saline followed by 20 μg) LPS-treated rats. The degree of anti-allodynic and thermal antinociception produced by morphine was determined by comparing the response thresholds prior to (i.e. that produced by i.t. LPS (40-min onset of hyperalgesic effect)) and following morphine administration. Behavioral response thresholds were assessed 30 min following morphine administration (70 min after the initial LPS injection), a time point that was shown to produce maximum antinociceptive efficacy in the hot plate test [5],...
and the percent maximum possible effect (% MPE) was calculated, as indicated below. An experiment was also designed to determine whether the anti-allodynic and antinociceptive effects produced by morphine could be blocked by pretreatment with the non-selective opioid receptor antagonist naloxone (1 mg kg$^{-1}$, s.c.).

2.4. Statistical analysis

Data are presented as raw scores or converted to % MPE where % MPE = [(response value - baseline) / (cutoff - baseline)] × 100% for thermal thresholds. For mechanical thresholds data are converted to % return to baseline defined as [(baseline - threshold) / baseline] × 100% because the % MPE equation can not be used due to instances when the baseline and the cutoff are the same value due to the nature of the stimulus (non-noxious). All data are expressed as means±S.E.M. Statistical analysis on thermal thresholds was performed using repeated measures one-way analysis of variance followed by the Dunnett’s test for post hoc comparisons. Statistical analysis on mechanical thresholds was performed using the non-parametric Friedman’s repeated measures analysis of variance on ranks followed by Wilcoxon signed rank test for post hoc comparisons. $EC_{50}$ values produced by i.t. morphine were obtained by regression analysis and interpolation calculated using Graphpad software (Prism 3.0).

3. Results

3.1. Thermal hyperalgesia and mechanical allodynia following intrathecal administration of LPS

The nociceptive effects produced by i.t. administration of LPS were evaluated using both thermal (hot plate test) and mechanical (von Frey hair stimulation) behavioral testing parameters (Fig. 1). Two groups were examined: unprimed control rats received i.t. saline (Fig. 1A,B), and primed rats received i.t. LPS (2.0 μg; Fig. 1C,D) 24 h prior to a challenge injection of LPS (20 μg). A single i.t. injection of the endotoxin in unprimed rats altered neither mechanical nor thermal response thresholds (Fig. 1A,B). However, mechanical and thermal response thresholds were significantly decreased in LPS-primed rats (Fig. 1C,D). For mechanical response thresholds, the Friedman’s

![Graphs showing effects of intrathecally administered LPS in unprimed and primed rats.](image-url)
A non-parametric test demonstrated a statistical significance over time ($\chi^2(7)=25.67$, $P<0.001$) and post hoc comparisons using the Wilcoxon on ranks test demonstrated a significant decrease in the 50% threshold at 40–120 min after the challenge LPS injection. For thermal response thresholds, repeated measures analysis of variance (ANOVA) ($F(7,70)=4.21$, $P<0.001$) demonstrated a significant main effect of time. Post hoc comparisons using the Dunnett’s test revealed that the thermal threshold latencies of LPS primed rats were significantly decreased compared to baseline values at 40–100 min after the challenge injection of endotoxin.

Further experiments were performed to determine whether the LPS-induced decreases in thermal and mechanical response thresholds were dose-dependent. Fig. 2 demonstrates the effects of various challenge doses of i.t. LPS. All groups received the same priming dose (2.0 $\mu$g) of i.t. LPS. Baseline thermal latencies to response and mechanical response threshold were evaluated prior to the injection of LPS on the testing day. No differences were detected between baseline responses when compared between each group (the mean and S.E.M. for the combined groups was 29±2.7 s for thermal latencies to response and 9.8±1.4 g for the mechanical response threshold). For LPS-induced decreases in mechanical thresholds, challenge doses of endotoxin ranging from 0.2 to 20 $\mu$g produced dose-dependent decreases in mechanical response thresholds compared to their baseline response. The Friedman’s non-parametric test demonstrated a statistical significance over time ($\chi^2(3)=10.2$, $P<0.05$) and post hoc comparisons using the Wilcoxon on ranks test demonstrated a significant decrease in the 50% threshold for 0.2, 2 and 20 $\mu$g challenge doses compared at 40 min post injection to baseline thresholds. Dose-dependent endotoxin-induced decreases in thermal threshold latencies were also demonstrated, with both the 2- and 20-$\mu$g i.t. LPS challenge doses producing a significant decrease in thermal thresholds. For thermal response thresholds, repeated measures analysis of variance (ANOVA) ($F(3,24)=4.72$, $P<0.01$) demonstrated a significant effect. Post hoc comparisons using the Dunnett’s test revealed that the thermal threshold latencies of LPS primed rats were significantly decreased compared to baseline values at 40 min after the challenge injections of 2.0 and 20 $\mu$g of endotoxin. In addition, regression analysis of the dose–response curves identified LPS-induced mechanical allodynia at lower doses than those required to produce thermal hyperalgesia.

