Increasing of intrathecal CSF excitatory amino acids concentration following morphine challenge in morphine-tolerant rats

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Abstract

Excitatory amino acids (EAAs) are involved in the development of opioid tolerance. The present study reveals that an increasing of CSF EAAs concentration might be responsible for the losing of morphine’s antinociceptive effect in morphine tolerant rats. Male Wistar rats were implanted with two intrathecal (i.t.) catheters and one microdialysis probe, then continuously infused i.t. for 5 days with saline (1 μl/h; control group), morphine (15 μg/h), the NMDA antagonist, MK-801 (5 μg/h), or morphine (15 μg/h) plus MK-801 (5 μg/h). Each day, tail-flick responses were measured; in addition, CSF dialysates were collected and CSF amino acids measured by high performance liquid chromatography using a fluorescence detector. Morphine started to lose its analgesic effect on day 2 and this effect was overcome by MK-801. The AD50 (AD: analgesic dose) was 1.33 μg in control animals, 83.83 μg in morphine-tolerant rats (a 63-fold shift), and 11.2 μg (a 8.4-fold shift) in rats that had received MK-801 plus morphine. No significant differences were observed in CSF amino acid release between the groups from day 1 to day 5. On day 5, after basal dialysate collection, a 10-μg challenge of morphine was administered i.t., and CSF samples collected over the next 3 h. After morphine challenge, morphine-tolerant rats showed a significant increase in the release of glutamate and aspartate (131 ± 9.5% and 156 ± 12% of basal levels, respectively), and no antinociceptive effect in the tail-flick latency test, while MK-801/morphine co-infused rats showed no increase in morphine-induced EAA release and a partial antinociceptive effect (MPE = 40%). The present study provides direct evidence for a relationship between EAA release and a lack of an antinociceptive response to morphine, and shows that the NMDA antagonist, MK-801, attenuates both of these effects.

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1. Introduction

Opioids have been used clinically for decades to produce analgesia. Long-term opioid administration can lead to the development of tolerance. Koob and Bloom [22] described two possible mechanisms for drug tolerance: a within-system and a between-system adaptation. The effects of endogenous and exogenous opioids are mediated and modulated by a complex group of receptors. We have previously demonstrated that, in addition to the within-system mechanism, which involves receptor uncoupling and receptor down-regulation [41,43], a between-system mechanism is involved in the development of opioid tolerance, particularly involving NMDA receptors [16,17,28]. Trujillo and Akil [39] demonstrated that the non-competitive NMDA receptor antagonist, MK-801, attenuates opioid tolerance and dependence without affecting the antinociceptive effect of morphine, and, in a spinalized rat model, confirmed that the antagonism of morphine-induced tolerance is at the spinal site [14]. Kest et al. [21] showed that intrathecal (i.t.) administration of MK-801 inhibits morphine tolerance, direct evidence that the NMDA receptor antagonist prevents opioid tolerance at the
spinal level. Similarly, we also found that both competitive and non-competitive NMDA receptor antagonists inhibit morphine tolerance in a rat spinal model [43,44] and, using a $[3^H]$DAMGO binding assay, showed that NMDA antagonists prevent the reduction in the number of $\mu$-opioid receptor high-affinity sites seen in tolerant animals [43]. Larcher et al. [24] also proposed that activation of the NMDA-dependent pain facilitatory systems, the between-system mechanism, may be another possible mechanism of opioid tolerance [25].

The EAAs, glutamate and aspartate, have been shown to be involved in nociception transmission in the spinal cord [1,3,6,15,20], and a high concentration of glutamate binding sites has been found in the substantia gelatinosa (a region in which opioid receptors are known to exist) of the rat spinal cord [13,36]. Autoradiographic data confirmed the presence of a significant number of glutamate and NMDA-sensitive binding sites in the dorsal horn of the spinal cord [13,32]. In the spinal cord, releasable pools of EAAs have been found in opioid-sensitive primary afferent neurons [31,37]. Increased EAA release in the CNS is seen in naloxone-opioid receptor antagonists, morphine-tolerant animals [2,19]. We also demonstrated increased EAA release in the nucleus accumbens, locus coeruleus neurons, and the striatal system in naloxone-precipitated, morphine-tolerant rats [18]. Furthermore, Hong et al. [16] found that naloxone-precipitated withdrawal is attenuated by EAA antagonists. Chen and Huang [4,5] demonstrated that stimulation of $\mu$-opioid receptors enhances the NMDA receptor-mediated glutamate response. The above evidence suggested positive feedback control between opioid and EAA receptors, particularly NMDA receptors. Jhamandas et al. [19] proposed that an increased release of spinal EAAs might be observed in opioid-tolerant rats, but failed to detect any increased release in morphine-tolerant rats. Recently, we were able to demonstrate increased levels of the EAAs, glutamate and aspartate, in the CSF of terminally ill cancer patients who received i.t. morphine for pain relief, and loss of the analgesic effect of morphine at the effective dose after long-term administration [45]. The present study was designed to examine whether increased EAA release is responsible for the development of tolerance to morphine.

