Roles of extra-cellular signal-regulated protein kinase 5 signaling pathway in the development of spinal cord injury

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Abstract

Background: In consideration of characteristics and functions, extra-cellular signal-regulated protein kinase 5 (ERK5) signaling pathway could be a new target for spinal cord injury (SCI) treatment. Our study aimed to evaluate the roles of ERK5 signaling pathway in secondary damage of SCI.

Methods: We randomly divided 70 healthy Wistar rats into five groups: ten in the blank group, 15 in the sham surgery + BIX02188 (sham + B) group, 15 in the sham surgery + dimethyl sulfoxide (DMSO; sham + D) group, 15 in the SCI + BIX02188 (SCI + B) group, and 15 in the SCI + DMSO (SCI + D) group. BIX02188 is a specific inhibitor of the ERK5 signaling pathway. SCI was induced by the application of vascular clips (with the force of 30 g) to the dura on T10 level, while rats in the sham surgery group underwent only T9-T11 laminectomy. BIX02188 or DMSO was intra-thecally injected at 1, 6, and 12 h after surgery or SCI. Spinal cord samples were taken for testing at 24 h after surgery or SCI.

Results: Expression of phosphorylated-ERK5 (p-ERK5) significantly increased after SCI. Application of BIX02188 indeed inhibited ERK5 signaling pathway and reduced the degree of spinal cord tissue injury, neutrophil infiltration and proinflammatory cytokine expression, nuclear factor-κB (NF-κB) activation and apoptosis (measured by TdT-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling, expression of Fas-ligand, BCL2-associated X [Bax], and B-cell lymphoma-2 [Bcl-2]). Double immunofluorescence revealed activation of ERK5 in neurons and microglia after SCI.

Conclusion: ERK5 signaling pathway was activated in spinal neurons and microglia, contributing to secondary injury of SCI. Moreover, inhibition of ERK5 signaling pathway could alleviate the degree of SCI, which might be related to its regulation of infiltration of inflammatory cells and release of inflammatory cytokines, expression of NF-κB and cell apoptosis.

Keywords: Extracellular signal-regulated protein kinase 5; Mitogen activated protein kinase; Spinal cord injury; Nuclear factor-κB; Apoptosis

Introduction

In global, over one million individuals are living with spinal cord injury (SCI),[1] and this condition causes socioeconomic and personal burdens over a lifetime. After the primary mechanical injury, a complex sequence of inflammatory mediators enters the injury site and leads to demyelination,[2] glial scarring,[3] and neural cells apoptosis, eventually resulting in loss of neural circuitry.[4] In the secondary injury, pathophysiological changes combined with the limited regenerative potential of nervous system, cause critical persistent functional loss in future.[5]

With more attention paid to nerve regeneration, relevant signaling pathways have been new subjects of study. The mitogen-activated protein kinase (MAPK) signaling pathway participates in the regulation of gene expression, cell proliferation, and apoptosis,[6] and the mitogen extra-cellular signal-regulated kinase kinase 5/extra-cellular signal-regulated kinase 5 (MEK5/ERK5) pathway is the lesser studied segment in the MAPK family.

Studies about the effects of MAPK signaling pathways in SCI have been conducted. Xu *et al*[7] demonstrated that *in vivo* ERK1/2 and p38 MAPK in microglia/macrophages were activated within 1 h after SCI and persisted for at least 24 h. Genovese *et al*[8] injected PD98059, a specific inhibitor of ERK1/2, into SCI mice and found that inflammation, tissue damage, and neuronal apoptosis were substantially reduced and neural functions were improved. The inhibitor of *JNK* reduced phosphorylation of c-Jun, caspase-3 splitting, erythrocyte extra-vasation, and blood-brain barrier permeability, suggesting that inhibition of *JNK* decreased cell apoptosis and protected the vascular system.[9] Cao *et al*[10] found that the up-regulation of the Ras/Raf/ERK1/2 signaling pathway may contribute to the pathogenesis of SCI through both its impairment of the

spinal cord neurons development and causing neural circuit imbalances.

Although the ERK5 signaling pathway has been proved to participate in anti-apoptotic signaling, angiogenesis, cell survival, differentiation, and proliferation,[11-13] there have been few studies regarding its roles in SCI. However, in consideration of characteristics and functions, this pathway could be a new target for SCI treatment. In this study, we used BIX02188, a specific inhibitor of ERK5 to evaluate whether ERK5 activation participates in modulation of secondary injury.

Methods

Ethical approval

All experiment protocols were approved by the Animal Care and Use Committee of Peking University People's Hospital (No. 2017PHC014) and were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Animals

Male or female Wistar rats (250–300 g) were maintained with adequate access to food and water. The animals were provided by Experimental Animal Center of Peking University People's Hospital.

SCI model

Rats were anesthetized using isoflurane inhalation (3–4% induction, 1–2% maintenance, flow 0.6–0.8 L/min). SCI was induced by the application of vascular clip (force of 30 g) to the dura of T10 level via T9-11 laminectomy. The clip was rapidly released with the clip applicator, causing acute compression and lasting for 30 s. For sham surgery groups, animals were just subjected to T9-11 laminectomy.

Experimental design

Seventy healthy Wistar rats were randomly divided into five groups: ten in the blank group, 15 in the sham surgery + BIX02188 (sham + B) group, 15 in the sham surgery + dimethyl sulfoxide (DMSO; sham + D) group, 15 in the SCI + BIX02188 (SCI + B) group, and 15 in the SCI + DMSO (SCI + D) group. They were injected intra-thecally with 10 μL DMSO (1%) or 10 μL BIX02188 (1 μg/1 μL) at 1, 6, and 12 h after the surgical procedure. SCI animals were subjected to the surgical procedure with the aneurysm clip applied, and were treated intra-thecally with 10 μL DMSO (1%) or 10 μL BIX02188 (1 μg/1 μL) at 1, 6, and 12 h after SCI. Twenty-four hours after SCI or surgery, the spinal cord samples were removed and tested.

BIX02188

BIX02188 is a pharmacological inhibitor specific to the MEK5/ERK5 signaling pathway.[14] This inhibitor suppressed ERK5 phosphorylation in a dose-dependent manner. In this research, the concentration was 1 μg/μL

and the dose was 10 μL each time. Cells were cultured for a period of 24 h with this drug, showing that there were no cytotoxic effects. Moreover, it did not inhibit other related MAPKs: ERK2, JNK2, MEK1, and MEK2.

Myeloperoxidase activity

As the indicator of polymorphonuclear leukocyte accumulation, myeloperoxidase (MPO) activity was tested by enzyme-linked immunosorbent assay (ELISA). The assay was carried out by a MPO ELISA kit (Jiancheng Biological Company, Nanjing, China), following the instructions. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per minute at 37°C, and it was expressed as units of MPO per milligram of proteins.

Measurement of TNF-α and IL-1β levels by ELISA

Portions of spinal cord tissues were homogenized as described previously in phosphate buffered saline (PBS), and tumor necrosis factor (TNF)-α and interleukin (IL)-1β levels were evaluated. The assay was carried out by using a colorimetric commercial kit (Jiancheng Biological Company), following the instructions. All determinations were performed in gradient dilutions.

