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Original Article

Activation of spinal NF-$_{K}$B mediates pain behavior induced by plantar incision

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Abstract: A growing body of evidence indicates that the activation of nuclear factor kappa B (NF-$_{\kappa}$B) pathway was involved in neuropathic and inflammatory pain, however, the role of NF-$_{\kappa}$B in incisional pain is still unclear. Therefore, in this study, we investigated whether the activation of NF-$_{\kappa}$B in the spinal cord is involved in pain hypersensitivity after a plantar incision in the rat hind paw. After rats received a plantar incision surgery, mechanical allodynia and thermal hyperalgesia were determined by von Frey filaments and radiant heat, respectively. Western blot was used to determineNF-$_{\kappa}$B activation at different time points after incision. The NF-$_{\kappa}$B inhibitor pyrrolidinedithiocarbamate (PDTC) was administered intrathecally 30 min before hind paw plantar incision to determine the role of NF-$_{\kappa}$B in incision-induced pain. Our results showed that the expression level of NF-$_{\kappa}$B was significantly increased in spinal cord dorsal horn from 30 min to 3 days after the incision. Intrathecal pretreatment of PDTC attenuated incision-induced mechanical allodynia and thermal hyperalgesia. Furthermore, PDTC significantly reduced the expression level of c-Fos in the dorsal horn after plantar incision. Taken together, plantar incision-induced pain behaviors can be prevented by the NF-$_{\kappa}$B inhibitor. Our results suggest that the blockage of the NF-$_{K}$B signaling pathway might represent a valuable alternative for treating postoperative pain.

Keywords: Nuclear factor kppa B (NF-$_{\kappa}$B), plantar incision, analgesia

Introduction

Postoperative pain is perceived by patients as one of the most obnoxious aspects of surgical pain. It includes evoked and non-evoked pain. Non-evoked pain is short lasting and ongoing pain at rest in patients, and evoked pain is long lasting and movement-related pain, which is usually induced by coughing, mobilization and ambulation [1]. Although increasing studies focus on its pathologic mechanisms, optimal postsurgical pain therapy remains a challenge for physicians. Currently, opioid agonists are the mainstay of pain treatment after surgery, but opioid therapy is severely limited by side-effects at effective doses [2]. Therefore, there is an urgent need to explore the molecular mechanisms underlying postoperative pain.

It is well known that the activation of nuclear factor kappa B (NF-$_{\kappa}$B) pathway is a key event for the transmission and processing of nociceptive information [3]. A recent study showed that intrathecal pretreatment with NF-$_{\kappa}$B inhibitors significantly reduced mechanical allodynia and thermal hyperalgesia following unilateral hind-paw inflammation evoked by complete Freund's adjuvant [4]. Another study reported that intrathecal administration of NF-$_{\kappa}$B inhibitors, pyrrolidinedithiocarbamate (PDTC) and SN50, partially attenuated glycoprotein 120-induced allodynia, and PDTC reversed allodynia in a model of neuropathic pain induced by sciatic nerve inflammation [5]. In addition, it has been reported that repeated intraperitoneal administration of a specific inhibitor of IkappaB kinase also showed antiallodynic potency in a chronic constriction injury (CCI) model of neuropathic pain [6]. Although NF-$_{\kappa}$B is involved in neuropathic and inflammatory pain, the role of NF-$_{\kappa}$B in incisional pain is still unclear. Therefore, in this study, we investigated whether the activation of NF-$_{\kappa}$B in the spinal cord is involved in pain hypersensitivity after a plantar incision in the rat hind paw. We also investigated the effects of intrathecal injection of NF-$_{\kappa}$B inhibitor (PDTC),

before plantar incision on postsurgical mechanical allodynia and thermal hyperalgesia.

Materials and methods

Animals

Adult male Sprague-Dawley rats (weight, 250-300 g) were purchased from the Medical Experimental Animal Center (Zhejiang, China). They were housed in groups of three to four per cage and acclimatized to the laboratory conditions (12-h light/dark cycle; 22 ± 1°C room temperature) 1 week before experiments. Animals had free access to laboratory chow and tap water. The present study was performed with approval from the Animal Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. All surgery was performed under sodium pentobarbital anesthesia (Sigma, St. Louis, MO), and all efforts were made to minimize animal suffering and the number of animals.

