Melanocortin 4 Receptor Is Involved in the Development of Morphine Tolerance
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ABSTRACT

Background: Prolonged morphine treatment usually results in the development of analgesic tolerance. Melanocortin 4 receptor (MC4R) is involved in the development of morphine tolerance. The aim of this study was to examine the effects of an MC4R antagonist, HS014, on MC4R-mediated hyperalgesia and on microglia and cytokine expression in the spinal cord of rat during morphine tolerance.

Methods: Thirty rats were assigned randomly to the N group, M group, HM group, NM group and HN group (N=6 in each group). Rats received 5-day treatment with saline (N group) or morphine (M group). Rats were given an intrathecal injection (i.t.) of HS014 (HM group) or saline (NM group) at 15 minutes prior to the morphine challenge. In the HN group, rats were injected with HS014 at 15 minutes prior to the saline. To observe the effect of HS014 on the development of morphine tolerance, morphine-tolerant rats were assigned to M1 group, M2 group, M3 group and N group on day 6 (N=6 in each group). The morphine-tolerant rats in M1 group, M2 group and M3 group received morphine (10 mg/kg, i.p.), HS014 (5 μg, i.t.) and HS014 (5 μg, i.t.) followed 15 minutes later by injection of morphine (10 mg/kg, i.p.), respectively. Control rats (N group, N=6) were injected with saline under identical conditions. Hot-plate test and immunohistochemistry were used to examine the withdrawal latency and inflammatory cytokines.

Results: Morphine treatment (10 mg/kg, i.p. twice daily) over 5 days induced tolerance as reflected by a significant reduction of withdrawal latency from 29.67 ± 1.81 s to 8.67 ± 1.70 seconds in the hot-plate test. Repeated HS014 injection prior to morphine administration inhibited the development of morphine tolerance. Furthermore, a single administration of HS014 restored morphine analgesic potency in morphine-tolerant rats. Immunohistochemical staining showed that the administration of HS014 during the induction of morphine tolerance inhibited the activation of microglia, reduced the expression of proinflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α, and increased the expression of anti-inflammatory cytokines (IL-10) at the L5 lumbar spinal cord.

Conclusions: HS014 attenuates the development of antinociceptive tolerance following chronic administration of morphine by inhibiting microglial activation and reducing the expression of IL-1, IL-6 and TNF-α.
While morphine is used for various types of pain as a highly potent analgesic effect, its efficacy gradually subsides after prolonged administration, leading to the development of morphine tolerance (1). The underlying mechanisms for morphine tolerance remain largely unknown so far despite of extensive research over the years. Previous studies have shown that systemic morphine administration activates glial cells, including microglia and astrocytes (2-5). Microglia activation, enhanced expression of proinflammatory cytokines and decreased expression of anti-inflammatory cytokines are present in the lumbar spinal cord of rats after prolonged morphine treatment (4, 6). Inhibition of glial activation and proinflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α can attenuate the development of morphine tolerance in rats (4, 7).

Melanocortins, a group of peptide hormones derived from proopiomelanocortin in the pituitary gland, exert various physiological functions such as pigmentation, energy homeostasis and inflammation via binding to the melanocortin receptors (8). In the central nervous system, melanocortins are involved in the induction of neural plasticity (9). Melanocortin 4 receptor (MC4R) has been reported to play an important role in neuropathic pain and the development of morphine tolerance (10, 11). Vrinten et al. (12) have demonstrated the antiallodynic action of an MC4R antagonist SHU9119 when administered into the cisterna magna. Moreover, SHU9119 has also been shown to enhance morphine analgesic effects in neuropathic animal models (11). Previous studies suggested that neuropathic pain and the development of morphine tolerance shared similar mechanisms (13), thus prompting the idea that MC4R may be a potential target for the inhibition of morphine tolerance.

In the present study, we investigated the effect of MC4R antagonists on antinociceptive response, microglia activation and cytokines expression in the development of chronic morphine tolerance.

**MATERIALS AND METHODS**

**Animals**

Male Sprague-Dawley (SD) rats bred at Qingdao Drug Research Institute, China, weighing 220-250 g, were used for this study. The rats were housed in single cages under standard conditions (12-h light/dark cycle, 24 ± 2 °C; 50-70% humidity) with free access to food and water. All experimental procedures in this study were approved by the Bioethics Committee of the Institute of Pharmacology and were performed in accordance with the ethical guidelines for investigations of experimental pain in conscious animals (14).

