Peripheral K_{ATP} activation inhibits pain sensitization induced by skin/muscle incision and retraction via the nuclear factor-κB/c-Jun N-terminal kinase signaling pathway

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Abstract. The aim of the current study was to assess the effect of pinacidil activation of ATP-sensitive potassium (K_{ATP}) channels prior to skin/muscle incision and retraction (SMIR) surgery on peripheral and central sensitization, and investigate molecular interferential targets for preventive analgesia. Male Sprague-Dawley rats were randomly assigned to one of the following five groups: Control, incision (sham surgery), incision plus retraction (SMIR) group, SMIR plus pinacidil (pinacidil) group and the SMIR plus pyrrolidine dithiocarbamate (PDTC) group. The rats in the pinacidil and PDTC groups were intraperitoneally injected with pinacidil or PDTC, respectively, prior to the SMIR procedure. The mechanical withdrawal threshold (MWT) was determined. Western blotting was performed to detect the alterations in the subunits of the K_{ATP} channels, Kir6.1 and SUR2, levels of nuclear factor-κB (NF-κB) in the tissue around the incision and c-Jun N-terminal kinase (JNK) in the spinal cord. There was a significant increase observed in the levels of NF-κB and JNK following SMIR surgery compared with the control group, and a significant reduction in MWT and the levels of Kir6.1 and SUR2. Additionally, intraperitoneal administration of pinacidil inhibited the reduction in MWT, and Kir6.1 and SUR2 levels. SMIR was observed to result in increases in the levels of NF-κB and JNK. In addition, in the PDTC group, the alterations in MWT, NF-κB, JNK, Kir6.1 and SUR2 resulting from SMIR were blocked. The results of the current study suggest that the deteriorations in the microenvironment resulting from the SMIR procedure can induce peripheral and central sensitization, and that the activation of peripheral K_{ATP} by pinacidil prior to SMIR is able to inhibit peripheral and central sensitization via the NF-κB/JNK signaling pathway, thus resulting in preventive analgesia.

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Introduction

Postoperative pain is a common clinical symptom predominantly resulting from peripheral and central sensitization due to the persistent excitement of nociceptors. Current methods for analgesia lack efficacy; thus, research is required to elucidate how to reduce and ultimately eliminate postoperative pain. It has been demonstrated that intracellular signal transduction pathways serve important roles in the induction of peripheral and central sensitization. Nuclear factor κB (NF-κB) is a ubiquitous transcription factor that regulates the expression of numerous genes, resulting in mediation of the production of cytokines, chemokines and iNOS, which serve crucial roles in the development of inflammatory and neuropathic pain (1). Evidence suggests that persistent activation of c-Jun N-terminal kinase (JNK) in spinal astrocytes resulting from nerve injury and inflammation can induce central sensitization. JNK signaling serves an important role in regulating the pain threshold. The JNK cascade is a critical signaling pathway for the initiation and the maintenance of neuropathic pain (2).

Nociceptive responses to noxious stimuli are initiated at the peripheral nociceptor terminals (3). Ion channels serve a vital role in the onset and conduction of pain signals. Increased excitability of peripheral nociceptive sensory fibers resulting from the action of inflammatory mediators can alter the activity of ion channels, subsequently inducing inflammatory pain (4). The K_{ATP} channels, which consist of pore-forming (Kir6.1 or Kir6.2) and regulatory (SUR1 or SUR2) subunits, couple the intracellular metabolic state to membrane excitability. Recombinant K_{ATP} channels consisting of Kir6.1 and SUR2B subunits predominantly exist in vascular smooth muscle cells and endothelial cells (5). In addition, vascular dysfunction and vascular endothelial cells serve a contributory role in mechanical pain (6). It has been previously reported that SUR2 may be used as a therapeutic target of pinacidil (7). It has been demonstrated that the K_{ATP} channels are involved in the metabolism after a stress response, mediate analgesia and also participate in neuroprotection under metabolic stress (8,9). Thus, K_{ATP} is an important adaptive regulator for autoprotection. Activation of K_{ATP} channels has been implicated in mediating the antinociceptive effects subsequent to ventricular, intrathecal or epidural injection of K_{ATP} activators in various animal models (10,11). However,

the effect of activated peripheral K_{ATP} on peripheral and central sensitization remains to be fully elucidated.