Plantar incision

The plantar surgery was performed as previously described [7]. In brief, after aseptic preparation and draping, a 1-cm longitudinal skin incision was made on the plantar surface of the left hindpaw, starting 0.5 cm distal to the tibiotarsus, and extending toward the digits. The plantaris muscle was elevated with forceps and incised longitudinally, leaving muscle origin and insertion intact. After hemostasis with gentle pressure, the incision was closed with two interrupted horizontal mattress sutures of 5-0 nylon. All rats were allowed to recover from anesthesia and surgery for a period of at least 1 h. The incision was checked daily, and animals that exhibited any sign of wound infection or dehiscence were excluded from the study. Sham-operated rats underwent all procedures but were not incised.

Intrathecal drug administration

Intrathecal drug administration was performed as previously described [8]. In brief, a volume of 5 μl of drug solution or 10% DMSO was injected over a 30-s period into the subarachnoid space at the level of the L5 or L6 lumbar vertebra by using a 10 μl Hamilton syringe connected to a 30-gauge needle. The flick of the tail was considered indicative of a successful intrathecal administration.

Measurement of mechanical allodynia and thermal hyperalgesia

Mechanical allodynia was measured as indicated by the paw withdrawal threshold (PWT) in response to von Frey filaments using the up-down method, as previously described [9]. In brief, the rats were placed in individual plastic boxes on a metal mesh fl In. Before testing, rats were left 30 min to adapt the environment. The plantar surface of each hind paw was applied pressure from below with the electronic Von Frey filament via the mesh floor. The force applied at the time of paw withdrawal was recorded.

Paw withdrawal latency (PWL) was determined by assessing heat sensitivity in response to a radiant heat in accordance with the Hargreaves method [10]. In brief, rats were placed in perspex boxes on an elevated glass table. Under the glass table, a radiant heat source was focused on center of the plantar surface of hind paws. The heat was kept at a constant intensity, and the stimulus duration was set at 25 s to avoid tissue damage. The results were expressed as the amount of time in seconds that elapsed from onset of radiant heat application to withdrawal of the rat's hindpaw from the heat source. Heat stimuli was given for three times at an interval of 5-10 min.

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

Total RNA was isolated from the L5-L6 segment of the spinal cord using TRIzol® (BioTeke, Beijing, China) according to the manufacturer's instructions. The synthesis of first-strand cDNA was performed using High Capacity RNA-to-cDNA (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Green I and the iQCycler thermocycler (Bio-Rad, Hercules, CA, USA). The primers for c-Fos were sense, 5'-ATGATGTTCTCGGGTTTCAA-3'; antisense 5'-TGACATGGTCTTCACCACTC-3'; and for GAPDH were sense, 5'-AGGTTGTCTCCTGCGACTTCA-3', and antisense, 5'-TGGTCCAGGGTTTCTTACTCC-3'. Real-time PCR was carried out as follows: initial denaturation for 5 min at 95°C and 35 cycles of PCR consisting of 15 s at 94°C, 15 s at 59°C and 30 s at 72°C. Quantification was always normalized using endogenous control GAPDH.