Immunohistochemical localization of p-ERK5, glial fibrillary acidic protein, ionized calcium binding adapter molecule 1, NeuN, Fas-ligand, nuclear factor-κB sub-unit P65 Ser536, Bax, and Bcl-2

At 24 h after SCI or surgery, tissues were removed and fixed in 10% (w/v) PBS-buffered formaldehyde, and 5-mm sections were prepared from paraffin-embedded tissues. Sections were incubated overnight with anti-p-ERK5 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Fas-ligand (FasL) antibody (1:500; Abcam, Cambridge, UK), anti-nuclear factor-κB (NF-κB) p65 S536 antibody (1:1000; Abcam), anti-Bax antibody (1:50; Abcam), or anti-Bcl-2 antibody (1:50; Abcam). Sections were washed with PBS, and then incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit immunoglobulin G (IgG) and avidin-biotin-peroxidase complex. For double immunofluorescence, spinal tissues were incubated with a mixture of anti-p-ERK5 antibody and anti-NeuN antibody (1:500; Abcam), or anti-ionized calcium binding adapter molecule 1 (Iba1) (1:500; Abcam) antibody, or anti-glial fibrillary acidic protein (GFAP) antibody (1:500; Abcam) overnight at 4°C. The stained sections were examined under a fluorescence microscope, and images were captured with a charge-coupled device spot camera.

TUNEL assay

TdT-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick-end labeling (TUNEL) assay was conducted by using a TUNEL detection kit according to the manufacturer's instruction (Roche, California, USA). Briefly, sections were incubated with relevant reagents, and then were immersed in terminal deoxynucleotidyl transferase buffer containing deoxynucleotidyl transferase and biotinylated dUTP in a humid atmosphere at 37°C for 90 min. The sections were incubated at room temperature for 30 min with anti-

horseradish peroxidase-conjugated antibody, and signals were visualized with diaminobenzidine.

Western blot analysis for p-ERK5, FasL, NF-κB P65 Ser536, Bax, and Bcl-2

The tissue samples were homogenized in 50 mmol/L radio immunoprecipitation assay buffer containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail. Total proteins and nuclear proteins were extracted for the detection. Proteins were resolved by sodium dodecylsulphate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blocked overnight in 5% non-fat milk. Membranes were then incubated with anti-p-ERK5 antibody (1:100; Santa Cruz Biotechnology), FasL antibody (1:500; Abcam), anti-NF-κB p65 Ser536 antibody (1:1000; Abcam), anti-Bax antibody (1:1000; Abcam), anti-Bcl-2 antibody (1:1000; Abcam), and anti-β-actin antibody (1:5000; Abcam) at 4°C overnight. Membranes were then washed twice with TBST and probed with goat anti-rabbit IgG (1:1000; Abmart) at 37°C for 2 h. Finally, membranes were washed for several times to remove unbound secondary antibodies. The density of bands was measured with the Image J software (Rawak Software Inc., Stuttgart, Germany). Scanning densitometry was used for semi-quantitative analysis.

Light microscopy

Tissue segments were paraffin embedded and cut into 5-μm sections. Then they were deparaffinized with xylene, stained with hematoxylin and eosin, and studied using light microscopy. Damaged neurons were counted, and the histopathological changes of the gray matter were scored on a 6-point scale[15]: 0, no lesion observed; 1, gray matter contained 1 to 5 eosinophilic neurons; 2, gray matter contained 5 to 10 eosinophilic neurons; 3, gray matter contained more than ten eosinophilic neurons; 4, small infarction (less than 1/3 of the gray matter area); 5, moderate infarction (1/3 to 1/2 of the gray matter area); 6, large infarction (more than half of the gray matter area). The scores from all the sections of each spinal cord were averaged to give a final score for each mouse.

Statistical analysis

All values were expressed as mean ± standard error of n observations (n represented the number of animals studied). The results were analyzed by one-way analysis of variance followed by a Bonferroni post-hoc test for multiple comparisons, and $P < 0.05$ was considered significant. Histological scale data were analyzed by the Mann-Whitney U test and were considered statistically significant when $P < 0.05$.

Results

ERK5 expression increased after SCI and BIX02188 inhibited ERK5 expression

Spinal cord sections obtained from SCI rats revealed positive immunohistological staining for p-ERK5. Likewise, at 24 h after SCI or surgery, expression of p-ERK5 was investigated by Western blot. We observed a significant increase of p-ERK5 levels in mice subjected to SCI. By contrast, BIX02188 treatment prevented SCI-induced p-ERK5 increases [Figure 1A–D].

Inhibition of ERK5 reduced the severity of SCI

At 24 h after injury, we measured the severity of the trauma in the perilesional area, assessed by the presence of edema, as well as alteration of the white matter and infiltration of leucocytes. Significant damages were observed in the spinal cord tissues of SCI rats compared with rats of the blank group. It is noteworthy that remarkable protection against SCI was observed in mice of SCI + B group, also reflected in histological scores (SCI + B group $vs.$ SCI + D group, $Z = 1.000$, $P < 0.001$) [Figure 2A–D].

SCI-induced ERK5 activation in spinal neurons and microglia

To identify the cell types expressing p-ERK5 after SCI, we performed double immunostaining of p-ERK5 with several cell-specific markers: NeuN for neurons, GFAP for astrocytes, and Iba1 for microglia. It was found that p-ERK5 did not colocalize with GFAP, while the majority of the p-ERK5-IR cells were double-labeled with NeuN and Iba1, suggesting ERK5 signaling pathway activated in neurons and microglia, not in astrocytes [Figure 3A–C].

Effects of inhibition of ERK5 on neutrophil infiltration

The above-mentioned SCI histological pattern correlated with the influx of leukocytes. Therefore, we investigated the effect of ERK5 inhibition on neutrophil infiltration by measuring MPO activity. ELISA showed that MPO activity significantly increased at 24 h after SCI, compared with sham-operated group. Moreover, inhibition of ERK5 attenuated neutrophil infiltration induced by SCI [Figure 4A].

Inhibition of ERK5 modulated expression of TNF-α and IL-1β after SCI

To observe whether ERK5 inhibition modulated the inflammatory process through the regulation of pro-inflammatory cytokines expression, we analyzed TNF-α and IL-1β levels in spinal cord tissues by ELISA. A substantial increase in secretion of these two cytokines was found in samples of SCI mice [Figure 4B and 4C]. Spinal cord levels of IL-1β and TNF-α were significantly attenuated by treatment of BIX02188.

