Sparstolonin B selectively suppresses toll-like receptor-2 and -4 to alleviate neuropathic pain

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Abstract. It has been previously demonstrated that sparstolonin B (SsnB) inhibits toll-like receptor (TLR)-2 and TLR-4. The present study investigated the effect of SsnB on neuropathic pain (NP). A chronic constriction injury (CCI) model was constructed in rats and the protein expression of TLR-2 and TLR-4 was determined by western blot analysis. Rats were divided into the following three groups: Rats with sham surgery (control group); rats with CCI (model group); and rats with CCI and injection of SsnB (SsnB group). The mechanical withdrawal threshold (MWT) was measured by using Von Frey filaments. In addition, the mRNA and protein expression levels of nuclear factor-κB (NF-κB) were investigated by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively, and the concentrations of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 were determined by ELISA. Compared with control rats, the protein expression levels of TLR-2 and TLR-4 were increased in model rats ($P<0.001$). At 7 and 14 days after surgery, the MWTs in the model group were significantly reduced compared with the control group ($P<0.001$). However, the MWTs in the SsnB group were significantly increased compared with the model group ($P<0.001$). The results also demonstrated that the mRNA and protein expression levels of NF-κB, and the protein expression levels of TNF-α and IL-6, were increased in model group compared with the control group ($P<0.001$). Furthermore, these increases in expression were all reduced in the SsnB group compared with the model group. Therefore, the results indicate that SsnB may alleviate NP via suppression of TLR-2 and TLR-4, and may be a potential drug for the treatment of NP.

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Introduction

Neuropathic pain (NP) is defined as a type of direct or indirect pain that results from lesions or diseases affecting the somatosensory nervous system (1,2). Based on the location of lesions, NP is classified into the following two types: Peripheral NP, triggered by damage to the peripheral nervous system; and central NP, triggered by spinal cord injury, stroke or multiple sclerosis (3,4). The most important feature of NP is the manifestation of maladaptive plasticity (5). Upon clinical examination, the prevalence of NP in USA was estimated to be 9.8% in 2004-2005 (6). In addition to the obvious suffering induced by pain, patients with NP are also reported to suffer with depression, disordered sleep and impairments in body function (7). Therefore, NP affects the physical function and the quality of life of individuals.

Toll-like receptors (TLRs) are a family of receptors that are associated with pathogen invasion and are involved in the innate immune response (8). Activation of the TLRs triggers downstream signals resulting in the secretion of cytokines and chemokines, and inflammatory response initiation (9,10). A previous review has concluded that TLR is involved in the induction and maintenance of chronic pain via initiation of central immune signaling (11). Among TLRs, TLR-2 and TLR-4 exhibit the widest distribution, broadest pathogen recognition and maximum release of cytokines and chemokines (12). A previous study reported that TLR-2 contributes to spinal nerve injury-induced pain hypersensitivity (13). In addition, a TLR-4 antagonist was demonstrated to relieve mechanical allodynia in mice with neuropathic pain (14).

Sparstolonin B (SsnB) is a novel compound that was originally isolated from a Chinese medicine termed *Sparganium stoloniferum*. SsnB is a polyphenol whose structure is similar to that of isocounmarins (15). An increasing number of studies have investigated the functions of SsnB. Liu *et al* reported that SsnB suppresses cell proliferation, migration and inflammation in rat vascular smooth muscle (16). In addition, Wang *et al* (17) demonstrated that SsnB inhibits lipopolysaccharide-induced inflammation in 3T3-L1 adipocytes. Another study also indicated that SsnB acts as a selective TLR-2 and TLR-4 antagonist, which subsequently reduces inflammation in cardiomyocytes (18).