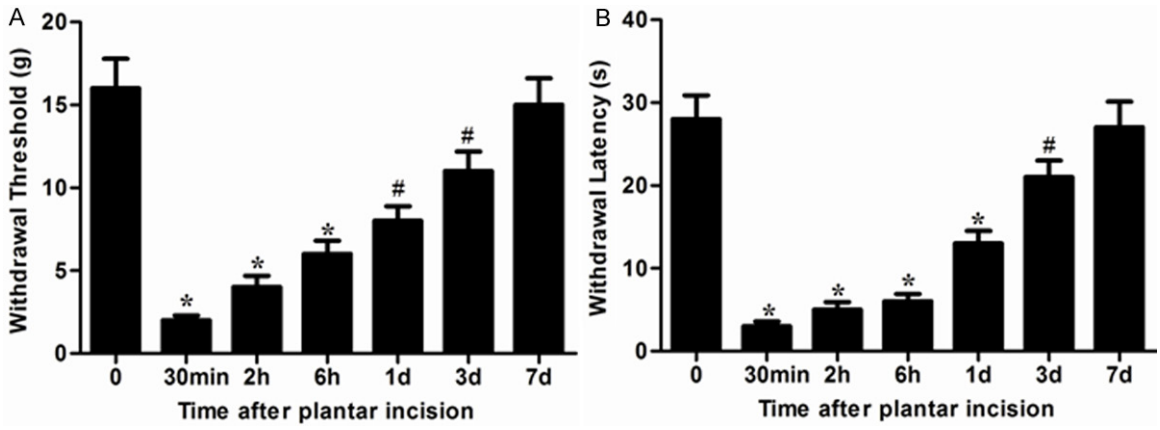

Figure 1. Plantar incision induced mechanical allodynia and thermal hyperalgesia. The hind paw withdrawal threshold (A), and the hind paw withdrawal latency (B) were developed after incision. Data were expressed in mean ± SEM. n = 6, *P < 0.05 compared with the control mice.

Western blot

The animals were killed using isoflurane anesthesia (Sigma, St. Louis, MO, USA) and decapitation. The L5-L6 region of the spinal cord was collected and sonicated in PBS containing 1:1000 protease inhibitors (Sigma, St. Louis, MO, USA). Then the equivalent amounts of proteins (30 μg) were separated by using 8% SDS-polyacrylamide gel (PAGE) electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Boston, MA, USA). Membranes were saturated in blocking solution (5% non-fat dry milk, 0.1% Tween 20 in PBS) for 1 h at room temperature and then incubated (overnight, 4°C) with primary antibodies directed against NF-$_{\kappa}$B p65, c-Fos or anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the blocking solution The membranes were extensively washed with Tris-Buffered Saline Tween-20 (TBST) and incubated with the secondary antibody conjugated for 1 h at room temperature. Finally, the membranes were exposed to reagents from the Enhanced Chemiluminescence (ECL) Detection Kit (Abcam, Cambridge, UK) and x-ray film for visualization of protein bands.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis between two samples was performed using unpaired Student's t-test. Statistical comparison of more than two groups was performed using one-way analysis of variance (ANOVA) followed by a Tukey test. The significance of any differences in thermal latency and mechanical threshold in behavior test was assessed using two-way ANOVA. $P < 0.05$ was considered to be statistically significant.

Results

Plantar incision induced a time-dependent activation of NF-$_{\kappa}$B in spinal dorsal horn

As expected, plantar incision resulted in a significant reduction of mechanical withdrawal threshold to von Frey stimulation (**Figure 1A**); and it also caused a significant decrease in the paw withdrawal latency to noxious thermal stimuli (**Figure 1B**). Furthermore, to investigate whether NF-$_{\kappa}$B is activated after plantar incision, we used Western blot to examine the expression of NF-$_{\kappa}$B p65 in the spinal dorsal horn at different postoperative time points. As shown in **Figure 2**, incisions significantly increased the expression level of NF-$_{\kappa}$B p65 in the L5-L6 spinal segments. The expression of NF-$_{\kappa}$B p65 increased at 30 min, peaked between 6 h and 3 d, and returned to basal level at 7 day.

