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Original Article

PKC regulates postoperative pain via ERK and mTOR pathways

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Abstract: The aim of this study was to investigate the role of protein kinase C (PKC) in the treatment of acute and chronic postoperative pain. First, mouse acute and chronic pain models were established *via* IL-6 injection and plantar incision, respectively. After Enzastaurin injection and model construction, a specific inhibitor of PKC, mRNA levels of mTOR were detected, as well as extracellular signal-regulated kinase (ERK) and PKC at different time points by quantitative real-time polymerase chain reaction (qRT-PCR). Moreover, phosphorylation levels of mTOR, ERK, and PKC were also accessed by Western blot. Mouse acute and chronic pain models were established and ERK and mTOR pathways were analyzed in sensory neurons and found to be significantly inhibited *via* PKC inhibition. This was capable of relieving postoperative pain in mice. Enzastaurin injection at the incision site could improve mechanical pain *via* inhibiting mTOR and ERK pathways, which provides a new target for the treatment of postoperative pain.

Keywords: PKC, ERK and mTOR pathways, postoperative pain

Introduction

Acute pain caused by surgery and chronic pain during postoperative incision recovery is common clinical condition. Chronic pain is a well-recognized complication in multiple surgeries including cardiothoracic surgery, craniotomy, fracture surgery, hernia repair, etc. Postoperative chronic pain has a great impact on the quality of life for patients, which brings heavy physiology and economic burdens [1-3]. Despite improvements in postoperative pain management, the incidence of postoperative pain still remains high. It is of great importance to clarify the underlying mechanism of postoperative pain, thereby searching for effective treatments.

Interleukin-6 (IL-6), as a cytokine, is immediately produced after infection and tissue damage, which is capable of promoting host defense by stimulating acute phase reaction, hematopoiesis, and immune response. Dysregulation of IL-6 synthesis has a significant impact on the formation of chronic inflammation [4]. Moreover, IL-6 is closely related to the development and progression of pain. Relative studies have found that serum level of IL-6 is significantly elevated after surgery [5]. IL-6 may be involved in the postoperative chronic pain, which induces nascent protein synthesis in primary neurons and axons, thereby contributing to increased pain sensitivity [6, 7].

It has been proven that mammalian target of rapamycin and its downstream effector molecules are associated with the development of chronic inflammation, neuropathic pain, and cancer pain [8]. mTOR is widely expressed in primary sensory axons, dorsal root ganglia (DRG), and dorsal horn neurons. Inhibition of mTOR has a remarkable effect on relieving pain in experimental models of inflammatory and neuropathic pain.

Protein kinase C (PKC) and extracellular signal-regulated kinase 1/2 (ERK 1/2) are considered to be related to the pathogenesis of neuropathic pain [9]. PKC is mainly expressed in the cornu dorsale medullae spinalis, medullary dorsal horn, and superficial neurons, which participate in regulation of nociceptive information and

chronic pain [10, 11]. Phosphorylated ERK is believed to be associated with pain. Studies have confirmed that inhibition of ERK phosphorylation can reduce the pain induced by infraorbital nerve during chronic constrictive injury [12]. Phosphorylated ERK promotes central sensitization by altering activities of glutamate receptors and potassium channels [13]. ERK also plays a key role in the development of mechanical pain responses after trigeminal nerve injury. In this study, the effects of mTOR and ERK pathways were analyzed on acute and chronic postoperative pain in mice.

Materials and methods

Experimental animals

Adult male ICR mice (8-10 weeks old) were utilized in this study. Mice were maintained in the environment at specific pathogen free (SPF) level, with 8 mice in each group. This study was approved by the Animal Ethics Committee of Beijing University of Chinese Medicine Animal Center.

Behavioral determinations

Mice were placed in a special custom cage with wire netting at the bottom floor. The minimum stimulation of the mice to produce the lift foot reflex was recorded by an electronic von Frey anesthesiometer. The procedure was repeated three times with an interval of 3-4 minutes. The average of 3 records was taken as the paw withdrawal mechanical threshold (PWMT).

Construction of acute pain model

The acute pain model was constructed by IL-6 injection in mice. Before injection, baseline mechanical threshold of the left hind paw was determined. For acute sensitization experiment, 25 μL of IL-6 (Abcam, Cambridge, MA, USA) was injected in the left hind paw of mice from control group, whereas 25 μL of IL-6 and 10 μg of Enzastaurin was injected in the left hind paw of mice from treatment group. PWMT was recorded 1, 3, 24, 48, and 72 hours after injection, respectively.