**Intrathecal Catheter Implantation and Drug Administration**

Intrathecal (i.t.) catheters were implanted as described previously by Yaksh and Rudy (15). Briefly, the rats were anesthetized with 10% chloral hydrate (400 mg/kg, i.p.). After exposing to the cisternal membrane, i.t. polyethylene catheters (PE-10: OD 0.5 mm, ID 0.25 mm, Anilab, Ningbo, China) were inserted via an incision in the cisterna magna and advanced caudally to the lumbar enlargement of the spinal cord (approximately 7-8 cm deep). The catheter was fixed firmly under the skin and was sealed effectively. After the surgery, the rats were housed individually, and were allowed to recover for at least 7 days before habituation and behavioral testing. Animals with neurological deficits (signs of paralysis) or infection were excluded from experiments. The proper location of the catheter was confirmed by assessing sensory and motor blockade after intrathecal injection of 10 μl of 2% lidocaine. At the end of the study, location of the catheter was examined by postmortem dissection. An initial injection of saline (10 μl) was delivered through the catheter, and the catheter was then tightened. Subsequently, for individual experiments, drugs (in 5 μl solution) as specified individually were slowly injected through the catheter within 1-2 minutes, with 5 μl saline injected into the control rats, followed by 10 μl saline to flush the catheter.

To establish the role of HS014 in the development of morphine tolerance, thirty rats were assigned randomly to the N group, M group, HM group, NM group and HN group (N=6 in each group). Rats received 5-day treatment with saline (N group) or morphine (M group). Rats were given intrathecal injection of 5 μg of HS014 (HM group) or saline (NM group) at 15 minutes prior...
to the morning morphine challenge. In the HN group, rats were injected with 5 μg of HS014 at 15 minutes prior to the saline. Morphine was administered intraperitoneally (10 mg/kg) twice daily at 8 AM and 3 PM for five consecutive days.

In order to determine the influence of HS014 injection on morphine tolerance, morphine-tolerant rats were established by consecutive injection of morphine for 5 days. Morphine-tolerant rats were assigned to M1 group, M2 group, M3 group, and N group on day 6 (N=6 in each group). The morphine-tolerant rats in M1 group, M2 group and M3 group received morphine (10 mg/kg, i.p.), HS014 (5 μg, i.t.) and HS014 (5 μg, i.t.) followed 15 minutes later by injection of morphine (10 mg/kg, i.p.), respectively. Control rats (N group, N=6) were injected with saline under identical conditions.

Thermal Test
Antinociceptive response to morphine was determined by the hot-plate test. Animals were placed on a hot metallic plate surface maintained at 55 °C. The time until the occurrence of either licking of the hind paws, shaking, or jumping off the surface was recorded as reaction time, with the cutoff time setting at 60 seconds. Withdrawal latencies to thermal stimulation were determined at 30 minutes after the second injection of morphine.

Immunohistochemistry
Five days after the thermal test, the rats were euthanized by deep anesthesia of 10% chloralhydrate. Rats were then perfused with phosphate-buffer saline. Laminctomy was performed from the lower edge of the twelfth thoracic vertebrae to the sacral vertebrae. The L5 lumbar spinal cord was collected, frozen immediately in liquid nitrogen and stored at -80 °C. The tissues were fixed in 4% paraformaldehyde at 4 °C overnight until the tissues sink to the bottom. The samples were fixed in 10% paraformaldehyde for 12 hours and then were washed three times with 0.1 mol/L phosphate-buffer saline (PBS) for 120 minutes. Then the sample was dehydrated from 70% to 80%, 90%, 95% and 100% ethanol, and then was hyalinized with dimethylbenzene and imbedded with paraffin wax.

The spinal cord was cut into 5 μm-thick sections using a Microm cryostat (Microm International GmbH, Walldorf, Germany). The tissue sections were stained for assessment of OX-42, TNF-α, IL-1, IL-6, and IL-10 expression according to respective immunohistochemistry kit supplied by Wuhan Boster Biological Technology (Wuhan, China). The details are as described previously by Chu (10). Three sections per rat and three squares located at the same area of the dorsal horn in each section were counted. The total number of positive cells in each dorsal horn section was calculated under the high microscope (400 × magnification) for evaluation of OX-42, TNF-α, IL-1, IL-6, and IL-10-immune response (IR) by an experimenter who did not know which treatment the rat was receiving.

Statistical Analysis
All statistical analyses were performed using the SPSS 13.0 software. Values from the thermal test were represented as means ± SEM, and values from cell counts as means ± SD. A sample size of 6 rats per group was selected according to our preliminary results. All quantitative data were examined for their distribution before comparision, such as normally distributed variables (withdrawal latency), the number of OX-42, cytokines number. Behavior tests were analyzed by repeated measures one way ANOVA. Post-drug time course measures for thermal hyperalgesia were analyzed by two-way repeated measures ANOVA (post hoc, Newman–Keuls), while the TNF-α, IL-1β, IL-6, and IL-10 contents were analyzed by one-way ANOVA (Dunnett multiple comparisons). Differences among means were considered to be statistically significant if P<0.05.