2. Materials and methods

2.1. Animal model: implantation of intrathecal catheters and the microdialysis probe

Male Wistar rats (400–450 g) were used. Two i.t. catheters and a microdialysis probe were inserted via the atlanto-occipital membrane into the i.t. space at the level of the lumbar enlargement of the spinal cord, and externalized and fixed to the cranial aspect of the head. The rats were then returned to their home cages for a 4-day recovery period. Each rat was housed individually and maintained on a 12-h light/dark cycle with food and water freely available. Rats were excluded from the study if they showed evidence of gross neurological injury or the presence of fresh blood in the CSF. The use of animals in this study conformed to the Guiding Principles in the Care and Use of Animals of our Institute, and was approved by the Care and Use Committee of our Institute.

2.2. Construction of the microdialysis probe

The microdialysis probe was constructed as described previously [30], using two 7 cm PE5 tubes (0.008 in. inner diameter, 0.014 in. outer diameter) and a 4-cm cuprophan hollow fiber (300 $\mu$m outer diameter, 200 $\mu$m inner diameter, 50 kDa molecular weight cut-off; DM-22, Eicom, Kyoto, Japan). To make the probe firm enough for implantation, a Nichrome-Formavar wire (0.0026 in.; A–M system, Everett, WA, USA) was passed through a polycarbonate tube (194 $\mu$m outer diameter, 102 $\mu$m inner diameter; 0.7 cm in length) and the cuprophan hollow fiber (active dialysis region), and connected to a PE5 catheter with epoxy glue. The fiber was then bent in the middle section of the cuprophan hollow fiber, forming a “U”-shaped loop. The two ends of the dialysis fiber, consisting of silastic tubes, were sealed with silicon sealant. The dead space of the dialysis probe was 8 $\mu$l. During in vitro measurements, the recovery rate of the dialysis probe was 40% at an infusion rate of 5 $\mu$l/min. Using this technique, it was possible to measure levels of CSF amino acids for up to 12 days after implantation.

2.3. Antinociception test and tolerance induction

Tail-flick latency in the hot water immersion test (52 $\pm$ 0.5 °C) was used to measure the antinociceptive effect. At this temperature, the mean tail-flick latency was approximately 2.2 $\pm$ 0.3 s in naive rats. An automatic cutoff was set at 10 s to prevent tissue injury. The rats were placed in a plastic restrainer for drug injection and the antinociception test. Continuous morphine infusion (15 $\mu$g/h, i.t.) [12] for 5 days was used for tolerance induction, while co-infusion of MK-801 (5 $\mu$g/h, i.t.) and morphine (15 $\mu$g/h, i.t.) was used to examine the effect of MK-801 on the development of morphine tolerance. Rats receiving saline or MK-801 (5 $\mu$g/h, i.t.) alone were used as controls. Tail-flick responses were examined before drug infusion and daily after the start of infusion for 5 days. All drug infusions were at the rate of 1 $\mu$l/h via a mini-osmotic pump (model 2001; Alzet, Palo Alto, CA, USA) implanted in the interscapular region. The tail-flick dose–response curves for morphine were also measured before and after induction of morphine tolerance. Tolerance was defined as a decrease in, or loss of, the antinociceptive effect of morphine infused i.t. at a rate of 15 $\mu$g/h. Since initial studies showed that the tail-flick latency returned to the
baseline level (< 2.5 s) 3 h after discontinuation of drug infusion, morphine challenge (10 μg via the other i.t. catheter) on day 5 was performed at this time. Each tail-flick latency was an average of four measurements over a 6-min testing period in each rat. The latency response was converted into the maximum percent effect (MPE) using the equation:

$$\text{MPE} (%) = \frac{\text{Test response time} - \text{Basal response time}}{\text{Cut off time} - \text{Basal response time}} \times 100\%$$

Latencies less than the baseline or higher than the cut-off time were assigned MPE values of 0% or 100%, respectively.