Effects of treatment of ERK5 inhibition on the NF-κB subunit P65 Ser536

We evaluated NF-κB by immunohistochemical and Western blot analysis. Spinal cord sections from SCI + D mice exhibited positive staining for NF-κB P65 Ser536, while fewer P65 Ser536-IR cells were observed in the SCI + B group. Similarly, we observed a significant increase of NF-κB levels in SCI rats by Western blot. By contrast, BIX02188 treatment prevented SCI-induced NF-κB up-regulation [Figure 5A–E].
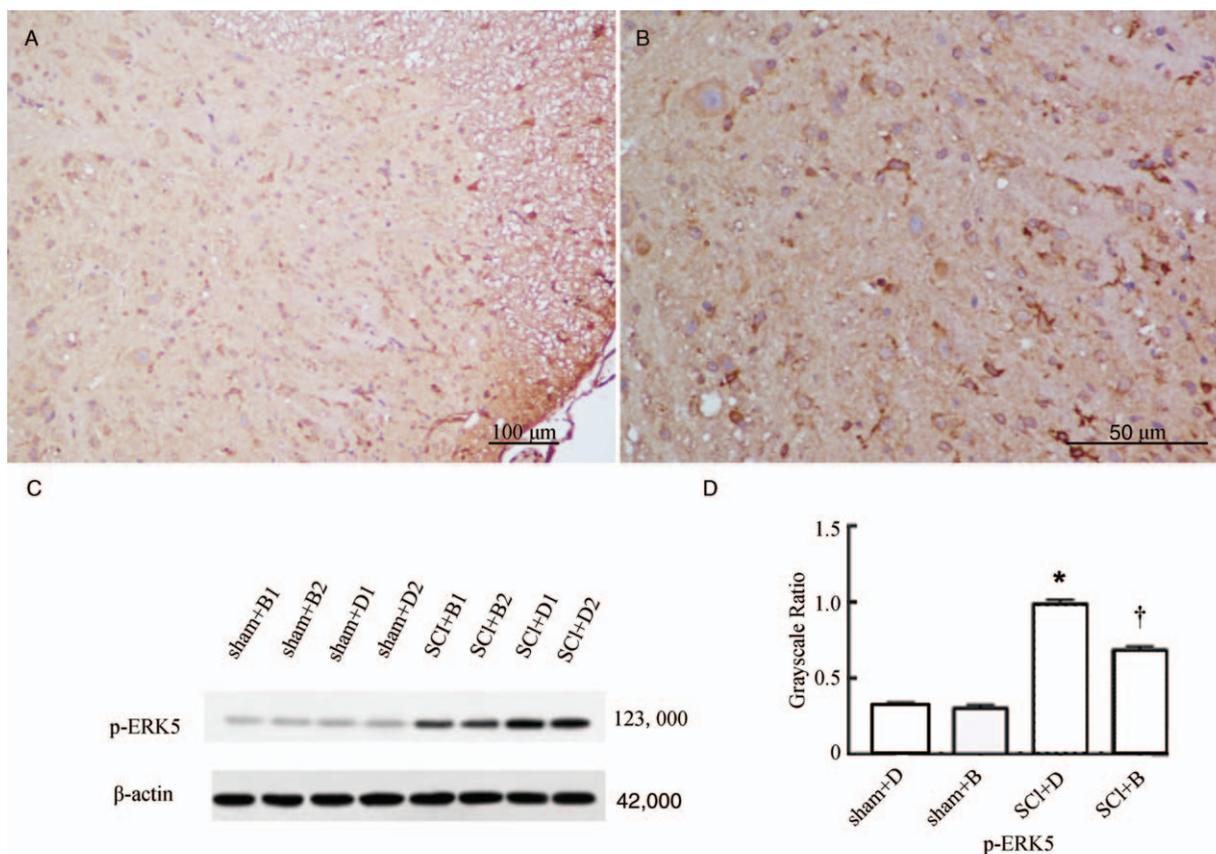
Figure 1: Significantly increased ERK5 expression after SCI and BIX02188 inhibition of ERK5 expression. (A and B) Immunohistological staining showing spinal cord sections obtained from SCI mice exhibiting positive staining (in neurons and neuroglia) for p-ERK5 24 h after injury. (C) At 24 h after SCI, expression of p-ERK5 in the spinal cord homogenates was investigated by Western blot (1: sample 1; 2: sample 2). (D) A significant increase in p-ERK5 levels was observed in the spinal cord of mice subjected to SCI, and BIX02188 treatment prevented the SCI-induced p-ERK5 increase. Data were represented as mean ± standard error; $n = 15$ per group. *$P < 0.05$ compared with the sham and SCI + B groups. †$P < 0.05$ compared with the sham and SCI + D groups. ERK5: Extra-cellular signal-regulated protein kinase 5; SCI: Spinal cord injury; Sham + B: Sham surgery + BIX02188 group; Sham + D: Sham surgery + dimethyl sulfoxide group.

Effects of inhibition of ERK5 on apoptosis measured by TUNEL assay

We measured TUNEL-like staining in perilesional spinal cord tissues. Almost no apoptotic cells were detected in the spinal cord from sham-operated rats. At 24 h after SCI, spinal cord tissues demonstrated a marked appearance of dark brown apoptotic cells. By contrast, tissues from SCI + B mice demonstrated few apoptotic cells [Figure 6A–D].

Effects of BIX02188 on immunohistochemical localization and expression of Fas-ligand

Spinal cord sections from SCI rats exhibited positive staining for Fas-ligand and BIX02188 reduced the degree of positive staining. A significant increase of Fas-ligand level was observed in SCI + D rats by Western blot. By contrast, inhibition of ERK5 blocked Fas-ligand expression induced by SCI [Figure 7A–E].

Immunohistochemical and Western blot analysis for Bax and Bcl-2

Spinal cord sections obtained from SCI rats exhibited positive staining for Bax, while BIX02188 reduced the degree of positive staining for Bax. At 24 h after SCI, the pro-apoptic protein Bax was also investigated by Western blot. Bax levels substantially increased in SCI + D mice, but inhibition of ERK5 prevented the SCI-induced Bax expression [Figure 8A–D]. In addition, spinal cord sections from sham-operated rats demonstrated Bcl-2 positive staining (data not shown), whereas the staining significantly reduced in SCI rats. Moreover, BIX02188 attenuated the loss of positive staining for Bcl-2 in SCI rats. Expression of Bcl-2 significantly decreased in spinal cords of SCI rats. BIX02188 blunted the SCI-induced inhibition of anti-apoptotic protein expression [Figure 9A–D].