RESULTS
Development of Morphine Tolerance
The baseline withdrawal latency (before morphine administration) remained constant throughout the entire testing procedure in the N group (Figure 1). On day 1 and 2, morphine administration induced significant analgesia in the M group compared to the N group (P<0.001). On day 4 of morphine administration, a significant decrease in the analgesic efficacy was observed, indicating that 5-day intrathecal administration of morphine induced morphine tolerance. On the other
hand, the withdrawal latency of the M group did not differ significantly from that of the N group. Saline delivered via the intrathecal catheter prior to morphine (i.p.) did not significantly alter the effect of morphine (P > 0.05).

Effect of HS014 Administration on Morphine-Induced Antinociception
Repeated administration of HS014 (5 μg, i.t.) did not alter the withdrawal latency in rats in the N group (Figure 1). Two-way ANOVA revealed significant effect of intrathecal injection of HS014 (withdrawal latency: F1, 29 = 40.081, P < 0.001) and interaction between HS014 treatment and time (withdrawal latency: F5, 29 = 6.383, P < 0.001). HS014-induced changes in development of morphine tolerance are shown in figure 1. Morphine-induced analgesia was similar between the M group and the HM group on day 1 and 2 (P > 0.05). However, the withdrawal latency reduced significantly in the M group after day 3, while the withdrawal latency was markedly extended after day 3 in the HM group compared to the initial effect of morphine administration alone (P < 0.05).

Effect of Single I.T. HS014 Injection on Withdrawal Latency in Morphine-Tolerant Rats
After five days of morphine administration, the rats were tolerant to morphine as shown above. On day 6, morphine (10 mg/kg, i.p.), HS014 (5 μg, i.t.), or HS014 (5 μg, i.t.) was administered to the morphine-tolerant rats at 15 minutes prior to morphine, with saline injection as the control. HS014 (5 μg, i.t.) injected at 15 minutes prior to morphine resulted in a significant increase in the withdrawal latency (P < 0.05) compared to morphine alone (Figure 2), while injection of HS014 alone showed no antinociceptive effect (P > 0.05) on morphine-tolerant rats (Figure 2).

Effect of HS014 on Microglia (OX-42) Immunoreactivities in the Dorsal Horn of the Lumbar Spinal Cord of Morphine-Tolerant Rat
The expression of OX-42 at L5 lumbar spinal cord in M group was significantly higher compared to the N group (Figure 3). HS014 had no effect on the expression of OX-42 in the N group. In contrast, HS014 injection during the induction of morphine tolerance significantly attenuated the morphine-induced upregulation of OX-42 (Figure 3).

Effect of HS014 on IL-1, IL-6, IL-10, and TNF-α Expression in Rat Spinal Cords
In the M group, chronic administration of morphine significantly increased the expression of IL-1, IL-6, and TNF-α but decreased the expression of IL-10 in L5 lumbar spinal cord compared to the N group (Table). HS014 had no effect on the expression of IL-1, IL-6, TNF-α or
Figure 3. Effect of HS014 on Glial Activation in the Spinal Cord of Morphine-Tolerant Rats Determined by OX-42 Expression.
The arrow points to an OX-42 positive cell. Data are expressed as means ± SD. *P < 0.05 compared with N group; #P < 0.05 compared with M group.

IL-10 in the N group. However, HS014 treatment during the induction of morphine tolerance significantly attenuated the morphine-induced upregulation of IL-1, IL-6, and TNF-α and downregulation of IL-10 expression (Table).

DISCUSSION

The present study investigated the contribution of HS014 (MC4R antagonist) to the development
of morphine-induced tolerance and its biochemical mechanisms. Results showed that administration of HS014 decreased the development of morphine tolerance and its associated hyperalgesia. This finding was attributed to the modulation of blockade of MC4 receptors on morphine-induced microglial activation and inflammatory cytokines secretion. The findings of this study shed light on the mechanisms underlying the development of morphine tolerance, and provided a potential target (MC4R) as a new strategy for the treatment of morphine tolerance.

Since morphine is still the most effective drug for the treatment of chronic pain, it is very important to maintain and/or resume the potency of opioid analgesia. Reduction or loss of analgesic potency has been a major limitation in the repeated use of morphine, making chronic pain obstinate in clinical practice.