In the present study, the K_{ATP} opener, pinacidil, was used to precondition rats following skin/muscle incision and retraction (SMIR) (12), in order to establish the effects of direct activation of peripheral K_{ATP} on pain sensation. The role of the NF-κB/JNK signaling pathway in postoperative peripheral and central sensitization was investigated, and the possible molecular targets for preoperative activation of K_{ATP} for preventive analgesia were discussed.

Materials and methods

Animal grouping. Male Sprague-Dawley rats (n=30) weighing 200-250 g were obtained from the Experimental Animal Center at Nantong University (Nantong, China), and housed in temperature-controlled rooms and received water and food, *ad libitum*. The current study was approved by the Experimental Animal Protection and Ethics Committee of Nantong University (Jiangsu, China).

The rats were randomly assigned to the following five groups (six rats per group): Control group, incision (sham surgery) group; incision plus retraction (SMIR) group; SMIR plus pinacidil (pinacidil) group; and the SMIR plus pyrrolidine dithiocarbamate (PDTC) group. The rats in the control group did not receive any treatment. The rats in the sham surgery group had an incision made through the skin and muscle. The rats in the SMIR group underwent 1-h retraction subsequent to skin/muscle incision. The rats in the pinacidil group received an intraperitoneal injection with pinacidil (25 μg/kg; D9035-250MG; Sigma-Aldrich, St. Louis, MO, USA) 30 min prior to the SMIR procedure. The rats in the PDTC group received an intraperitoneal injection of PDTC (100 mg/kg; P-8765; Sigma-Aldrich) 30 min prior to the SMIR procedure.

Behavioral assessments. Prior to the initiation of the experiment, all rats were adapted to the testing conditions for a minimum of 2 days. The room temperature and humidity remained stable for all experiments. To quantify mechanical allodynia, the mechanical withdrawal threshold (MWT) was determined using von Frey filaments (range, 1.4-26 g; North Coast Medical, Inc., Morgan Hill, CA, USA) (13). Briefly, each rat was placed in a Plexiglas® box (Xiyangyang, Inc., Shenzhen, China) with a wire mesh floor. Following habituation for 30 min to this environment, the von Frey filament was pressed perpendicular to the plantar surface of both hind paws and held for no more than 4 sec. A positive response was noted if the rats exhibited paw withdrawal, flinches or licking. If there was no response (negative), the next heavier filament was tested. Each trial was repeated five times. At each 30-sec interval the 50% threshold was determined by the 'up and down' method (13).

Establishment of the SMIR model. Rats were anesthetized by an intraperitoneal injection of Nembutal (40 mg/kg; P3761; Sigma-Aldrich), and laid supine under sterilized conditions. A 1.5-2-cm incision was made in the medial side of the right hind limb approximately 4 mm medial to the saphenous vein to reveal the muscle of the thigh. An incision (7-10 mm long) was then made in the superficial muscle layer of the thigh. The superficial muscle was then retracted 2 cm by spreading blunt scissors within the muscle incision site. This retraction was maintained for 1 h. During the retraction period, the incision site was covered with gauze moistened with sterile saline to prevent dehydration of the surgical site. Following the SMIR procedure, the incision was covered with gauze coated with gentamycin (Yantai Justaware Pharmaceutical Co., Ltd., Yantai, China) to avoid infection. The establishment of the injury site during the 1 h retraction period of the SMIR surgery is presented in Fig. 1 (14).