### 3.2. Morphine effects on LPS-induced hyperalgesia and allodynia

The potential antinociceptive effects of morphine were examined in LPS-treated (primed) compared to control rats. Fig. 3 demonstrates the dose–response curves obtained for morphine-induced antinociception on the hot plate test in both groups following various routes of morphine administration. Subcutaneous administration of morphine produced similar antinociceptive dose–response curves in both LPS-treated and saline groups, where ED$_{50}$ values for the control group were 1.91 (1.44–6.7) mg kg$^{-1}$ and 0.95 (0.90–0.97) mg kg$^{-1}$ for the LPS group (Fig. 3A). Similarly no difference in the antinociceptive effect induced by i.c.v. morphine was seen between saline and...
LPS-treated rats (Fig. 3C). Regression analysis confirmed that no significant difference in morphine-induced antinociception was evident between LPS-treated and control rats for either peripheral or i.c.v. administration of the opioid. However, the dose–response curve for the antinociceptive effects produced by i.t. administration of morphine were shifted significantly to the left in LPS-treated rats compared to saline control rats (Fig. 3B). ED_{50} values calculated for i.t. morphine-induced antinociception were 0.054 (0.032–0.071) μg in LPS versus 2.01 (1.83–2.79) μg in control rats, representing a 40-fold shift in potency.

Morphine administered by i.t. (Fig. 4A), s.c. (Fig. 4B) or i.c.v. (Fig. 4C) routes in saline-pretreated/LPS-challenged rats produced no significant changes in paw withdrawal thresholds in the von Frey test, when compared to baseline values obtained prior to LPS administration (Fig. 4, right column). In contrast, all three routes of morphine administration (Fig. 4A–C) increased mechanical response thresholds in i.t. LPS-treated (pretreated and challenge injections consisted of LPS) rats compared to mechanical thresholds following LPS (≈P<0.05). The effects of i.c.v. morphine were modest (Fig. 4C, left column), as the mechanical threshold following morphine administration remained significantly attenuated compared to baseline values (≈P<0.05). The anti-allodynic effects produced by higher doses of i.c.v. morphine could not determined due to the appearance of motor side-effects.

The anti-hyperalgesic and anti-allodynic effects of i.t. morphine are also illustrated in Fig. 5. Baseline thermal (bottom panel) and mechanical thresholds (top panel) were recorded for all three groups prior to LPS injection (time: 0 min). Each group was assessed for hyperalgesia and allodynia after LPS treatment but prior to drug treatment (time: 40 min). There was no difference between baseline values for all groups, and LPS treatment (first arrow) produced an equivalent reduction in mechanical thresholds and hot plate latency for all groups. Mechanical (Fig. 5B) or thermal (Fig. 5E) thresholds were shown to be significantly elevated 30 min following i.t. morphine administration (3 μg) compared to response values following LPS administration (≈P<0.05), an effect that was effectively antagonized by pretreatment with s.c. naloxone (1 mg kg⁻¹; Fig. 5C,F).

4. Discussion

In this study, we demonstrate the incidence of thermal hyperalgesia and tactile allodynia following spinal administration of endotoxin. Expression of LPS-induced hyperalgesia and allodynia is dependent on prior exposure to LPS via a priming dose. The severity of nociceptive responses elicited by LPS is dose-dependent. We also demonstrate that i.t., but not i.c.v. or s.c., morphine is more potent in LPS-treated compared to control rats when
Fig. 4. Anti-allodynic effects of morphine (MS) in LPS-treated (left column) and control (right) rats in the mechanical von Frey threshold test. Baseline (BL) thresholds assessed prior to challenge administration of LPS. At 40 min following administration of LPS, von Frey thresholds were significantly decreased compared to baseline responses. Anti-allodynic effects were produced by either subcutaneous (s.c., panel A), intrathecal (i.t., panel B) or intracerebroventricular (i.c.v., panel C) administration of morphine in LPS-treated rats. No effect on mechanical response thresholds was evident following either s.c., i.t. or i.c.v. morphine compared to baseline thresholds in saline-treated rats (right column). Data are expressed as means ± S.E.M. for n=5–6 per group; *P<0.05 compared to baseline response, ≈P<0.05 compared to threshold following LPS administration.