Currently, the interest is centered on the investigation of morphine tolerance. However, the mechanisms of the development of morphine tolerance are not completely identical. For example, 5-hydroxytryptamine exerts opposite action on the development (16) and maintenance (17) of opioid tolerance while the activation of GABA (18) and dopamine (19) receptors is only involved in the development of tolerance. Therefore, it is necessary to investigate the mechanisms underlying morphine tolerance. Accumulating evidence suggests that MC4R plays an important role in morphine tolerance and neuropathic pain (12, 16). Recently, expression of MC4R is also found in dorsal root ganglion (DRG) neurons (17, 18) and microglia (3), which is involved in the development and maintenance of neuropathic pain. Similar mechanisms are implicated in the development of morphine tolerance and neuropathic pain. Previous studies by our group have shown that MC4R induced hyperalgesia and allodynia by activation of p38 MAPK in DRG neurons (16). The present study found that morphine tolerance was markedly delayed by pretreatment with HS014, which provided further evidence that MC4R contributed to the development of morphine tolerance. This study also found that HS014 could attenuate the decreased analgesic effect of morphine caused by tolerance. Based on these findings, we hypothesize that MC4R enhances activity of the opioid systems.

Morphine has been demonstrated to activate spinal glial activity, enhance production of TNF-α and nitric oxide, and inhibit microglial chemotaxis (5, 19). Morphine tolerance is associated with spinal microglial and astroglial activation (3, 11, 20). Inhibition of spinal glial activation can reverse the development of morphine tolerance in rats (5). Consistently, the present study has shown that morphine tolerance could induce microglial activation in the spinal cord that is associated with increased expression of OX-42-IR, and HS014 could suppress morphine-induced spinal microglial activation, suggesting that MC4R may be involved in the establishment of spinal microglial plasticity.

Inflammatory cytokines and MC4R both play an important role in the development and maintenance of morphine tolerance (21). The chronic administration of morphine leads to upregulation of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α (4, 22). In our previous study (10), we have demonstrated that activation of MC4R in periaqueductal gray after peripheral nerve injury participated in pain facilitation by regulating the glial activation and inflammatory cytokines secretion. In the present study, we found that HS014 inhibited the upregulation of these proinflammatory cytokines (IL-1, IL-6, and TNF-α) in the spinal cord induced by repeated morphine administration. These results further support the theory mentioned above and in-

### Table. Comparison of Cytokine Expression in the Spinal Cord (number, mean±SD).

| Group    | TNF positive cell | IL-1 positive cell | IL-6 positive cell | IL-10 positive cell |
|----------|-------------------|--------------------|--------------------|---------------------|
| N (N=6)  | 28.4±1.1          | 48.5±0.5           | 38.0±0.5           | 69.9±1.2            |
| M (N=6)  | 99.3±2.1*         | 97.8±1.3*          | 68.1±0.5*          | 71.2±1.3*           |
| HM (N=6) | 61.0±1.3†         | 64.3±1.2‡          | 49.1±0.7†          | 126.8±2.2‡          |
| NM (N=6) | 92.6±1.4§         | 96.9±0.7§          | 69.1±0.7§          | 70.4±0.9§           |
| HN (N=6) | 26.1±0.8**        | 49.4±0.5**         | 38.4±0.7**         | 70.0±1.0**          |

*P<0.001, #P<0.001, **P>0.05 compared to normal saline group (N); ◇P>0.05, ☆P<0.001 compared to morphine group (M).
This effect may be mediated by inhibiting microglial activation, and reducing the expression of IL-1, IL-6, and TNF-α. Therefore, it can be proposed that MC4R could be an effective therapeutic target to restore the potency of morphine following the development of tolerance.

CONCLUSIONS

HS014 has shown a profound attenuating effect on the development of antinociceptive tolerance following chronic administration of morphine.