Western blotting analysis. A total of three days subsequent to SMIR surgery, the rats in each group were anesthetized as described above; peripheral muscle and L3-5 segments of the spinal cord were removed and homogenized on ice in sodium dodecyl sulphate sample buffer (10 ml/mg tissue), containing a cocktail of proteinase and phosphatase inhibitors (Sigma-Aldrich), using a hand-held pestle. The protease inhibitor cocktail (cat no. P2714) contained AEBSF, E-64, bestatin, leupeptin, aprotinin and sodium EDTA, and the phosphatase cocktail (cat no. P5726; all Sigma-Aldrich) contained sodium orthovanadate, sodium molybdate, sodium tartrate and imidazole. The cell lysates were collected and transferred to a 1.5-ml centrifuge tube. Subsequent to centrifugation at 10,000 x g for 18 min at 4˚C , the protein was extracted, boiled and denatured for 5 min and stored at 4˚C. Subsequently, equal amounts (40 μg per lane) of total protein from each sample were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 5 and 10% separating gels (Beyotime Institute of Biotechnology, Jiangsu, China), sequentially, and transferred to polyvinylidene difluoride membranes (Merck Millipore, Shanghai, China). The required protein volume per lane was calculated by dividing the total protein amount loaded per lane by the protein concentrations. Thereafter, the membranes were incubated overnight at 4˚C with one of the following primary monoclonal antibodies against NF-κB (rabbit; diluted 1:1,000; cat no. sc-372; Cell Signaling Technology), p-JNK [goat; diluted 1:200; cat no. sc-12882; Santa Cruz Biotechnology, Inc. (Dallas, TX, USA)], Kir6.1 (rabbit; diluted 1:50; cat no. sc-20808; SUR2 (rabbit; diluted 1:50; cat no. sc-25684) and GAPDH (mouse; diluted 1:20,000; cat no. sc-365062), all Santa Cruz Biotechnology, and then with the corresponding horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, cat no. RPN4301; donkey anti-goat IgG, cat no. RPN510; goat anti-mouse IgG, cat no. RPN998, all diluted 1:3,000; GE Healthcare Life Sciences, Chalfont, UK) at room temperature for 2 h. All antibodies used for western blotting were purchased from Santa Cruz Biotechnology, Inc, unless otherwise stated. Following three washes for 10 min with TBS-T (Beijing Biosntech Biotechnology Co., Ltd., Beijing, China) at room temperature, the intensity of the visualization signal was detected using an enhanced chemiluminescence substrate kit (cat no. WP20005; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the protein levels were quantified using Image J software (version 1.40; National Institutes of Health, Bethesda, MD, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an endogenous internal

reference gene. The relative expression of each target protein was calculated as the ratio of the intensity of the target protein band to that of GAPDH. Each determination was performed 3 times with tissues from different rats.

Statistical analysis. All statistical analyses were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA), and all data were expressed as the mean ± standard deviation. Pairwise differences between the two groups were compared by Student's t-test and one-way analysis of variance to compare the differences among a minimum of three groups. Graphs were drawn using Excel (Microsoft Corporation, Redmond, WA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Alterations in NF-κB p65 levels around the incision site in the control, sham and SMIR groups. In comparison with the NF-κB p65 level in the control group, the sham surgery and SMIR groups were significantly increased following surgery ($P<0.05$ and $P<0.01$, respectively). In addition, the NF-κB p65 level in the SMIR group 3 days subsequent to surgery was significantly greater than that of the sham group ($P<0.01$) (Fig. 2).

Alterations in MWT. It has been previously reported that SMIR is able to induce mechanical allodynia in rats, however it was previously reported to have no effects on hot or cold hyperalgesia (12) As presented in Fig. 3, compared with the control group SMIR induced no significant alterations in the MWT of rats on the first day subsequent to surgery. However, the MWT was significantly reduced 3, 7 and 12 days subsequent to SMIR surgery. No significant reduction in MWT was observed in the rats from the pinacidil group or PDTC group in comparison with the rats in the control group ($P>0.05$). Administration of pinacidil and PDTC reversed the SMIR-induced reduction in MWT following SMIR surgery (Fig. 3).

Alterations in spinal levels of JNK. Compared with the control group, the JNK level was observed to be significantly increased in the SMIR group on days 3 and 7 after surgery ($P<0.05$) (Fig. 4).