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2.4. CSF sample collection and measurement of amino acid levels

After recovery, rats were transferred to a free-moving animal system and one end of the externalized microdialysis probe was connected to a syringe pump (CMA-100) for the collection of CSF samples. The dialysis system was perfused with artificial cerebrospinal fluid (aCSF), consisting of 151.1 mM Na+, 2.6 mM K+, 122.7 mM Cl−, 21.0 mM HCO3−, 0.9 mM Mg2+, 1.3 mM Ca2+, 2.5 mM HPO42−, and 3.5 mM dextrose, bubbled with 5% CO2 in 95% O2 to adjust the final pH to 7.3. All CSF sample collections followed a standard procedure of a 30-min washout period, followed by a 30-min sample collection period, at a flow rate of 5 μl/min. Thirty microliters of dialysate was collected before pump implantation, then daily for 5 days after implantation. On day 5, at 3 h after discontinuation of i.t. drug infusion, two consecutive CSF samples were collected before morphine challenge (basal level), and another four samples at 30, 60, 90 and 180 min after challenge. The dialysates were collected in polypropylene tubes on ice, then frozen at −80 °C until assayed.

Amino acid levels were analyzed by high performance liquid chromatography using a fluorescence detector (Gibson model 121, set at 428 nm) as described previously [40]. In brief, amino acids were assayed by precolumn derivatization with o-phthalaldehyde/β-butylthiol (OPA) reagent and iodoacetamide/methanol scavenger. Derivatization was performed by adding 4 μl of OPA reagent to 40 μl of sample, shaking the mixture, then allowing it to react...
for 2 min. Four milliliters of reagent B (185 mg of iodacetamide/ml of methanol) was added and the mixture allowed to react for another 2 min. The derivatized sample was then injected onto a C18 reversed phase column and eluted at a flow rate of 0.45 ml/min. A linear gradient from 100% eluent A [0.1 M sodium acetate buffer, pH 6.8/acetonitrile (80:20)] to 100% eluent B [acetonitrile/double-distilled water (80:20)] was used to separate the amino

Fig. 3. Effect of morphine challenge (10 μg, i.t.) on aspartate (A) and glutamate (B) levels in CSF dialysates after various drug treatments. Animals were treated with the standard protocol and infusion halted on day 5, then, 3 h later, two consecutive basal CSF dialysate samples (30 min each) were obtained just before morphine challenge. The average concentrations of aspartate (A) and glutamate (B) in these two basal dialysates were used as the 100% level. Another four dialysate samples were collected at 30, 60, 90, and 180 min after morphine challenge and the aspartate and glutamate levels at each time-point expressed as the percentage change from the basal levels. The insert in the left top of each figure shows the percentage change in the area under the curve (AUC) at 180 min compared to the baseline AUC. (*P<0.05 compared to the saline group; #P<0.05 compared to the morphine group).
acids. All solvents were vacuum filtered through a 0.22-μm membrane (Millipore) and degassed by sonication before use. External standard solutions, containing $10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-5}$ M standard amino acids, were run before and after each sample group.

2.5. Data and statistical analysis

The data are presented as the mean ± S.E.M. The dose–response data were analyzed using a linear regression program. Values for the analgesic dose for 50% of dose–response data were analyzed using a linear regression program. Values for the analgesic dose for 50% of dose–response data were analyzed using a linear regression program. As shown in Fig. 1A, the average baseline tail-flick latency in the hot water immersion test for naïve rats was $2.2 ± 0.15$ s ($n = 38$). As in our previous study [43], morphine started to lose its analgesic effect on day 2 of continuous infusion (15 μg/h, i.t.) and continued infusion of MK-801 alone (5 μg/h, i.t.) did not produce any analgesic effect or motor block, but co-administration of MK-801 with morphine reduced tolerance (Fig. 1A). As shown in Fig. 1B, the dose–response curve on day 5 revealed that the AD$_{50}$ for morphine was 1.33 μg in saline-treated rats, 83.8 μg and morphine-tolerant rats (a 63-fold shift), and 11.2 μg in co-infused rats (an 8.4-fold shift).