References

1. Chu LF, Clark DJ, Angst MS. Opioid tolerance and hyperalgesia in chronic pain patients after one month of oral morphine therapy: a preliminary prospective study. J Pain 2006; 7: 43-8.
2. Horvath RJ, DeLeo JA. Morphine enhances microglial migration through modulation of P2X4 receptor signaling. J Neurosci 2009; 29: 988-1005.
3. Mika J. Modulation of microglia can attenuate neuropathic pain symptoms and enhance morphine effectiveness. Pharmacol Rep 2008; 60: 297-307.
4. Raghavendra V, Tanga FY, DeLeo JA. Attenuation of morphine tolerance, withdrawal-induced hyperalgesia, and associated spinal inflammatory immune responses by propentofylline in rats. Neuropsychopharmacology 2004; 29: 327-34.
5. Song P, Zhao ZQ. The involvement of glial cells in the development of morphine tolerance. Neurosci Res 2001; 39: 281-6.
6. Merighi S, Gesi S, Varani K, Fazzi D, Stefanelli A, Borella PA. Morphine mediates a proinflammatory phenotype via mu-opioid receptor-PKC epsilon-Akt-ERK1/2 signaling pathway in activated microglial cells. Biochem Pharmacol 2011; 84: 487-96.
7. Starowicz K, Obata I, Przewlocki R, Przewlocka B. Inhibition of morphine tolerance by spinal melanocortin receptor blockade. Pain 2005; 117: 401-11.
8. Gantz I, Fong TM. The melanocortin system. Am J Physiol Endocrinol Metab 2013; 284: E468-74.
9. Darlington CL, Gilchrist DP, Smith PF. Melanocortins and lesion-induced plasticity in the CNS: a review. Brain Res Brain Res Rev 1996; 22: 245-57.
10. Chu H, Sun J, Xu H, Niu Z, Xu M. Effect of periaqueductual gray melanocortin 4 receptor in pain facilitation and glial activation in rat model of chronic constriction injury. Neurou Res 2012; 34: 871-88.
11. Wen YR, Tan PH, Cheng JK, Liu YC, Ji RR. Microglia: a promising target for treating neuropathic and postoperative pain, and morphine tolerance. J Formos Med Assoc 2011; 110: 487-94.
12. Vrenten DH, Gijzen WH, Kalkman CJ, Adan RA. Interaction between the spinal melanocortin and opiate systems in a rat model of neuropathic pain. Anesthesiology 2003; 99: 449-54.
13. Mao J, Price DD, Mayer DJ. Experimental mono- neuropathy reduces the antinociceptive effects of morphine: implications for common intracellular mechanisms involved in morphine tolerance and neuropathic pain. Pain 1995; 61: 353-64.
14. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983; 16: 109-10.
15. Takah TL, Rudy TA. Chronic catheterization of the spinal subarachnoid space. Physiol Behav 1976; 17: 1031-6.
16. Way EL, Loh HH, Shen F. Morphine tolerance, physical dependence, and withdrawal of brain 5-hydroxytryptamine. Science 1968; 162: 1290-2.
17. Neal BS, Sparber SB. Mianserin attenuates naloxone-precipitated withdrawal signs in rats acutely or chronically dependent upon morphine. J Pharmacol Exp Ther 1986; 236: 157-65.
18. Raghavendra V, Kalkan SK. Reversal of morphine tolerance and dependence by melatonin: possible role of central and peripheral benzodiazepine receptors. Brain Res 1999; 834: 178-81.
19. Cook CD, Barret AC, Syvanthong C, Picker MJ. The dopamine D3/D2 agonist 7-OH-DPAT attenuates the development of morphine tolerance but not physical dependence in rats. Psychopharmacology (Berl) 2000; 152: 93-104.
20. Chu H, Xia J, Yang Z, Gao J. Melanocortin 4 receptor induces hyperalgesia and allodynia after chronic constriction injury by activation of p38 MAPK in DRG. Int J Neurosci 2012; 122: 74-81.
21. Carniglia L, Durand D, Caruso G, Lasauga M. Effect of NDF-alpha-MSH on PPAR-gamma and -beta expression and anti-inflammatory cytokine release in rat astrocytes and microglia. PLoS One 2013; 8: e57313.
22. Trajillo KA. Effects of noncompetitive N-methyl-D-aspartate receptor antagonists on opiate tolerance and physical dependence. Neuropsychopharmacology 1995; 13: 101-7.
23. Gok YS, Hulsebosch CE. Remote astrocytic and microglial activation modulates neuronal hyperexcitability and below-level neuropathic pain after spinal injury in rat. Neuroscience 2009; 163: 895-903.
24. Tumati S, Largent-Milnes TM, Kerestes A, Ren J, Rokske WR, Vanderah TW, et al. Repeated morphine treatment-mediated hyperalgesia, allodynia and spinal glial activation are blocked by co-administration of a selective cannabinoid receptor type-2 agonist. J Neuroinflammation 2012; 9: 244: 23-31.
25. Limori E, Gaspari L, Panerai AE, Sacerdote P. Differential morphine tolerance development in the modulation of macrophage cytokine production in mice. J Leukoc Biol 2002; 72: 43-8.
26. Tai YH, Wang YH, Wang JJ, Tao PL, Tung CS, Wong CS. Antispasticity suppresses neuroinflammation and up-regulates glutamate transporters in morphine-tolerant rats. Pain 2006; 124: 77-86.