Alterations in NF-κB p65, Kir6.1 and SUR2 levels around the incision site. Compared with the control group, the NF-κB p65 level was observed to be significantly increased ($P<0.01$) on day 3 following surgery; whereas the Kir6.1 and SUR2 levels were significantly reduced in the SMIR group ($P<0.01$). No significant alterations were observed in the levels of NF-κB p65, Kir6.1 and SUR2 between the pinacidil and PDTC groups. The NF-κB p65 level in the pinacidil group and PDTC group was significantly reduced compared with that of the SMIR group ($P<0.01$). The Kir6.1 and SUR2 levels were significantly increased in the pinacidil group and PDTC groups in comparison with the SMIR group ($P<0.05$, $P<0.01$) (Fig. 5).

Alterations in spinal levels of NF-κB p65 and JNK. Compared with the control group, the levels of NF-κB and JNK were significantly increased in the SMIR group ($P<0.01$). In addition, the levels of NF-κB and JNK in the pinacidil and PDTC groups were significantly reduced, compared to those in the SMIR group (NF-κB, $P<0.01$; JNK, $P<0.05$) (Fig. 6).

Discussion

It has been identified that formalin-induced animal models of inflammatory nociception are unable to simulate the microenvironment around the incision site (15). However, SMIR has been previously reported to be able to evoke mechanical allodynia in rats, however produced no effects on hot or cold hyperalgesia (12). NF-κB is a ubiquitous transcription factor that is important in inflammatory pain and neuropathic pain (1). As presented in Fig. 2, the NF-κB levels around the incision site were significantly increased following surgery compared with control group. In addition, the NF-κB levels around the incision site were increased in the SMIR group on day 3 after surgery compared with those in the sham group. It was suggested that these effects were due to the extent of the alterations to the microenvironment resulting from surgery. This observation indicated that the incision and retraction procedure was able to significantly upregulate the NF-κB levels compared with the incision alone. As presented in Fig. 3, compared with the control group, there were no significant alterations in the MWT of rats undergoing SMIR on the first day after surgery. However, the MWT was significantly reduced on days 3, 7 and 12 subsequent to SMIR surgery. This indicates that it requires time for alterations in the microenvironment around the incision to activate central sensitization. SMIR surgery was able to result in the microenvironmental deterioration of peripheral nociceptor terminals around the incision site, inducing an increase in excitability of the nociceptors, resulting in peripheral and central sensitization. Thus, the SMIR model can provide a more accurate reflection of the microenvironment around the incision site.

In the current study, it was identified that deterioration of the microenvironment resulting from SMIR surgery upregulated the levels of NF-κB around the incision and NF-κB and JNK expression in the spinal cord, which inhibited the expression of Kir6.1 and SUR2. The results indicated that NF-κB, JNK and K_{ATP} participate in peripheral and central sensitization induced by SMIR surgery. These results thus indicate that the deterioration of the microenvironment at peripheral nociceptor terminals may act as a trigger for NF-κB and JNK signaling and K_{ATP} activity, which is an adaptive mechanism for autoprotection.

Preventive analgesia inhibits peripheral and central sensitization using multimodal analgesic techniques, including non-steroidal anti-inflammatory drugs, α2 agonists, local anesthetics, ketamine and α2δ ligands and opioids, which can reduce the occurrence of opioid-adverse events (16). It has been demonstrated that functional K_{ATP} channels are present in nociceptors (17). The opening of the K_{ATP} channels may inhibit the nociceptive responses induced by noxious stimuli by dampening the hyper-excitability of the nociceptors, and it has been identified that K_{ATP} has no significant effect on the resting membrane conductance of dorsal root ganglion neurons (18). Pinacidil is a sulfonylurea receptor agonist that opens the SUR2 potassium-sensitive ATP channel (7). It has