Construction of chronic postoperative pain model

Preoperative PWMT was detected in all mice. Mice were anesthetized with 40-50 mg/kg sodium pentobarbital by intraperitoneal injection. A 5 mm-longitudinal incision was cut on the plantar skin, fascia, and muscle of the mice. Mice in sham operation group were anesthetized without performing surgery. PWMT was recorded on the postoperative 1st, 3rd, 7th, and 14th day, respectively. Mice in treatment group received Enzastaurin injection immediately after the surgery, followed by the injection once a day. Behavioral determinations of mice from both groups were performed at the same day.

Sample collection

Mice were sacrificed after intraperitoneal injection of 40-50 mg/kg sodium pentobarbital for anesthesia. Trigeminal ganglia of mice were quickly dissociated under aseptic conditions and preserved in liquid nitrogen for the following experiments.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissues by TRIzol method (Invitrogen, Carlsbad, CA, USA) and then transcribed into complementary Deoxyribose Nucleic Acid (cDNA). The reverse transcription reaction was carried out in strict accordance with the instructions of SYBR Green Real Time PCR Master Mix (TaKaRa, Dalian, China). Primer sequences used in this study were as follows: ERK, F: 5'-GTCCAACCACAAGCTTTATC-3'; R: 5'-CCATATTCCAACGCAGCGCA-3'; mTOR, F: 5'-TCCCTGGCCTAGAAGACAGC-3'; R: 5'-TCGAATTCCCCTAAGGCATT-3'; PKC, F: 5'-GCTGTTTTCCCCGTTCTTCT-3'; R: 5'-TGTTTAAGGGTTTTGATCGG3'.

Western blot

Radioimmunoprecipitation assay (RIPA) solution was used to extract the total protein (Roche, Basel, Switzerland). Protein sample was separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After membranes were blocked for 1 h, the membranes were incubated with primary antibodies (p-mTOR, p-ERK, p-PKC, dilution at 1:1000, Abcam, Cambridge, MA, USA) for 2 hours at room temperature. The membranes were then washed with Tris Buffered Saline-Tween (TBST) and followed by the incubation of the Horse Reddish Peroxidase (HRP)-labeled secondary antibody

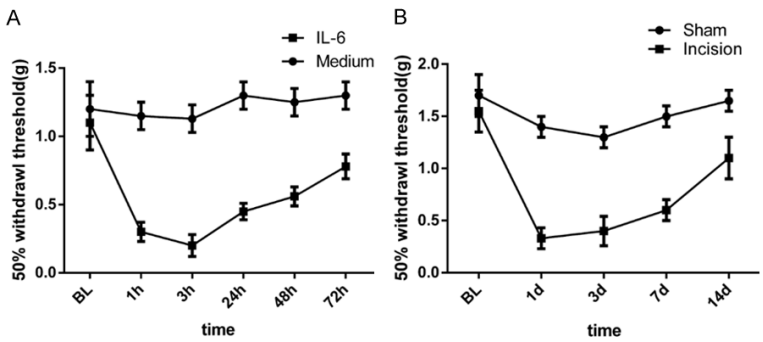

Figure 1. Behavioral changes of mice in acute and chronic pain models. A. PWMT was detected in mice of treatment group after 25 μL of IL-6 injection for 1, 3, 24, 48, and 72 hours, respectively. B. PWMT was detected in mice of sham operation group after plantar incision for 1, 3, 7 and 14 days, respectively.

(dilution at 1:5000, Abcam, Cambridge, MA, USA). The protein blot on the membrane was exposed by chemiluminescence.

Statistical analysis

All statistical analyses were conducted on statistical product and service solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). GraphPad Prism 6.0 (La Jolla, CA, USA) was introduced for image editing. All measurement data are expressed as mean ± standard deviation. Independent-sample t-test was used to compare the differences of mRNA and phosphorylation levels of mTOR, ERK, and PKC at different days after plantar incision in mice or between two groups. $P < 0.05$ was considered statistically significant.

Results

Construction of acute and chronic postoperative pain models in mice

The acute postoperative pain model was constructed in mice *via* IL-6 injection. PWMT was measured after IL-6 injection for 1 hour. The data showed that PWMT in control group was decreased more than that of the treatment group, indicating a stronger acute sensitization. PWMT in the control group was gradually increased after injection for 3, 24, 48, and 72 hours, which was not able to reverse to the baseline PWMT (**Figure 1A**).