Discussion

Our study showed that ERK5 was activated in neurons and microglia after SCI, a finding different from those of previous studies. Obata et al[16] found that peripheral nerve injury increased ERK5 phosphorylation in spinal microglia, as well as in both damaged and undamaged dorsal root ganglion neurons. Similarly, Sun et al[17] pointed out that ERK5 and microglia were activated in the spinal cord after spinal nerve ligation. However, Liu et al[18] found that ERK5 protein was expressed in neurons in cultures of embryonic cortical neurons. According to our double immunofluorescence figures, we concluded that
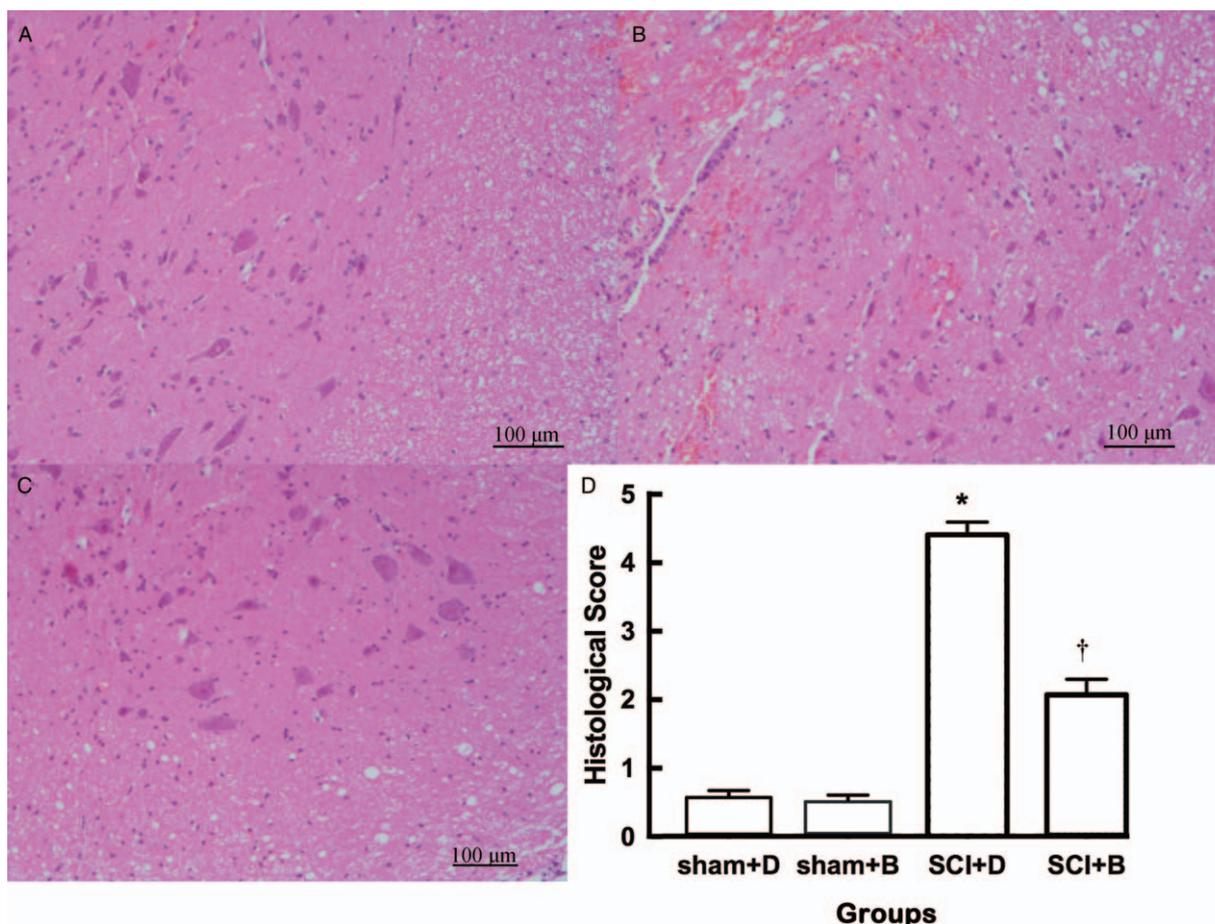
Figure 2: Effects of inhibition of ERK5 on histological alterations in the spinal cord tissue 24 h after injury. (A) The normal spinal cord tissue from blank group mice. (B) Significant damage to the spinal cord was observed in the spinal cord tissue from SCI + D mice. (C) Significant protection against the spinal cord injury was observed in mice in the SCI + B group. (D) The histological score was made by an independent observer. Data represent mean ± standard error; $n = 15$ per group. $^*P < 0.05$ compared with the sham and SCI + B groups. $^†P < 0.05$ compared with the sham and SCI + D groups. ERK5: Extra-cellular signal-regulated protein kinase 5; SCI: Spinal cord injury; Sham + B: Sham surgery + BIX02188 group; Sham + D: Sham surgery + dimethyl sulfoxide group.

after SCI, the ERK5 signaling pathway was activated in both neurons and microglia and participated in the process of neural injury and repair. Considering previous studies of ERK5 signaling pathway were almost all about peripheral nerve injuries, this kind of central nervous system (CNS) damage involves more severe and complicated pathophysiological processes, which may lead to the results difference.

In our research, the specific inhibition of BIX02188 on ERK5 signaling pathway plays a key role for the study of regulation mechanisms.[14] This inhibitor suppressed ERK5 phosphorylation by inhibiting the catalytic function of MEK5 enzyme. Moreover, it was shown to block MEF2-driven gene expression, a downstream target of MEK5/ERK5. As noted before, because of the efficacy and specificity, BIX02188 could provide effective tools for understanding the roles of the MEK5/ERK5 pathway relative to other closely related MAP kinases, and may provide a starting point for potential therapeutic treatments.[19]

We found that inhibition of ERK5 not only alleviated the degree of histological damage, but also reduced neutrophils infiltration, pro-inflammatory cytokines production, NF-κB activation and apoptosis. These effects might demonstrate roles of the ERK5 signaling pathway in SCI. In fact, rats in SCI + B group had a better locomotion recovery than those in SCI + D group, but our research mainly focused the explanation of possible mechanisms, so we did not show the outcomes of functional scores.