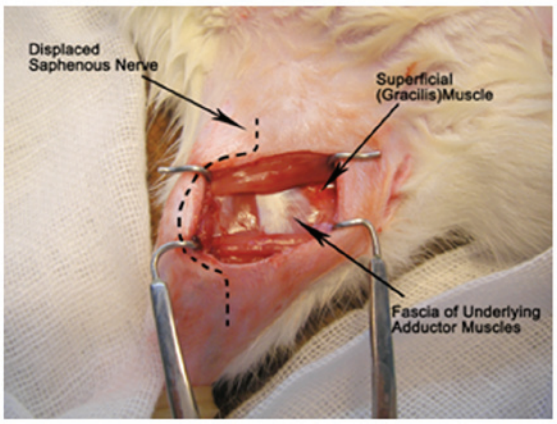

Figure 1. Model of skin/muscle incision and retraction-evoked pain in rat. Figure adapted from reference (14).

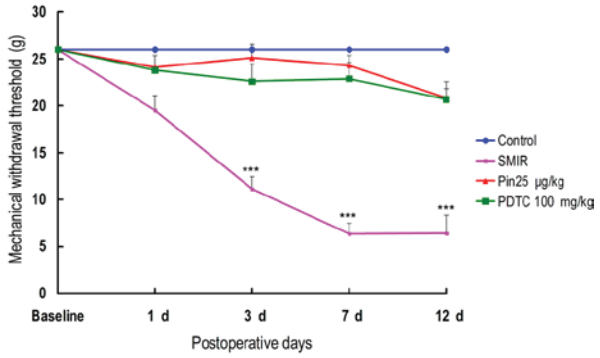

Figure 3. Comparison of mechanical withdrawal threshold in control, sham, pinacidil and PDTC groups of rats (n=6). ***P<0.001 vs. control group. PDTC, pyrrolidine dithiocarbamate.

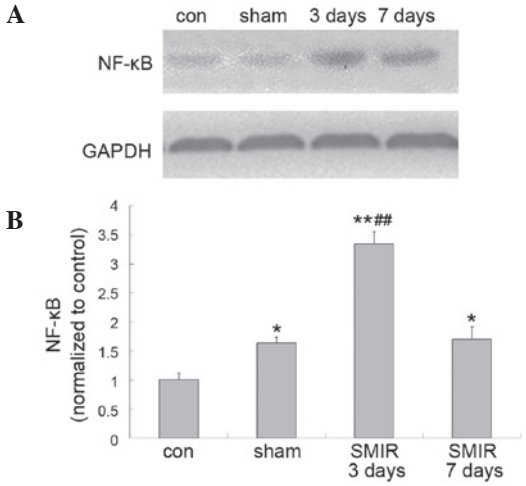

Figure 2. Comparison of NF-κB p65 protein levels around the incision site in the control, sham and SMIR rats. (A) Western blotting analysis of NF-κB p65 levels in different groups; (B) normalized NF-κB p65 levels determined by western blotting. *P<0.05, **P<0.01 vs. control group; ##P<0.01 vs. sham group. NF-κB, nuclear factor κB; SMIR, skin/muscle incision and retraction; con, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

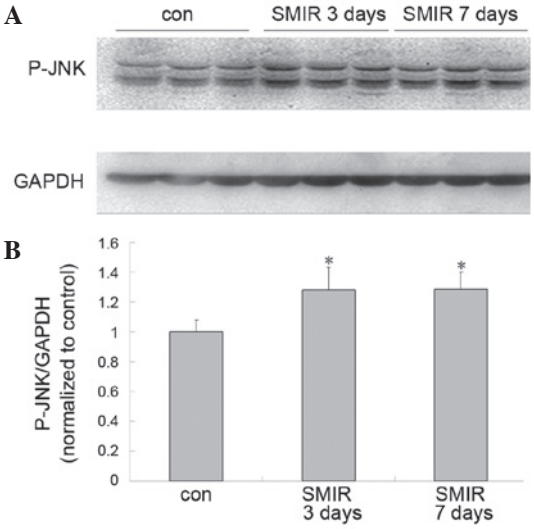

Figure 4. Comparison of p-JNK protein levels in the spinal cord in control and SMIR rats. (A) Western blotting of p-JNK levels in control and SMIR groups; (B) normalized p-JNK levels determined by western blotting. *P<0.05 vs. control group. p-, phosphorylated; JNK, c-Jun N-terminal kinase; SMIR, skin/muscle incision and retraction; con, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

