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Activation of the spinal extracellular signal-regulated kinase 5 signaling pathway contributes to morphine physical dependence in rats☆

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ABSTRACT

The activation of mitogen-activated protein kinases (MAPKs) has been observed in synaptic plasticity processes of learning and memory in morphine dependence. However, the role of extracellular signal-regulated protein kinase 5 (ERK5), a member of MAPKs, has not been studied yet in morphine dependence. To identify the function of ERK5 in the formation and development of morphine physical dependence, morphine withdrawal-like behavioral test and western blot technique were used in this research. Morphine was subcutaneously injected by an intermittent and escalating procedure to induce physical dependence, which was measured by withdrawal symptoms. In this study, spinal ERK5 signaling pathway was remarkably activated by chronic morphine injection and naloxone-precipitated withdrawal. Intrathecal injection of BIX02188, a novel specific inhibitor of mitogen-activated protein kinases kinase 5 (MEK5), produced a dose- and time-dependent inhibition of the activation of spinal ERK5, without affecting activation of other MAPKs. Moreover, selective attenuation of spinal p-ERK5 expression by BIX02188 could significantly relieve morphine withdrawal symptom, accompanying with the decreased phosphorylation of cAMP response-element binding protein (CREB) in the spinal cord. These findings suggested that activation of the ERK5 signaling pathway might contribute to morphine physical dependence and its specific pharmacological inhibitor BIX02188 could be a potential therapeutic choice for alleviation of morphine withdrawal symptoms in the future.

Opiate, such as morphine, is one of the most commonly used drugs in clinic for treatments of severe and chronic pain. However, its usage for chronic pain is largely limited by the development of dependence which is induced by prolonged opiate exposure. Opioid dependence involves psychological dependence characterized by compulsive, out-of-control drug use despite serious negative consequences and relapse, while physical dependence is characterized by withdrawal symptoms produced after discontinuation or reduction in the use of opiate [2]. Mechanisms of opiate dependence are complex and involve factors at the levels of receptors, ion channels, the cells and neural networks [17]. Previous studies have shown that diverse neurotransmitters, receptor systems and intracellular signaling proteins are closely related to opiate dependence, especially mitogen-activated protein kinases (MAPKs) [1,7,18,29]. MAPKs are crucial components of signaling cascades that regulate numerous physiological processes during development and pathogenesis [6,31]. The MAPK family includes extracellular signal-regulated protein kinase 1/2 (ERK1/2), p38MAPK, c-jun N-terminal kinase (JNK), and extracellular signal-regulated protein kinase 5 (ERK5). Although ERK5 contains a Thr-Glu-Tyr dual phosphorylation motif of the ERK1/2-type MAP kinase, it has a unique loop-12 structure and an unusually large C-terminal non-kinase domain, making ERK5 quite different from ERK1/2 [35].

It has been recently reported that ERK5 cascade contributed to pain hypersensitivity. Phosphorylation of ERK5 in rat spinal cord microglia remarkably increased nerve injury, however, antisense knockdown of ERK5 could depress nerve injury-induced hyperalgesia [24]. Our previous study found that inflammatory pain induced by injection of complete Freund's adjuvant into rat hindpaw produced notable ERK5 activation in dorsal root ganglion and spinal dorsal horn neurons [32]. Considering that morphine dependence and pathological pain may share common cellular mechanisms [20], we hypothesized that ERK5 might play an important role

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in morphine dependence. Moreover, compelling evidences have indicated that ERK1/2 plays an important role in morphine dependence and naloxone-precipitated withdrawal. Chronic morphine administration could provoke sustained ERK1/2 activation in central amygdale and spinal cord [3,16]. Inhibition of the spinal ERK1/2 activation by intrathecal injection of U0126 not only decreases the scores of morphine withdrawal, but also attenuates withdrawal-induced allodynia [3]. Interestingly, U0126, an ERK1/2-specific inhibitor, can also inhibit the ERK5 pathway [12,21,24]. So our attentions begin to turn to the role of ERK5 activation in morphine dependence which has not been studied yet.

Numerous previous studies have revealed that multiple sites of brain are closely related to morphine physical dependence including the periaqueductal gray, the dorsal thalamus, locus coeruleus, amygdale and nucleus accumbens [2,16,22]. However, many reports have demonstrated that the spinal cord, a connection between the peripheral and central nervous systems, plays an important role in mediating the development of physical dependence to opiates [3,10,19,26]. Accordingly, the aim of the present study was to determine whether the activation of spinal ERK5 contributes to morphine physical dependence, and whether intrathecal injection of BIX02188, a latest specific inhibitor of mitogen-activated protein kinases kinase 5 (MEK5), could produce a dose- and time-dependent inhibition of the activation of spinal ERK5, without affecting activation of other MAPKs *in vivo*.

Adult, male Sprague–Dawley rats (200–250 g) were housed on a 12/12 h light/dark cycle, with free access to food and water. The animals were provided by Experimental Animal Center of Xuzhou Medical College. All experiment protocols were approved by the Animal Care and Use Committee of Xuzhou Medical College and were in accordance with the Declaration of the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (publication no. 80–23, revised 1996).

One week prior to the experiments, for intrathecal drug administration, a polyethylene PE 10 catheter was implanted in the lumbar subarachnoid space of rat during deep pentobarbital sodium (40 mg/kg, intraperitoneal) anesthesia according to the method described previously [34]. Correct intrathecal placement was confirmed by injection of 10 μl 2% lidocaine through the catheter. After 30 s of this injection, the catheter was judged to be intrathecal if paralysis and dragging of bilateral hind limbs occurred. Animals with signs of motor dysfunction were excluded. The rats were housed individually after surgery and allowed to recover 7 days before test.