We observed a significant increase of MPO, TNF-α, and IL-1β in spinal cord tissues after trauma. Moreover, remarkable reduction of MPO, TNF-α, and IL-1β was found after the treatment of BIX02188. TNF-α and IL-1β exist in normal spinal cord tissues, and the expression increases rapidly after SCI, indicating that they participate synergistically in this process and might lead to apoptosis.[20] After the treatment of IL-1 inhibitor in acute SCI models of mice, expression of IL-1β and NF-κB decreased significantly, which suggested inhibitor of IL-1 receptor could down-regulate expression of IL-1β and NF-κB to alleviate local inflammation. In conclusion, the ERK5 pathway participates in the regulation of neutrophils infiltration and pro-inflammatory cytokine production and in further affects NF-κB expression and cell apoptosis.
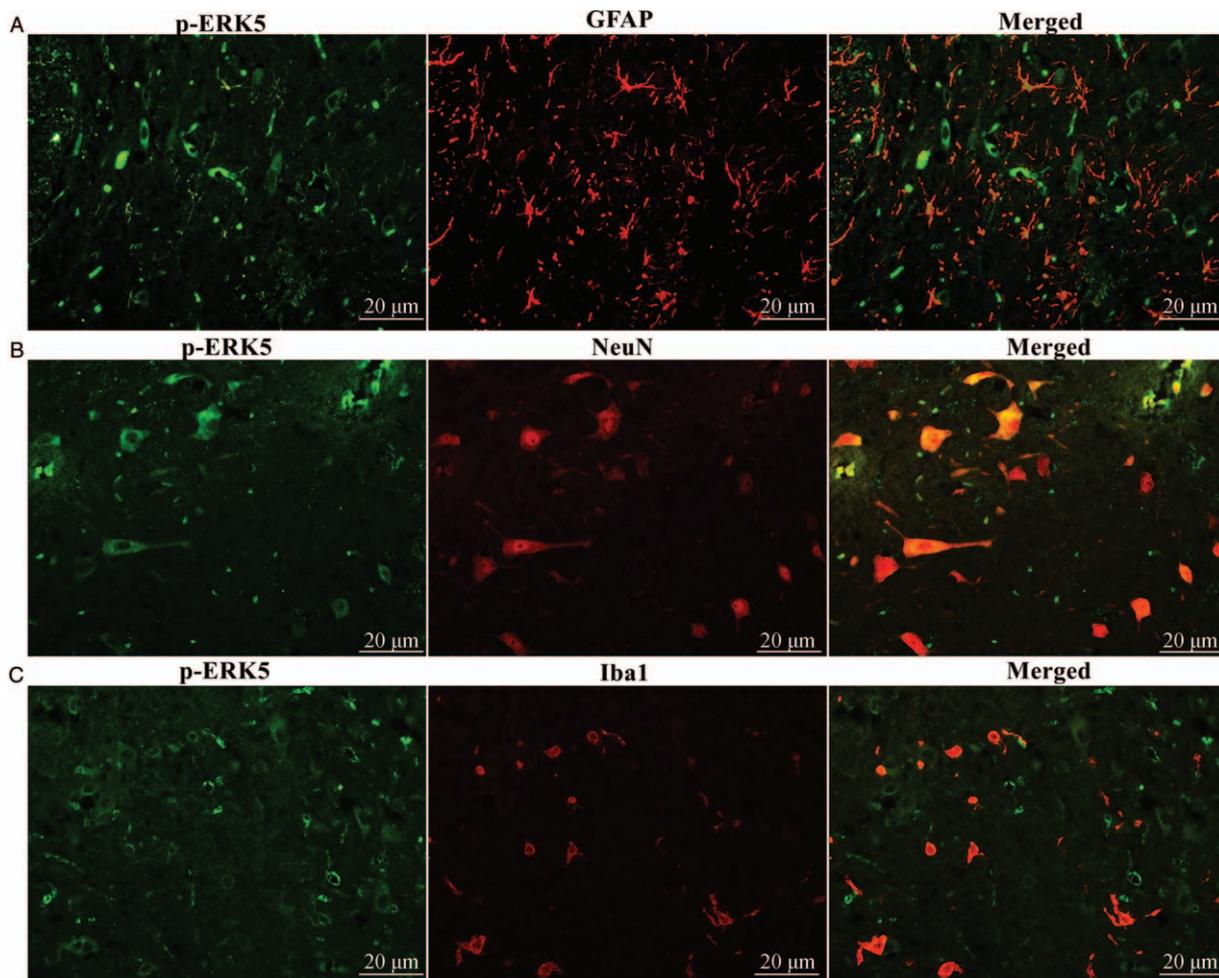
Figure 3: Double immunostaining of p-ERK5 with several cell-specific markers: (A) for astrocytes and GFAP; (B) for neurons and NeuN; (C) for microglia and Iba1. Green represents cells with p-ERK5. Red represents cells with GFAP, NeuN, and Iba1, respectively. ERK5: Extra-cellular signal-regulated protein kinase 5; GFAP: Glial fibrillary acidic protein; Iba1: Ionized calcium-binding adapter molecule 1.
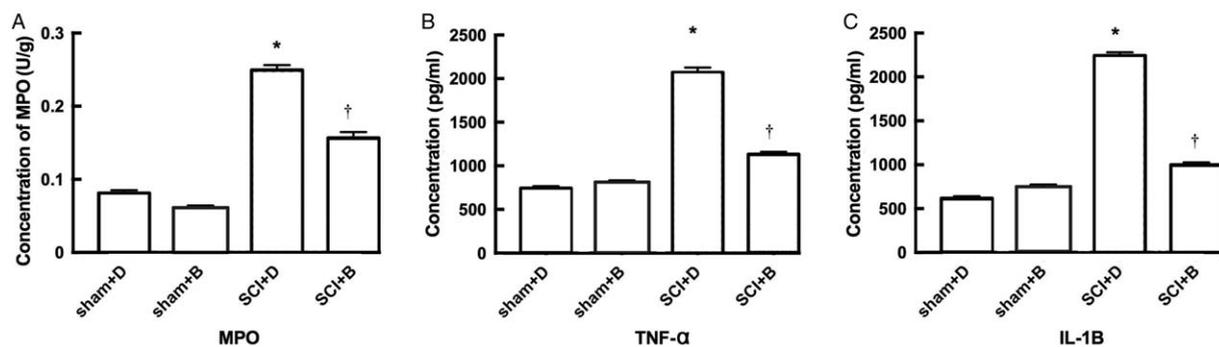

Figure 4: Effects of inhibition of ERK5 on MPO activity and spinal cord TNF-α and IL-1β levels. (A) After injury, MPO activity in spinal cord from SCI mice was significantly increased at 24 h in comparison with the sham groups. (B and C) In addition, a substantial increase in TNF-α and IL-1β production was found in spinal cord tissues from SCI mice 24 h after SCI. Intra-thecal treatment with BIX02188 significantly attenuated neutrophil infiltration and TNF-α and IL-1β levels into the spinal cord. Data represent mean $\pm$ SE; $n = 15$ per group. $^*P < 0.05$ compared with sham group and SCI + B group. $^\dagger P < 0.05$ compared with the sham and SCI + D groups. ERK5: Extra-cellular signal-regulated protein kinase 5; MPO: Myeloperoxidase; SCI: Spinal cord injury; Sham + B: Sham surgery + BIX02188 group; Sham + D: Sham surgery + dimethyl sulfoxide group.