been demonstrated that pinacidil does not readily cross the blood brain barrier under normal physiological conditions in the rat (19). To assess the effect of activated peripheral K_{ATP} on peripheral and central sensitization resulting from deteriorations to the microenvironment of peripheral nociceptor terminals around the incision site, the following experiments were conducted. The minimum effective dose of 25 μg/kg pinacidil was injected intraperitoneally into the rats 30 min prior to SMIR, according to the pre-experiment results. This resulted in activation of peripheral K_{ATP}, and then the levels of Kir6.1, SUR2 and MWT was analyzed. It was identified that pinacidil exerted an inhibitory effect on the SMIR-induced reduction of MWT, Kir6.1 and SUR2 levels around the incision, increases in NF-κB levels around the incision and increases in NF-κB and JNK levels in the spinal cord. These results suggested that pinacidil activated peripheral K_{ATP} and blocked the subsequent microenvironmental deterioration around the incision site induced by the SMIR procedure, thus preventing peripheral and central sensitization. The present study indicated that microenvironmental deterioration of peripheral nociceptor terminals around the incision site is an initial step of peripheral and central sensitization. Activation of peripheral K_{ATP} prior to central sensitization inhibits NF-κB expression around the incision; thus, an inflammatory microenvironment that motivates central excitatory is unable to be formed, thus NF-κB and JNK expression in the spinal cord is inhibited to prevent hyperalgesia. It has been demonstrated that activation of K_{ATP} can regulate cell metabolism and electrical activity, increase ATP synthesis, reduce oxyradicals, prevent calcium overload and maintain mitochondrial function, thus ameliorating the effects on the microenvironment (14,20-23). It is hypothesized that activated K_{ATP} preconditioned by pinacidil exerts a protective effect on the microenvironment by regulating cell activity, increasing ATP synthesis, reducing oxyradicals and preventing calcium overload to suppress microenvironmental deterioration resulting from SMIR surgery. Kir6.1/SUR2B is the major isoform of K_{ATP} channels in vascular smooth muscles, and vascular