Morphine HCl (the First Pharmaceutical Factory of Shenyang, China) and naloxone (Sigma) were dissolved in 0.9% physiological saline, respectively. BIX02188, kindly provided from Boehringer Ingelheim (Ridgefield, CT), was dissolved in 1% DMSO (10 μg/10 μl, 1 μg/10 μl and 0.1 μg/10 μl, respectively).

Rats were randomly divided into five groups as follows: group 1, saline treated rats; group 2, chronic morphine treated rats; group 3, naloxone-precipitated withdrawal rats; group 4, morphine treated animals which received naloxone 60 min after the administration of 1% DMSO and group 5, morphine treated animals receiving naloxone 60 min after the administration of BIX02188. Rats were subcutaneously injected with morphine (bid, for 5 day, 10, 20, 30, 40, 50, 50 mg/kg). On day 6, 4 h after injection of morphine 50 mg/kg, rats were injected with naloxone (4 mg/kg, intraperitoneal) to precipitate morphine withdrawal syndrome. Rats were administrated with an equivalent volume of saline as a control.

The animals were habituated the test environment in a glass cylinder 30 min prior to naloxone injection. After injecting, signs of withdrawal were scored in a 60 min at 15 min intervals. Ten accepted behavioral characteristics of the rat withdrawal syndrome were assessed. The absolute frequency of five episodic behaviors (jumping, teeth chatter, writhing, wet-dog shakes, and rearing) were recorded and scored as the following: 0 = no incidents; 1 = 1–5 incidents; 2 = 6–10 incidents; and 3 = more than 10 incidents. The severity of the other five behaviors, ptosis, lacrimation, piloerection, irritability, and diarrhea, was assessed using a four-point scale: 0 = absent; 1 = mild; 2 = moderate; 3 = severe. Finally, the scores for each time period were added together [25,38].

Rats were sacrificed immediately after behavioral characteristic examinations. The lumbosacral spinal cords of rats were quickly extracted under deep isoflurane anesthesia and stored in liquid nitrogen. Tissue samples were homogenized in 50 mM RIPA buffer (20 μl/mg), containing protease inhibitor cocktail and phosphatase inhibitor cocktril. Lysates were centrifuged at 14,400 × *g* for 60 min at 4 °C, and the concentration of protein in each sample (supernatant) was determined by the Lowry method. Samples with equal amounts of protein were then separated by 10–20% PAGE, and the resolved proteins were electrotransferred to nitrocellulose membrane. Membranes were incubated with 5% nonfat milk with for at least 60 min at room temperature and incubated with the following primary antibodies: p-ERK5, ERK5, p-ERK1/2, total ERK1/2, p-p38, p38, p-JNK, JNK (1:1000; Cell Signaling Technology), p-CREB, CREB (1:500; SAB) and antibody for β-actin (1:1000; Sigma) at 4 °C overnight. The membranes were extensively washed with TBST and incubated for 2 h with the secondary antibody conjugated with alkaline phosphatase (1:1000) at room temperature. The immune complexes were detected by using a NBT/BCIP assay kit (Sigma). Western blot densitometry analysis of signal intensity was performed using Adobe Photoshop software (Adobe, San Jose, CA), and phosphorylation levels of MAPKs from densitometry were normalized to total MAPKs. The blot density from control groups was set as 100%.

For statistical analysis, GraphPad Prism 5 (GraphPad Software, USA) was used. Data were expressed as mean ± SEM. Statistical comparison of more than two groups was performed using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. A value of $P < 0.05$ was considered as statistically significant.

In the present study, MAPK activation was evaluated by detecting the expression of p-MAPKs by western blot analysis. Firstly, we analyzed the time course of spinal p-ERK5 expression after intrathecal injection of BIX02188 (10 μg). The reduction of p-ERK5 was detected at 30 min, and the most significant reduction was detected at 1 h after intrathecal injection of BIX02188. The suppression of p-ERK5 lasted at least up to 4 h and returned to baseline levels at 8 h after intrathecal injection of BIX02188 ($P < 0.001$ at 0.5 h, 1 h, 2 h, $P < 0.05$ at 4 h, *vs.* 0-h; Fig. 1A). To further determine the influence of BIX02188 on the activation of MAPKs, 1 h time point was used to perform dose–response tests. Intrathecal injection of BIX02188 (0.1, 1, 10 μg), not 1% DMSO, induced a dose-dependent decrease in spinal p-ERK5 expression ($P < 0.001$, BIX02188 10 or 1 μg group *vs.* 1% DMSO group; Fig. 1B), while not blocking the phosphorylation of ERK1/2, p38MAPK and JNK.

There was no significant withdrawal sign in saline treatment rats, on the other hand, morphine–naloxone group and morphine–vehicle–naloxone group had pronounced withdrawal signs. Intrathecal injection of BIX02188 significantly attenuated naloxone-precipitated withdrawal symptoms in a dose-dependent manner (Fig. 2A and B). Western blot assay revealed that spinal p-ERK5 increased in chronic morphine treatment rats and there was a further increase in the activation of ERK5 after naloxone-precipitated withdrawal. Intrathecal injection of BIX02188, not DMSO, reduced the p-ERK5 expression in the spinal of morphine withdrawal rats (Fig. 2B).

Western blot result showed that p-CREB was highly expressed in the spinal of morphine dependence and withdrawal rats. Intrathecal injection of BIX02188, not DMSO, could significantly inhibit the increase of p-CREB. However, the total CREB expression was not altered in any state (Fig. 3).