Many experiments have suggested that NF-κB was crucial for regulation of many genes responsible for generation of mediators and proteins in secondary inflammation associated with SCI.[21] However, the precise mechanisms by which inhibition of the ERK5 signaling pathway suppresses NF-κB activation are not known. Many studies use ERK1/2 inhibitors, including PD98059 and U0126, to examine the roles of ERK1/2 signaling pathway in neuronal survival. Interestingly, these two inhibitors have also been shown to block the activation of ERK5.[22,23]
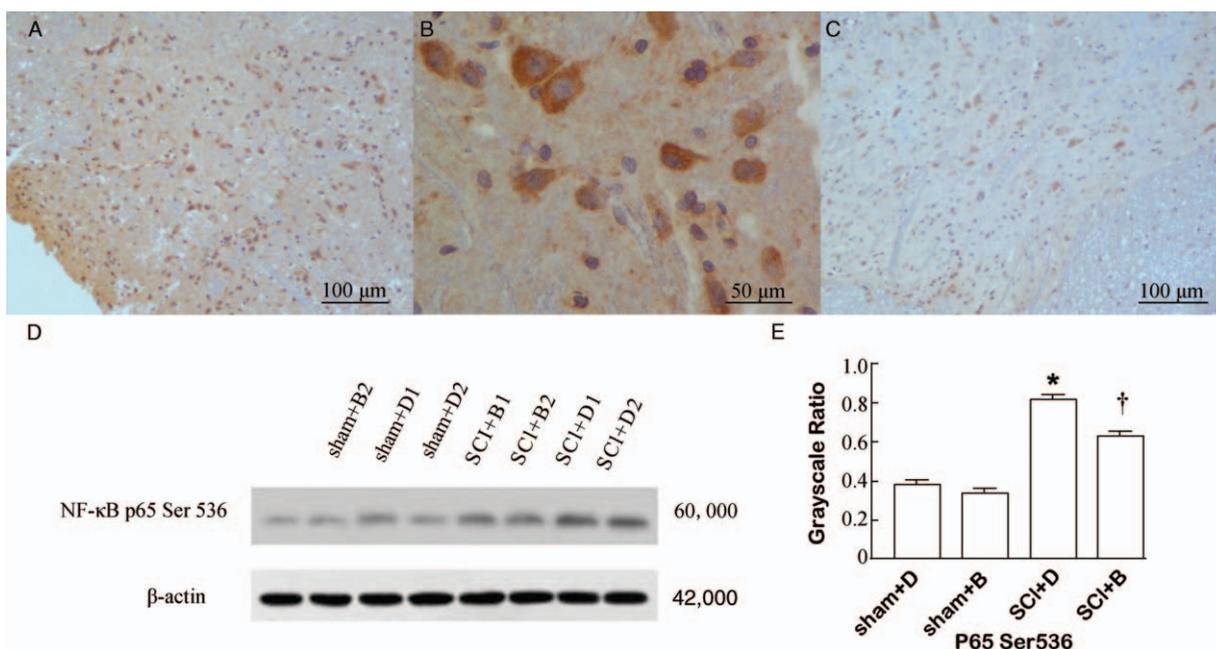
Figure 5: Effects of inhibition of ERK5 on the NF-κB P65 Ser536. (A and B) Immunohistological staining showed that spinal cord sections obtained from SCI mice exhibited positive staining for NF-κB P65 Ser536 24 h after injury. (C) Less P65 Ser536-IR cells were observed for spinal cord sections obtained from the SCI + B group. (D) At 24 h after SCI, expression of P65 Ser536 in the spinal cord homogenates was investigated by Western blot (1: sample 1; 2: sample). (E) A significant increase in P65 Ser536 levels was observed in the spinal cord from mice subjected to SCI, and BIX02188 treatment prevented the SCI-induced P65 Ser536 up-regulation. Data represent mean ± standard error; $n = 15$ per group. [*]$P < 0.05$ compared to the sham and SCI + B groups. [†]$P < 0.05$ compared to the sham and SCI + D groups. ERK5: Extra-cellular signal-regulated protein kinase 5; SCI: Spinal cord injury; Sham + B: Sham surgery + BIX02188 group; Sham + D: Sham surgery + dimethyl sulfoxide group.
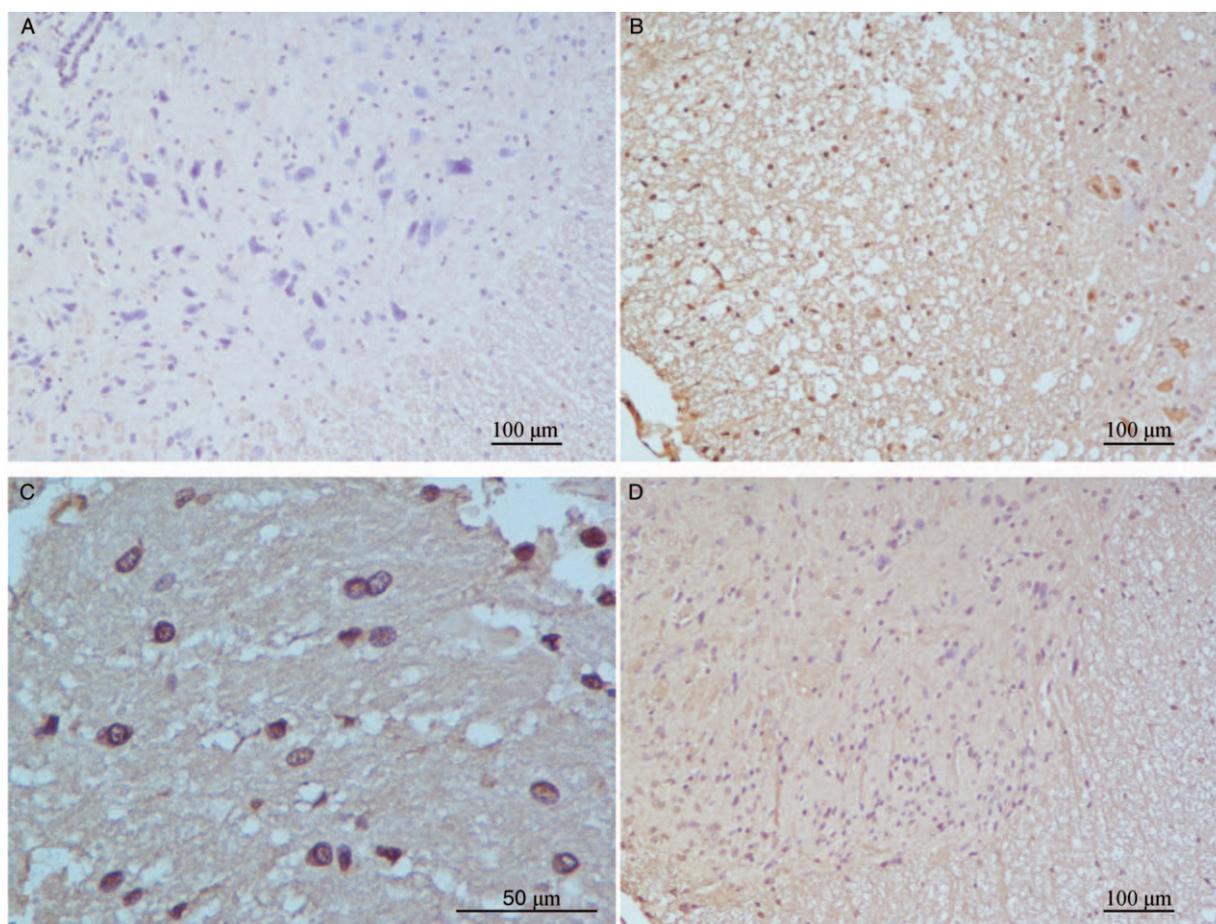