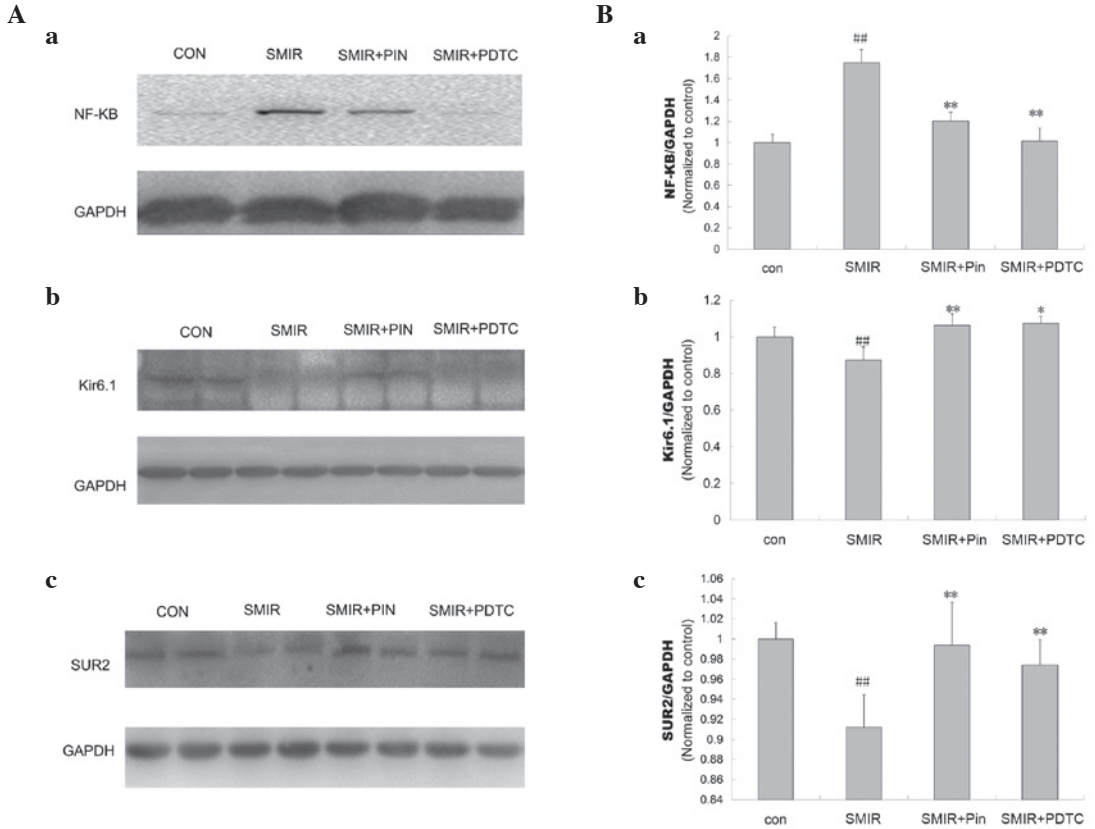

Figure 5. Comparison of NF-κB p65, Kir6.1 and SUR2 protein level around the incision site in control, SMIR, Pin and PDTC rats. (A) Western blotting of (a) NF-κB p65, (b) Kir6.1 and (c) SUR2 levels in different groups; (B) normalized (a) NF-κB p65, (b) Kir6.1 and (c) SUR2 levels determined by western blotting. $^{\#\#}$P<0.01 vs. control; *P<0.05, **P<0.01 vs. SMIR. NF-κB, nuclear factor κB; SMIR, skin/muscle incision and retraction; Pin, pinacidil; PDTC, pyrrolidine dithiocarbamate; con, control; GADPH, glyceraldehyde 3-phosphate dehydrogenase.