Next, the chronic postoperative pain model was constructed in mice *via* plantar incision. No significant difference in preoperative PWMT was found between sham operation group and treatment group ($P > 0.05$). PWMT was remarkably decreased in mice of treatment group on the first day after surgery, which was gradually increased in a time-dependent manner. However, PWMT did not reverse to the baseline on the postoperative 14th day (**Figure 1B**).

Increased PKC phosphorylation level and activated mTOR and ERK pathways in mouse trigeminal ganglia

After successful construction of the pain model in mice, mRNA levels of mTOR were detected and ERK and PKC were analyzed in mice at different time points after surgery. The data indicate that mRNA levels of mTOR, ERK, and PKC were increased in mouse trigeminal ganglia of treatment group compared with those of the sham operation group in a time-dependent manner (**Figure 2A-C**). Moreover, phosphorylation levels of mTOR, ERK, and PKC in mouse trigeminal ganglia were detected by Western blot. Specifically, p-mTOR expression achieved the peak on the postoperative 3rd day, which was slightly decreased on the postoperative 14th day. Protein expression of p-ERK showed a sharp increase after the surgery, which was then decreased in a time-dependent manner. It was also observed that protein level of p-PKC was significantly increased on the first day after the surgery, which remained to be overexpressed 14 days later ($P < 0.05$, **Figure 2D**, **2E**).

Inhibition of PKC reduced mechanical pain in mice

To explore the effect of PKC inhibitor on the acute pain in mice, IL-6 and Enzastaurin were injected in mice. As shown in **Figure 3A**, mechanical pain in mice of treatment group who received Enzastaurin administration was remarkably attenuated than that of the control group. The effect of PKC inhibitor on the chronic pain in mice was next explored. Enzastaurin was injected around the plantar incision. The data show that mice in the sham operation group presented mechanical pain for at least 10 days. PWMT in mice of sham operation group was slightly increased, which was not able to reverse to baseline. In contrast, Enzastaurin injection remarkably inhibited mechani-