Inhibition of NF-$_{\kappa}$B prevented pain behaviors induced by plantar incision

Plantar incision activated the NF-$_{\kappa}$B pathway and induced pain behavior. Therefore, we speculated that inhibition of NF-$_{\kappa}$B would alleviate the pain behavior. As shown in **Figure 3**, pretreatment with NF-$_{\kappa}$B inhibitor PDTC (1 or 5 μg in 10% DMSO), 30 min before plantar incision,

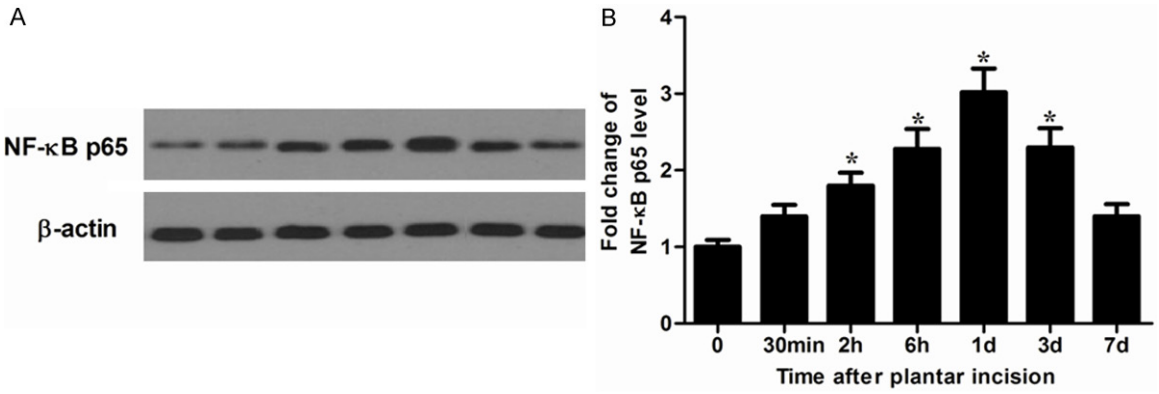

Figure 2. Plantar incision induced the activation of NF-$_{K}$B in spinal dorsal horn. The representative protein image of NF-$_{K}$B p65 was assayed at 0, 30 min, 2 h, 6 h, 1 day, 3 day and 7 day time-points after plantar incision. Incisions significantly increased the expression level of NF-$_{K}$B p65 in the ipsilateral L5-L6 spinal segments. Data were expressed in mean ± SEM. *P < 0.05 compared with the control mice.

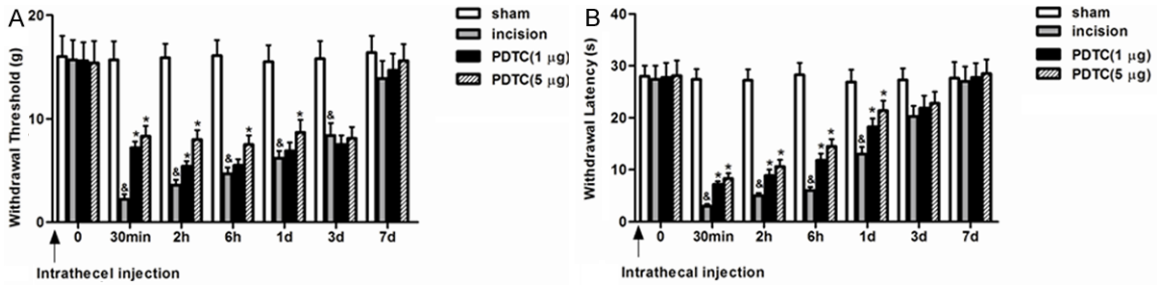

Figure 3. Effects of NF-$_{K}$B inhibitor PDTC pretreatment on withdrawal threshold to von Frey stimulation and withdrawal threshold to thermal stimulation before and after plantar incision. Rats were pretreated with intrathecal bolus of saline (n = 8), sham (10% DMSO in saline; n = 8), 1 μg (n = 8), or 5 μg (n = 8) PDTC. A. PDTC attenuates plantar incision-induced mechanical hypersensitivity. B. PDTC prevents incision-induced thermal hyperalgesia. Data are represented as the mean ± SEM. &P < 0.05 versus sham; *P < 0.05 versus incision.

prevented mechanical allodynia and thermal hyperalgesia in a dose-dependent manner. PDTC significantly elevated withdrawal latency and withdrawal threshold from 30 min to 1 d after plantar injection, as compared with the DMSO-incision group.