As shown in Fig. 2, morphine challenge (10 μg i.t.) had a significant anti-nociceptive effect in the saline infusion group, but no effect in morphine-tolerant rats. However, co-infusion of MK-801 with morphine significantly reversed the reduction in morphine analgesia. Chronic morphine infusion resulted in a slight, but not significant, increase in the release of the EAsas, aspartate and glutamate, during the 5-day infusion. Other amino acids (glycine, asparagine, glutamine, citrulline, arginine, alanine, histidine, taurine, and serine) also showed no significant difference in release during the 5-day infusion compared to the basal levels in any of the groups. Surprisingly, morphine challenge (10 μg, i.t.) on day 5 significantly increased aspartate and glutamate levels in the CSF dialysates during the 180-min sample collection period in morphine-tolerant rats (Fig. 3A,B). Co-infusion of MK-801 not only completely blocked this morphine-evoked aspartate and glutamate release, but also produced a slight antinociceptive effect (MPE = 40%) (Figs. 2 and 3). In saline-infused controls, morphine challenge resulted in a slight, but not significant, decrease in EAA release. No differences were observed in the release of other amino acids compared to the basal CSF levels in any of the three groups.

3. Results

As shown in Fig. 1A, the average baseline tail-flick latency in the hot water immersion test for naïve rats was $2.2 ± 0.15$ s ($n = 38$). As in our previous study [43], morphine started to lose its analgesic effect on day 2 of continuous infusion (15 μg/h, i.t.). Infusion of MK-801 alone (5 μg/h, i.t.) did not produce any analgesic effect or motor block, but co-administration of MK-801 with morphine reduced tolerance (Fig. 1A). As shown in Fig. 1B, the dose–response curve on day 5 revealed that the AD$_{50}$ for morphine was 1.33 μg in saline-treated rats, 83.8 μg and morphine-tolerant rats (a 63-fold shift), and 11.2 μg in co-infused rats (an 8.4-fold shift).

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4. Discussion

As in our previous report [43], continuous i.t. infusion of morphine-induced antinociceptive tolerance, shown by a right shift in the morphine dose–response curve, and the degree of tolerance was attenuated 7.5-fold by co-administration of MK-801. As in a previous report [19], the present study did not find any significant increase in the EAAs, glutamate and aspartate, concentration in the CSF during the 5-day tolerance induction period. However, in morphine-tolerant rats, increased amounts of glutamate and aspartate were seen in the CSF dialysate after morphine challenge, and no antinociceptive effect was observed. At the same dose, morphine produced an antinociceptive effect in morphine-naïve rats. Co-infusion of MK-801 with morphine significantly attenuated the morphine tolerance and inhibited the morphine challenge-evoked increasing of EAAs level. In terms of pharmacokinetics, Maeda et al. [26] found that intravenous co-injection of MK-801 and morphine did not affect the concentration of morphine in either the serum or the brain, suggesting that the effect of MK-801 on morphine tolerance was not due to a change in morphine pharmacokinetics. The dissociation of the time course of the reduction of morphine’s antinociceptive effect and increasing of the CSF EAAs concentration might be due to the tolerance had been developed maximally on day 5 before the morphine challenge.

The cellular mechanisms involved in the morphine-induced increase of spinal EAAs concentration in morphine-tolerant rats remain to be investigated. At least two possibilities exist. One is that the opioid may activate protein kinase C (PKC), which then phosphorylates glutamatergic receptors, particularly NMDA receptors, while the other is that it may down-regulate glutamate transporter function. Chen and Huang [4] demonstrated that, in trigeminal dorsal horn neurons, activation of μ-receptors can potentiate NMDA receptor activity via PKC. Activation of NMDA receptors is known to increase the intracellular Ca$^{2+}$ concentration and to stimulate PKC and exocytosis, which leads to increased release of EAAs [35]. The increased extracellular glutamate concentration is counterbalanced by glutamate transporter activity in neurons and glial cells which terminates glutamatergic signaling and thus protects neurons from glutamate toxicity [10]. Expression of glutamate transporter GLT-1 mRNA is significantly decreased in the CNS of morphine-dependent rats [33]. Mao et al. [29] also demonstrated that chronic i.t. administration of morphine induces down-regulation of glutamate transporter proteins (EACC1 and GLAST) in the dorsal horn of the spinal cord, and found that glutamate transporter activators attenuated the development of morphine tolerance. Moreover, PKC activation plays a crucial role in morphine tolerance and down-regulates glutamate transporter activity and expression [7,38]. Taken together, these results suggest that glutamate transporter...
down-regulation and PKC activation might be involved in the morphine challenge-induced increasing of the spinal CSF EAA concentration in tolerant rats.