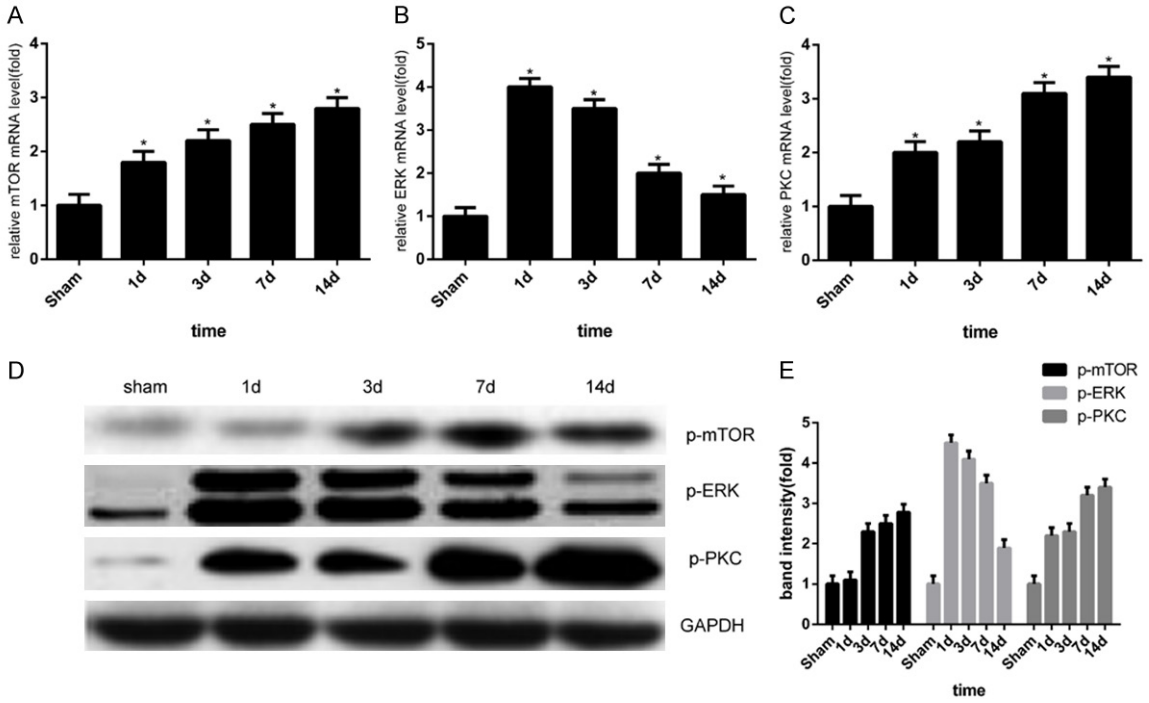

Figure 2. Increased phosphorylation of PKC and activation of mTOR and ERK pathways were observed in mouse chronic pain model. The mRNA levels of mTOR (A), ERK (B) and PKC (C) in the trigeminal ganglia at 1, 3, 7, and 14 days after plantar incision in mice were detected by RT-PCR. (D, E) Phosphorylation levels of mTOR, ERK and PKC in the nerve tissues at 1, 3, 7, and 14 days after the plantar incision in mice were detected by Western blot (*$P < 0.05$).

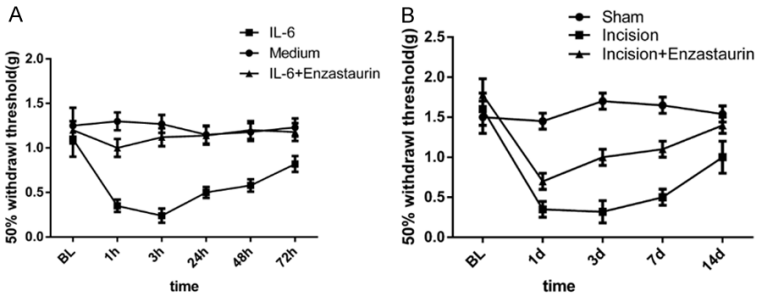

Figure 3. Enzastaurin injection can relieve mechanical pain induced by plantar incision. A. A total of 25 μL of IL-6 and 10 μg of Enzastaurin were injected into mice of treatment group, and PWMT was measured at 1, 3, 24, 48, and 72 hours, respectively. Baseline of PWMT was measured before injection in each group. B. PWMT was measured before surgery, 1, 3, 7, and 14 days after surgery.

cal pain in mice of the treatment group (**Figure 3B**).

Inhibition of PKC could inhibit the activation of mTOR and ERK pathways in chronic postoperative pain model

To further investigate the underlying mechanism of PKC inhibitor in the regulation of chronic postoperative pain in mice, mRNA levels of mTOR and ERK in mouse trigeminal ganglia on the first day after the surgery. qRT-PCR results demonstrated that mRNA levels of mTOR and ERK in mice of treatment group were decreased more than those of the sham operation group (**Figure 4A**, **4B**). Furthermore, mRNA level of PKC in mice of treatment group was also remarkably decreased, indicating that Enzastaurin could effectively inhibit the transcription and translation of PKC (**Figure 4C**). Lower phosphorylation levels of mTOR and ERK were found in mice of the treatment group compared with those of the sham operation group (**Figure 4D**, **4E**). These results elucidate that Enzastaurin administration is an efficient approach in relieving postoperative pain.

Discussion

Postoperative chronic pain is one of the thorniest clinical problems. The somatic mechanical pain may even cause mental disorders in some patients [14]. Unfortunately, treatment of