Figure 6: Effects of inhibition of ERK5 on apoptosis in spinal cord after injury (A) Almost no apoptotic cells were detected in the spinal cord from sham-operated mice at 24 h after the trauma. (B and C) SCI mice demonstrated marked appearance of dark brown apoptotic cells. (D) Tissues obtained from mice treated with BIX02188 demonstrated few apoptotic cells. ERK5: Extra-cellular signal-regulated protein kinase 5; SCI: Spinal cord injury.
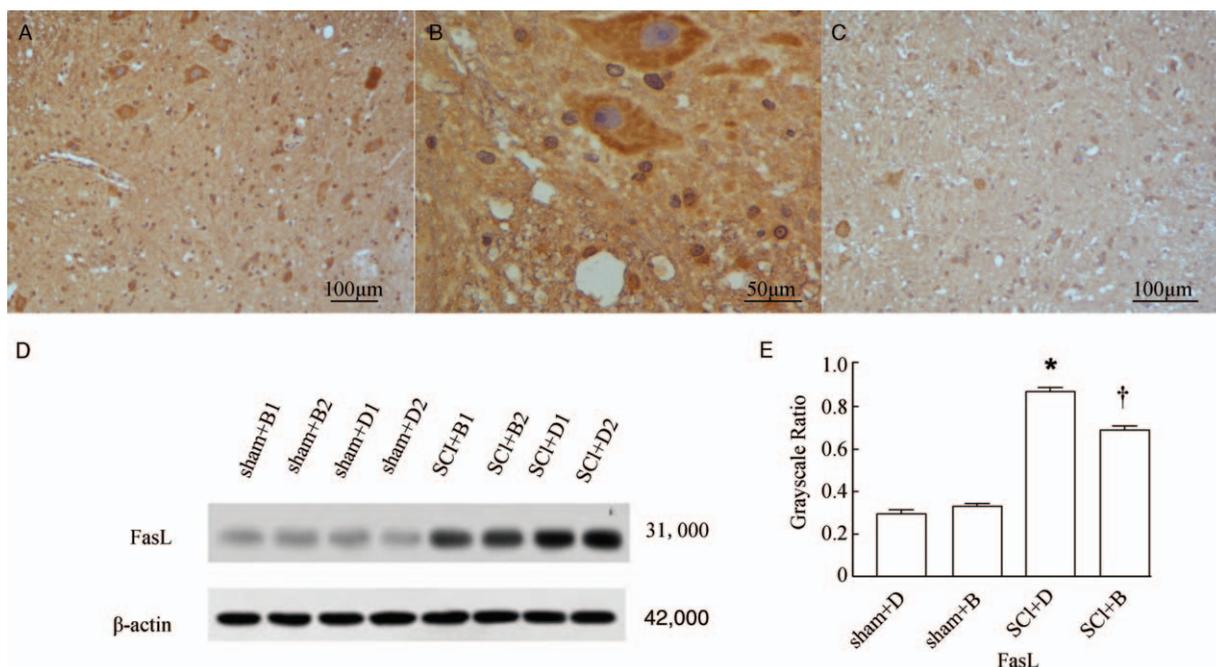
Figure 7: Effects of inhibition of ERK5 on Fas-ligand. (A and B) Immunohistological staining showed that spinal cord sections obtained from SCI mice exhibited positive staining for Fas-ligand 24 h after injury; (C) Fewer FasL-IR cells were observed for spinal cord sections obtained from the SCI + B group; (D) At 24 h after SCI, expression of Fas-ligand in spinal cord homogenates was investigated by Western blot (1: sample 1; 2: sample 2). (E) A significant increase in Fas-ligand levels was observed in the spinal cord from mice subjected to SCI, and BIX02188 treatment prevented SCI-induced Fas-ligand expression. Data represent mean ± standard error; $n = 15$ per group. [*]$P < 0.05$ compared with the sham and SCI + B groups. [†]$P < 0.05$ compared with the sham and SCI + D groups. ERK5: Extra-cellular signal-regulated protein kinase 5; SCI: Spinal cord injury; Sham + B: Sham surgery + BIX02188 group; Sham + D: Sham surgery + dimethyl sulfoxide group.
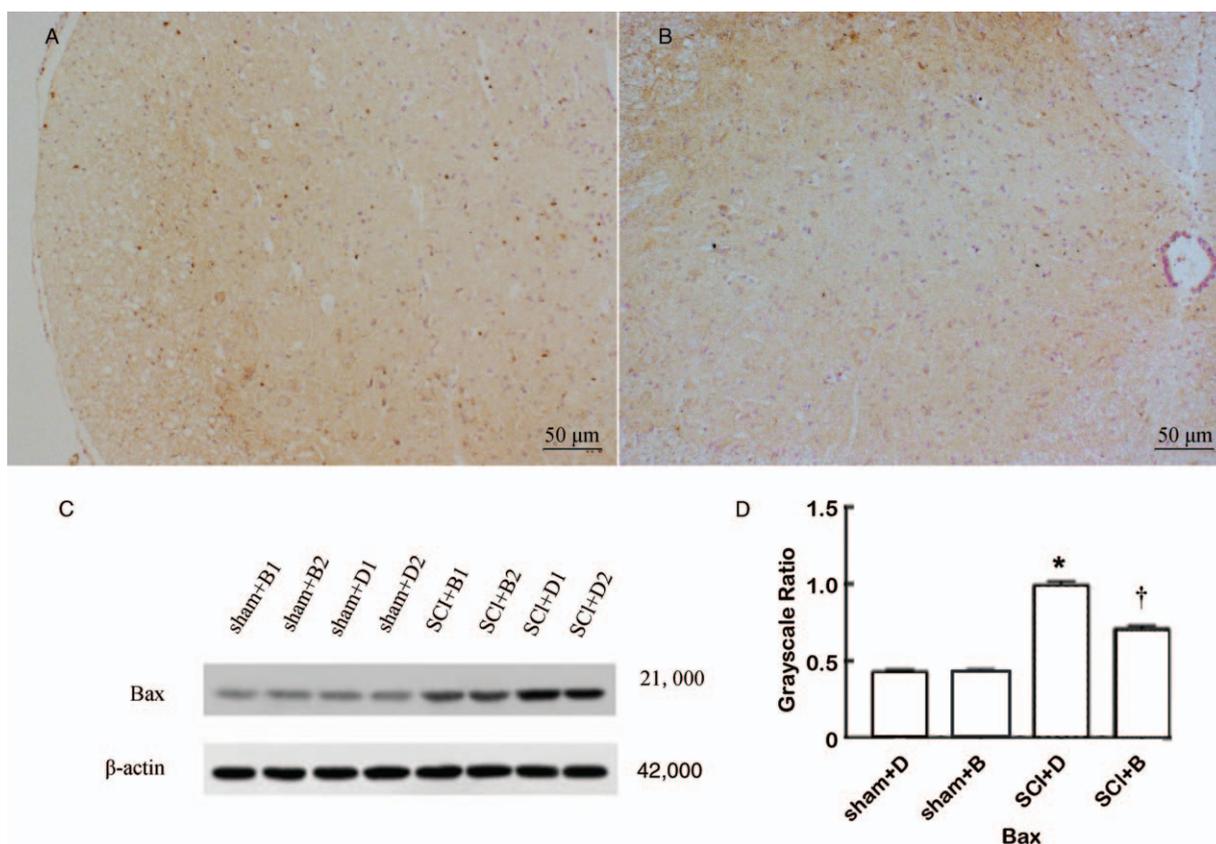