As well as conformational changes in opioid receptors [41,42], activation of NMDA receptors has long been considered to play an important role in opioid tolerance [27,28,39,43]. In behavioral studies, i.t. injection of EAs or NMDA results in spinal nociceptor sensitization, and thus in hyperalgesia and allodynia [1,23,34]. Several studies have demonstrated that opioid tolerance is associated with nociceptive sensitization and the subsequent hyperalgesia [24,27,28]. Larcher et al. [24] proposed that opioids may activate the NMDA receptor-dependent pain facilitatory system. The present study and others provide support for a positive feedback regulation between opioid and NMDA receptors [24,25,28]. Opioids may increase the synaptic EAs level and activate NMDA receptors, thus reducing the nociceptive threshold and the opioid analgesic effect. The present observations show that the NMDA antagonist, MK-801, not only attenuated morphine tolerance, but also blocked morphine-evoked EAs increase. This morphine-evoked increasing of spinal CSF EAs level may activate NMDA receptors, which sensitize nociceptors in the spinal cord, thus attenuating the analgesic effect of morphine. Similarly, in our recent study [45], we demonstrated increasing of glutamate and aspartate concentration in the CSF of terminal cancer pain patients receiving long-term i.t. injection of morphine for pain relief, and this was accompanied by a reduction in the analgesic effect of morphine. Moreover, in another study using a [3H]MK-801 binding assay [44], we found increased NMDA receptor binding activity in the spinal cord of morphine-tolerant rats. Koyuncuoglu et al. [23] also demonstrated increased [3H]glutamate binding in opioid-tolerant rats. Taken together, these results imply that, in addition to down-regulation of opioid receptor function, opioid tolerance may involve upregulation of NMDA receptor activity. According to Crain and Shen [8,9], at different doses, opioids can act via two opposing mechanisms, using either a Gs- or a Gi-protein for signaling. Chronic morphine induction may therefore enhance tolerance and desensitization of the Gi-protein pathway, while leaving the Gi-protein signal transduction pathway functional and even possibly dominant, therefore, morphine challenge may work via Gi-protein signal transduction, resulting in an excitatory effect of morphine on the EAA system.

In conclusion, long-term i.t. infusion of morphine results in the development of tolerance and a reduction in its antinociceptive effect. In addition to opioid receptor–G-protein uncoupling and receptor down-regulation, the pain facilitatory system, particularly NMDA receptors, is involved in, and enhances, synaptic nociceptive signal transduction in morphine tolerant rats. Several studies have suggested that the development of nociceptive sensitization (thermal hyperalgesia or allodynia) may be related to the increasing of EAs concentration and the subsequent activation of EAA receptors in the dorsal horn of the spinal cord [11,34,46]. In our previous study [43], we demonstrated that i.t. co-infusion of an NMDA antagonist with morphine inhibits the development of antinociceptive tolerance and the reduction in the number of high-affinity opioid receptors by a post-synaptic mechanism. The present study, as our previous observations in terminal cancer pain patients [45], also demonstrate a correlation between the increasing of CSF EAs level with the reduction of morphine’s antinociceptive effect after morphine challenge in morphine-tolerant rats. Our results support the theory of Mao et al. [28] that morphine tolerance may be due to a combination of synaptic EAs accumulation and postsynaptic NMDA receptors activation. This increasing of spinal CSF EAs concentration may responsible, in part, for the reduction in the analgesic effect of morphine in tolerant rats which was partially prevented by the NMDA receptor antagonist, MK-801. The other possible mechanism, that is, the classic Gi-protein mediated action of opioids was tolerant, it unmasks the Gs action of opioids, the excitatory action, which is usually minor compared to the classic inhibitory Gi action.

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