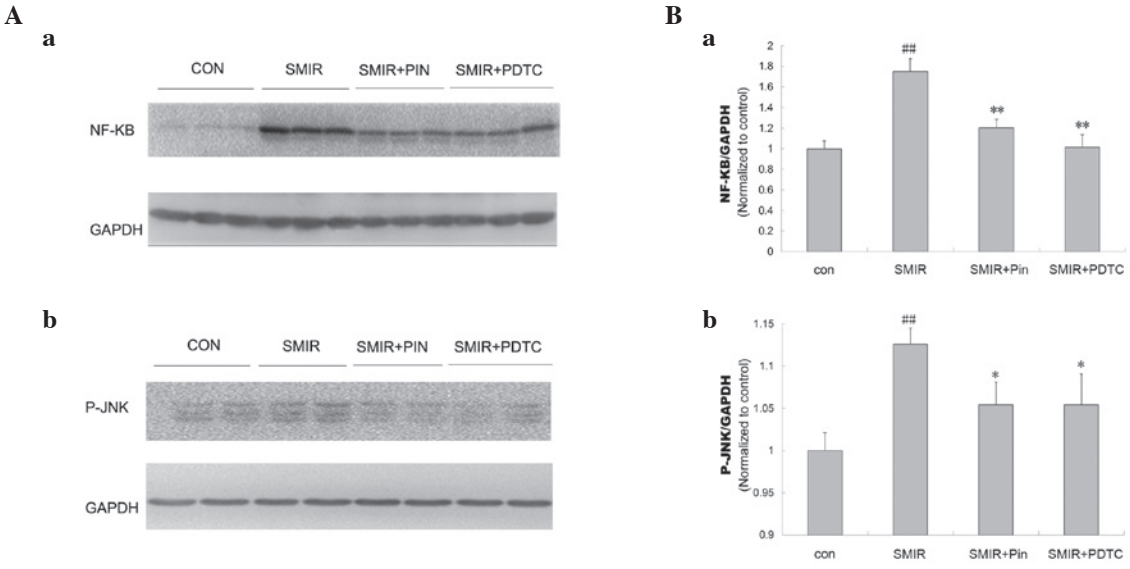

Figure 6. Comparison of the NF-κB and JNK expression levels in the spinal cord in control, SMIR, Pin and PDTC rats. (A) Western blotting analysis of (a) NF-κB and (b) JNK levels in the different groups; (B) normalized (a) NF-κB and (b) JNK levels determined by western blotting. $^{\#\#}$P<0.01 vs. control; *P<0.05, **P<0.01 vs. SMIR. NF-κB, nuclear factor κB; JNK, c-Jun N-terminal kinase; SMIR, skin/muscle incision and retraction; Pin, pinacidil; PDTC, pyrrolidine dithiocarbamate; con, control; GADPH, glyceraldehyde 3-phosphate dehydrogenase.

dysfunction and vascular endothelial cells serve a key role in mechanical pain (6,24). Thus the functional K_{ATP} channels in vascular endothelial cells serve a vital role in peripheral and central sensitization resulting from postoperative microenvironmental deterioration. Preconditioning of K_{ATP} may be an effective method of preventive analgesia.

NF-κB is a widely expressed transcription factor for genes involved in cell survival, inflammation, differentiation

and growth (25-29), and is necessary for the upregulation of the K_{ATP} channel (30). JNK is a stress-activated member of the mitogen-activated protein kinase family (31). Previous studies have demonstrated that hyperalgesia and allodynia are induced by tissue or nerve injury via the JNK pathway in primary sensory neurons and the spinal cord. Furthermore, this activation can maintain central sensitization (32,33). There are numerous common upstream molecules involved in the NF-κB/JNK signaling pathway, which participate in mediation of inflammation (34,35). In the current study, it was identified that deterioration of the microenvironment resulting from SMIR surgery upregulated the levels of NF-κB around the incision and NF-κB and JNK expression in the spinal cord, providing a basis for the activation of K_{ATP}, inhibiting the expression of Kir6.1 and SUR2. This indicated that the SMIR procedure was able to activate NF-κB/JNK signaling and inhibit K_{ATP} activity, inducing postoperative allodynia. PDTC, an antioxidant and a specific inhibitor of NF-κB, reversibly inhibits the nuclear translocation of NF-κB (36). In the current study, an 100 mg/kg intraperitoneal injection of PDTC was selected, administered 30 min prior to SMIR surgery according to a method described previously (37). It was identified that the rats in the PDTC group exhibited a significantly reduced NF-κB level, higher Kir6.1 and SUR2 levels around the incision and reduced spinal JNK levels compared with those in the SMIR group. The rats pretreated with PDTC additionally displayed significantly reduced MWT compared with those in the SMIR group. This observation suggests that the deteriorative microenvironment at peripheral nociceptor terminals inhibits peripheral K_{ATP} and activates JNK signaling via the NF-κB signaling pathway. In addition, activation of peripheral K_{ATP} exerting a preventative analgesic effect is suggested to be dependent on the NF-κB signaling pathway. Thus, the NF-κB/JNK signaling pathway is a potential molecular target for ameliorating the microenvironmental deterioration around the incision and preventing peripheral and central sensitization.

Taking the results of the current study and those of previous studies into account, it is suggested that alterations in the microenvironment provide a basis for the effect of activated K_{ATP}. Furthermore, preconditioning K_{ATP} can result in the amelioration of the microenvironment deteriorations to prevent peripheral and central sensitization via the NF-κB/JNK signaling pathway, thus providing a novel approach to preventive analgesia.

In summary, it was identified that the activation of peripheral K_{ATP} by pinacidil prior to surgery was able to ameliorate the microenvironmental deteriorations resulting from the SMIR procedure, thus preventing peripheral and central sensitization via the NF-κB/JNK signaling pathway. Thus, K_{ATP}/NF-κB/JNK may serve as an important molecular target for preventive analgesia.

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