Finally, morphine administered either spinally or systemically produced anti-allodynic effects in LPS-treated rats at doses that had no effect on baseline mechanical thresholds (and little or no effect on thermal thresholds) in control animals.
The central mechanisms underlying hyperalgesia elicited by a peripheral inflammatory injury have been extensively studied, confirming the relevance of various neurochemical systems and the necessity of neuronal plasticity in the development of hyperalgesia and chronic pain [10,14,15]. It is now well established from various types of central nervous system injuries (traumas, infections, ischemia, etc.), that the pro-inflammatory cytokines are rapidly produced in the brain [16]. This effect can also be produced by direct administration of endotoxin into the central nervous system. Thus, i.c.v. administration of endotoxin has been shown to increase circulating levels of IL-1 and IL-6 [19]. Moreover, there is intense monocyte recruitment and microglial activation after LPS injection [20,31,40].

In this study, we demonstrate that hyperalgesia and allodynia can also occur following spinal administration of an endotoxin. Other studies have reported the incidence of hyperalgesia following central administration of endotoxins. For example, i.t. administration of LPS was shown to induce thermal hyperalgesia [30] and increase the activity of dorsal horn neurons [41], and i.c.v. administration of LPS elicits both thermal hyperalgesia and a decrease in mechanical thresholds [4,50]. Littie is known regarding the sensory pathways within the central nervous system or the chemical mediators that are responsible for the nociceptive responses following a central inflammatory response, however, nitric oxide [30] and bradykinin [50] have been implicated. In addition, our own studies have demonstrated that i.c.v. LPS-induced thermal hyperalgesia and mechanical allodynia were correlated with an increased activation of microglia [4].

The present study demonstrates enhanced antinociceptive potency and anti-allodynic effects of morphine in a model where the inflammatory response is initiated within the central nervous system. It has been well established that the antinociceptive potency of systemically administered opioids [26,27,32] and spinal opioids [24,46] is enhanced in inflammatory states generated following a peripheral insult. Here we report for the first time the enhanced antinociceptive effectiveness of morphine in a central model of inflammation. It is also interesting to note that morphine, which normally has no effect on altering thresholds to innocuous stimuli, produces an anti-allodynic effect following either peripheral or spinal administration. The effects of supraspinal administration of morphine appeared to be only partially effective in producing anti-allodynic effects, however, this may be due to the confounding incidence of motor impairment associated with this route of administration. This is in agreement with previous studies demonstrating that i.t., s.c. or local administration of morphine into the paw does not sig-
immune-derived endogenous opioid peptides, such as inflammation. Taken together, i.t. LPS may increase the levels of enhanced effectiveness of morphine in a model of central tests that was completely blocked by naltrexone [55]. Knowledge, this is the first study to demonstrate the significant antinociceptive response in phasic nociceptive gesics during states of peripheral inflammation. To our and (iii) peripheral administration of LPS produces a peripherally administered opioids are more potent anal-
endotoxin (LPS) appears to be an effective method to rats to normal levels [49], and restores opioid efficacy in non-inflamed tissue [39]. In this latter study, numerous
produce the enhanced effectiveness seen in this study. It has been well established that antinociceptive synergy occurs following combined mu and delta opioid receptor activation [45]. The increase in opioid potency during inflammation may in part be attributed to an increase in the number of spinal opioid receptors. All three opioid receptors are differentially regulated following peripheral inflammation [1,2,12]. Importantly, in situ hybridization has shown that inflammation increases the percentage of mu-receptor expressing neurons in dorsal root ganglia [25].

Recently, it has been demonstrated that peripheral inflammation [18], like i.t. administration of LPS [4], produces an activation of spinal microglia. Thus, peripheral and central inflammation may produce a similar increase in cytokines and resultant changes in opioid peptide or receptors, as discussed above, that may contribute to enhanced morphine efficacy. However, these findings are not consistent with the observation that morphine analgesia is attenuated in neuropathic rats, despite similar observations of increased microglial activation in the spinal cords of neuropathic rats [11]. Recent finding in our lab and others suggest this discrepancy may be explained by differences in the influence of peripheral inflammation and nerve injury on spinal levels of nerve growth factor (NGF), and the ability of NGF to regulate anti-opioid peptides such as cholecystokinin (CCK). Importantly chronic spinal infusion of NGF both lowers the heightened spinal CCK levels in neuropathic rats to normal levels [49], and restores opioid efficacy in neuropathic rats [6].

In conclusion, intrathecal administration of bacterial endotoxin (LPS) appears to be an effective method to produce nociceptive behaviors associated with a central inflammatory response. We propose that spinal administration of LPS induces central inflammation, and produces a model of persistent pain of inflammatory origin. By providing a protocol for a behavioral measurement that is effective and reproducible, similar studies will likely aid better understanding of central inflammatory pathological states. Consequently, this model may provide an understanding of pain associated with inflammation within the central nervous system, a common process associated with many neurodegenerative diseases, headaches and migraine. It has been well established that both centrally and peripherally administered opioids are more potent analgesics during states of peripheral inflammation. To our knowledge, this is the first study to demonstrate the enhanced effectiveness of morphine in a model of central inflammation.
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