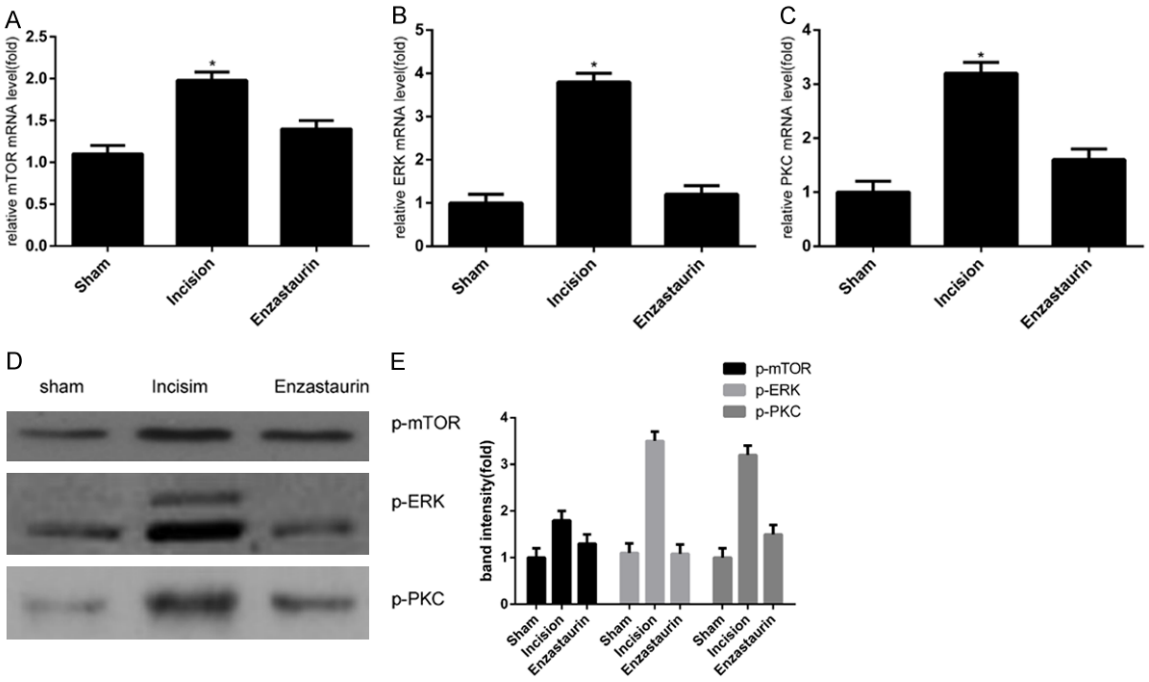

Figure 4. Enzastaurin injection inhibited activation of PKC/ERK/mTOR pathway. The mRNA expressions of mTOR (A), ERK (B) and PKC (C) were decreased after Enzastaurin injection. (D, E) Enzastaurin injection inhibited the phosphorylation levels of mTOR, ERK and PKC proteins (*$P < 0.05$).

chronic pain is unsatisfactory [15], which requires studies on searching for more efficient treatment approaches.

PKC is a serine/threonine kinase composed of approximately 15 different isozymes. PKC family includes classical kinases (α, βi, βII, γ), novel enzymes (δ, ε, η, θ) and atypical kinases (ξ, i/λ). Activation of classical and novel PKCs relies on diacylglycerol (DAG). Previous studies have confirmed that PKC has an effect on development of cardiac hypertrophy, heart failure, ischemic injury, diabetes, and cancers [16-18]. The effect of PKCs on postoperative chronic pain, however, is still unknown.

It has been reported that ERK and mTOR pathways are classical pathways that mediate peripheral mechanical pain. Therefore, these two signaling pathways may have a certain effect on postoperative mechanical pain [19, 20]. Studies have shown that ERK pathway is involved in multiple physiological processes, such as cell proliferation and growth, mutations of cancer cells and over-activation of various pathways. Downstream transcription factors in ERK pathway include FOS, STAT1, STAT 3, NF-kb, CREB, AP-1, and c-myc [21]. There are two signaling complexes in mTOR pathway, namely mTORC1 and mTORC2. Functionally, mTOR exerts a crucial role in the cellular processes *via* controlling cell metabolism and survival, such as tumorigenesis, metabolism, immune function, and aging [22-25]. In this study, acute and chronic postoperative pain models were generated as previously described [26-28]. Our data found that expression of PKC, ERK, and mTOR were remarkably increased after plantar incision in mice, suggesting that ERK and mTOR pathways may be involved in the process of postoperative mechanical pain in mice.

Enzastaurin is a selective PKCβ inhibitor which is a potent inhibitor of tumor angiogenesis. PKCβ inhibitor has been well studied in tumor-related diseases. Few reports, however, focus on the remission effect of Enzastaurin on postoperative chronic pain [29, 30]. Here, Enzastaurin could inhibit activation of ERK and mTOR pathways in the mouse pain model. Additionally, PWMT was remarkably higher in mice treated with Enzastaurin than that of negative controls. Therefore, Enzastaurin may serve as a new type of analgesic drug in relieving postoperative pain.

Conclusions

Enzastaurin injection at the incision site can improve mechanical pain *via* inhibiting mTOR and ERK pathways, which provides a new target for the treatment of postoperative chronic pain.

Disclosure of conflict of interest

None.

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