Figure 8: Immunohistochemistry and Western Blot Analysis for Bax. (A) Immunohistological staining showed Bax in spinal cord sections obtained from SCI mice exhibited positive staining for 24 h after injury. (B) BIX02188 reduced the degree of positive staining for Bax in the spinal cord of mice subjected to SCI. (C) At 24 h after SCI, expression of Bax in the spinal cord homogenates was investigated by Western blot (1:sample 1; 2:sample 2). (D) Bax levels were substantially increased in the spinal cords of mice subjected to SCI, while inhibition of ERK5 prevented the SCI-induced Bax expression. Data represent mean ± standard error; $n = 15$ per group. [*]$P < 0.05$ compared with the sham and SCI + B groups. [†]$P < 0.05$ compared with the sham and SCI + D groups. ERK5: Extra-cellular signal-regulated protein kinase 5; SCI: Spinal cord injury; Sham + B: Sham surgery + BIX02188 group; Sham + D: Sham surgery + dimethyl sulfoxide group.
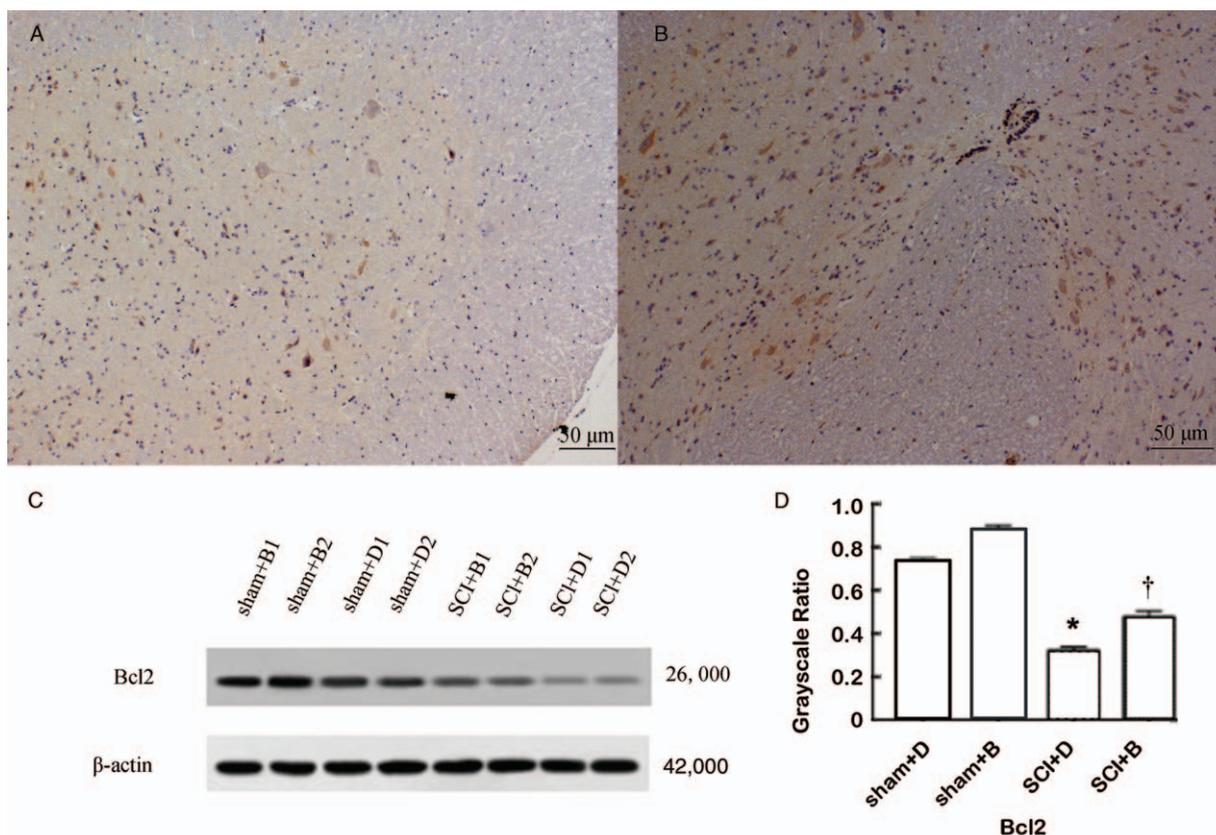
Figure 9: Immunohistochemistry and Western blot analysis for Bcl-2. (A) The Bcl-2 staining in SCI mice was significantly reduced. (B) BIX02188 attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI subjected mice. (C) At 24 h after SCI, expression of Bcl-2 in the spinal cord homogenates was investigated by Western blot (1:sample 1; 2:sample 2). (D) Twenty-four hours after SCI, Bcl-2 expression was significantly reduced in spinal cords of SCI mice, while treatment of mice with BIX02188 significantly blunted SCI-induced inhibition of anti-apoptotic protein expression. Data were represented as mean ± standard error; $n = 15$ per group. $^*P < 0.05$ compared with the sham and SCI + B groups. $^†P < 0.05$ compared with the sham and SCI + D groups. SCI: Spinal cord injury; Sham + B: Sham surgery + BIX02188 group; Sham + D: Sham surgery + dimethyl sulfoxide group.

Therefore, attention has been drawn to the roles of ERK5 in neuroprotection. ERK1/2 has been proved to stimulate NF-κB through the production of human heparin-binding epidermal growth factor-like growth factor (HbEGF).[24] However, increasing ERK5 activity had no effects on HbEGF transcription, suggesting that ERK5 may modulate NF-κB through a new mechanism. Gray et al[25] found that MEK5/ERK5 was required for Raf stimulation of NF-κB, and MEK5DD synergized with MEK1R4F to activate NF-κB. Moreover, NF-κB may serve as an integration point for ERK5 and ERK1/2 signaling. NF-κB also participates in the regulation of apoptosis with complicated mechanisms. On the one hand, expressions of pro-apoptotic and anti-apoptotic proteins are regulated by transcriptional functions of NF-κB; on the other hand, NF-κB interacts with relevant factors in apoptotic signaling pathways.

Apoptosis is an important mediator of SCI.[26,27] It exerts relevant effects in at least two phases: the initial phase, in which apoptosis accompanies necrosis in the degeneration of several cell types; and the later phase predominantly confined to white matter, mainly involving oligodendrocytes and microglia.[28] Chronologically, apoptosis occurs 6 h after injury at the lesion center and lasts for several days, associated with a steadily increased number of apoptotic cells. We demonstrated that inhibition of ERK5

with BIX02188 attenuated the degree of apoptosis after SCI, as measured by TUNEL assay.

FasL participates in apoptosis induced by a variety of chemical and physical insults.[29] FasL signaling participated in SCI according to a recent study.[30] Wang et al[10] reported that ERK5 signaling pathway promoted fibroblasts survival by down-regulating FasL expression via protein kinase B (PKB)-dependent inhibition of Forkhead box o3a (Foxo3a) activity. In our research, we confirmed that SCI led to substantial FasL activation, possibly contributing to the evolution of tissue injury. Furthermore, inhibition of ERK5 led to a reduction of FasL activation. Fas/FasL signaling pathway plays an important role in cell apoptosis, glial proliferation, and inflammations of neurologic diseases, and Fas mediates apoptosis of neurons and oligodendroglia in acute and sub-acute SCI.[31] Further studies are needed to clarify these mechanisms.

In mitochondria-dependent pathway, Bcl-2 family proteins are extremely important for apoptosis modulation. In mammals, Bcl-2 family contains pro-apoptotic protein and anti-apoptotic protein. When injury happens, up-regulation of pro-apoptotic protein and down-regulation of anti-apoptotic protein could lead to apoptosis.[32] Various studies have proposed that Bax, a pro-apoptotic gene, participates in developmental cell death[33] and CNS

injury.[34] Similarly, it has been shown that the administration of Bcl-xL fusion protein (Bcl-2 is the most expressed anti-apoptotic molecule in adult CNS) into injured spinal cords significantly increased neuronal survival, suggesting SCI-induced changes in Bcl-xL contribute considerably to neuronal death.[35] In our study, we identified up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2 in the process of SCI. The specific inhibition of ERK5 pathway by BIX02188 in the SCI models revealed features of apoptotic cell death after injury, showing that protection from apoptosis may be a pre-requisite for regenerative approaches to SCI. Therefore, further studies on the ERK5 activation and the influence of ERK5 inhibition on apoptotic process must be conducted to investigate the roles for ERK5 in neuronal cell death.

In conclusion, we demonstrated that inhibition of the ERK5 signaling pathway by a specific inhibitor alleviated the degree of SCI. Location of activated ERK5 and changes in relevant factors expression potentially explain possible mechanisms, implying that inhibition of the ERK5 pathway might be useful in the therapy of SCI, trauma, and inflammation.

Funding

Conflicts of interest

None.

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