Inhibition of spinal NF-$_{K}$B prevented spinal c-Fos expression induced by plantar incision

Spinal neuronal sensitization was involved in the development of hyperalgesia under different conditions. c-Fos protein has been used as the biologic mark for neuronal activation in the central nervous system, and used to examine the effectiveness of different analgesic regimens [11, 12]. To further confirm the analgesic effect of inhibition of NF-$_{K}$B on pain behavior induced by plantar incision, we assayed the expression of spinal c-Fos after pre-treating with NF-$_{K}$B inhibitor. DMSO, PDTC (1 or 5 μg) was intrathecally injected 30 min before the plantar incision. As shown in **Figure 4**, plantar incision induced the expression level of c-Fos in the dorsal horn, compared with the sham operation group. Inhibition of NF-$_{K}$B by intrathecal pre-treatment with PDTC partially inhibited the spinal c-Fos expression induced by plantar incision.

Discussion

In the present study, our findings demonstrate that plantar incision-induced hyperalgesia was accompanied by an increase of NF-$_{K}$B p65 in the spinal dorsal horn. Inhibition of spinal NF-$_{K}$B prevented plantar incision-induced pain behaviors. Moreover, NF-$_{K}$B inhibitor reduced spinal c-Fos expression induced by plantar incision. These results provide evidence that NF-$_{K}$B mediates pain behaviors induced by plantar incision.

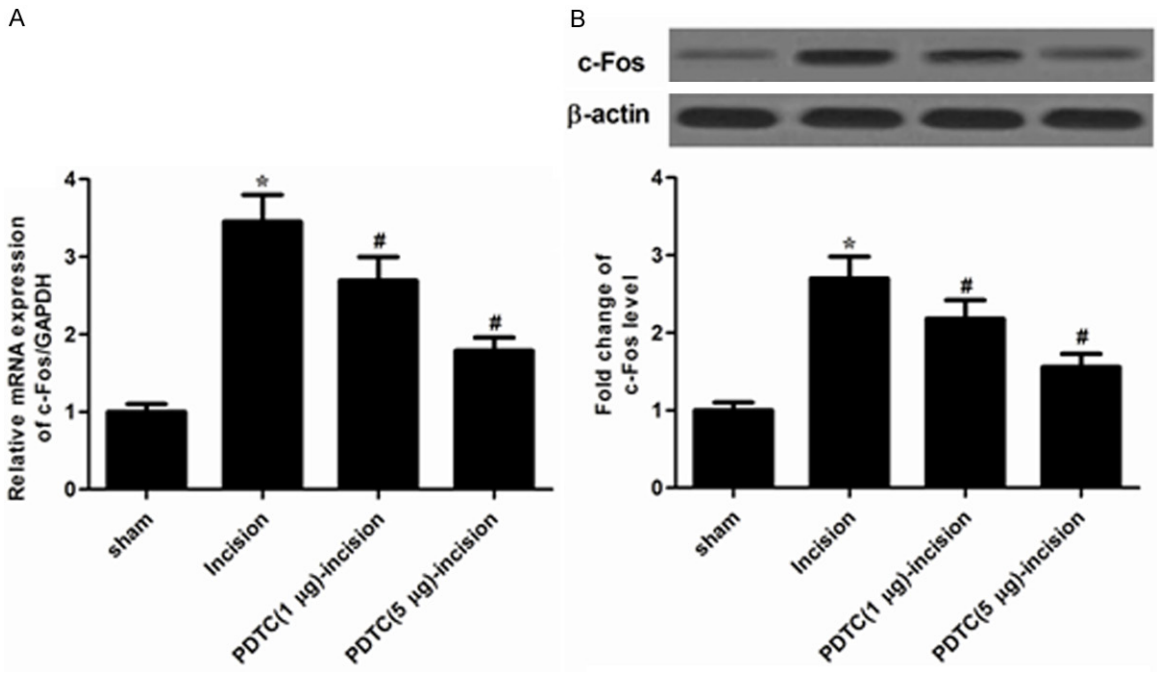

Figure 4. Inhibition of spinal NF-$_{\kappa}$B prevented spinal c-Fos expression induced by plantar incision. PDTC (1 and 5 μg) or DMSO was intrathecally injected at 30 min before plantar incision. The expression of Fos was assayed at the 2 h time-point after plantar incision. A. Inhibition of NF-$_{\kappa}$B by intrathecal pre-treatment with PDTC partially inhibited the spinal c-Fos mRNA expression induced by plantar incision. B. Inhibition of NF-$_{\kappa}$B by intrathecal pre-treatment with PDTC partially inhibited the spinal c-Fos protein expression induced by plantar incision. *P < 0.05 versus sham; #P < 0.05 versus incision.

Brennan et al. have developed a useful rat model of incisional pain that is characterized by increased mechanical sensitivity after a surgical incision made in the plantar aspect of rat hind paw, and this model exhibits a battery of nociceptive responses that parallel the time course of postoperative pain in humans [7, 13]. Therefore, we used this model to investigate whether the activation of NF-$_{\kappa}$B in the spinal cord is involved in pain hypersensitivity.

NF-$_{\kappa}$B is a ubiquitous transcription factor which regulates the expression for many genes that are important in central nervous system (CNS) injury [14-17]. Compelling evidences have demonstrated that the activation of NF-$_{\kappa}$B following tissue or nerve damage is related to the generation of chronic pain. After spinal nerve ligation (SNL), NF-$_{\kappa}$B was significantly increased in the ipsilateral lumbar dorsal horn [18]. In chronic postischemia pain (CPIP) rats, NF-$_{\kappa}$B was elevated in spinal cord of CPIP rats [19]. Consistent with the results of previous studies, in the present study, we found that plantar incision increased NF-$_{\kappa}$B p65 expression in spinal dorsal horn. Recently, several reports have suggested that NF-$_{\kappa}$B might also be related to exaggerated pain. For example, the NF-$_{\kappa}$B inhibitor PDTC prevented allodynia in models of pain evoked by intrathecal dynorphin or perisciatic zymosan administration [5, 20]. Blocking the activation of spinal NF-$_{\kappa}$B, either directly with ammonium PDTC or indirectly with S(+)-ibuprofen prevented the spinal nerve ligation (SNL)-induced allodynia [18]; and intrathecal-administration PDTC relieved mechanical allodynia in CPIP rats [19]. In this study, we found that spinal blocking NF-$_{\kappa}$B with PDTC prevented the mechanical and thermal hyperalgesia in a dose-dependent manner, which suggested that inhibition of spinal NF-$_{\kappa}$B prevented pain behavior induced by plantar incision in mice.

Fos protein is a product of the proto-oncogene, c-fos, which is rapidly, within a few hours, expressed in the neuronal nuclei of the spinal cord by a variety of stimuli presented to the peripheral tissues [21]. These stimuli include chemical, thermal, mechanical stimulation of the peripheral tissues, and electrical stimulation of the peripheral nerves [21-23]. It has been reported that Fos protein is induced in the

spinal dorsal horn after paw skin incision [24]. This is consistent with our data that plantar incision significantly increased the expression level of c-Fos protein in the spinal dorsal horn. Furthermore, NF-$_{\kappa}$B inhibitor reduced c-Fos expression induced by plantar incision. Meanwhile, NF-$_{\kappa}$B inhibitor also prevented plantar incision-induced pain behaviors. Our results suggest that the activation of NF-$_{\kappa}$B signaling pathway is required for the development of plantar incision-induced pain.

In conclusion, this study provides pharmacological and molecular evidence that activation of NF-$_{\kappa}$B signaling contributes to the pain hypersensitivity induced by plantar incision. Therefore, we suggest that NF-$_{\kappa}$B inhibitor could be used as an effective therapeutic agent to relieve postoperative pain and the blockage of the NF-$_{\kappa}$B signaling pathway might represent a valuable alternative for treating postoperative pain.

Disclosure of conflict of interest

None.

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