Due to the suffering that NP causes, the investigation and identification of novel reagents to alleviate pain sensitivity in patients with NP is required. Based on its ability to selectively

inhibit TLR-2 and TLR-4, the present study chose to investigate the potential effects of SsnB on NP. Through construction of a bilateral chronic constriction injury (CCI) model in rats, the effects of SsnB on mechanical withdrawal threshold (MWT) and the expression of nuclear factor-κB (NF-κB) were investigated. In addition, the effect on the protein expression levels of various proinflammatory factors was also investigated to identify the potential underlying mechanism.

Materials and methods

Construction of NP model. A total number of 30 (n=10 for each group) adult male Sprague-Dawley (SD) rats weighing 250-350 g (6-8 weeks old) were purchased from the Experimental Animal Center of Jilin University (Changchun, China). The rats were housed under a standard 12-h light/dark cycle at a temperature of 22˚C and a relative humidity of 55%. Food and water were available *ad libitum*. All animal studies were conducted in accordance with the Guidelines of the International Association for the Study of Pain (19). The experimental protocols were approved by the Ethics Committee of Beihua University (Jilin, China). All efforts were made to minimize the number and suffering of mice involved in the study.

A CCI model was established for NP according to procedures described previously (20). In brief, rats were administered intraperitoneally with 40-50 mg/kg sodium pentobarbital (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for anesthesia. Following separation of the biceps femoris by blunt dissection, the sciatic nerves were exposed and tied loosely using four ligatures (4.0 chromic gut) from sciatic trifurcation with 1 mm spacing. The desired degree of constriction estimated by observation by a dissecting microscope usually generated a brief twitch in the muscles around the exposure site. Subsequently, physiological saline was used to wash the exposed area. Identical surgery with ligation of the sciatic nerve was also performed on the opposite in both hind legs of the rats. Sham group surgery was performed in the same way but without ligation of the sciatic nerve. Following surgery, the rats were housed in cages with a solid floor to avoid exacerbation of the discomfort generated by surgery to hind paws. All surgical operations were conducted by the same individual.

Mechanical allodynia measurement. The pain threshold of MWT was measured by using Von Frey filaments (Stoelting Co., Wood Dale, IL, USA), as described previously (21). Sharp withdrawal was a response to mechanical stimulation generated by Von Frey filaments. In brief, rats were acclimatized in transparent plastic cages with a wire mesh floor for 30 min. Subsequently, a series of gradually increasing force (2, 4, 6, 8, 10, 15 and 20 g) were produced by Von Frey filaments and utilized to press the ipsilateral hind paw at the plantar surface. Each press lasted for 5-6 sec. If the rats lifted the hind paw immediately, it was recorded as a positive response. The same paw was stimulated 10 times under the same force before moving onto the next larger filament. The interval between filaments was ≥5 min. The MWT was recorded as the force that caused ≥6 positive responses out of 10 stimulations.

SsnB administration. SsnB was purified from the plant *Sparganium stoloniferum* according to the method described previously (15). The purity of SsnB was determined to be >99% by high-performance liquid chromatography as described previously (22). SsnB powders were dissolved in soybean oil as a stock solution. Rats were randomly divided into the following three groups: Rats with sham surgery (control group); rats with CCI (model group); and rats with CCI followed by intraperitoneal injection of SsnB (6 mg/kg; SsnB group). The rats in the control and model group received an intraperitoneal injection of soybean oil alone. Rats received intraperitoneal injection 2 h after surgery, and the injections were administered once a day for the next 13 days.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). At 14 days after modeling, dorsal root ganglia (DRG) isolated from ipsilateral dorsal rat spinal cords (L4-L6) was collected. Total RNA was isolated by TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and DNase I (Promega Corp., Madison, WI, USA), according to the manufacturers' protocol. Reverse transcription of the RNA was performed using PrimeScript First Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) with the following cycling parameters: 30˚C for 10 min, followed by 42˚C for 30 min and 95˚C for 5 min, according to the manufacturer's protocol. The cDNA was diluted 1:10 with nuclease-free water and the expression of NF-κB was detected utilizing the SYBR ExScript RT-PCR kit (Takara Biotechnology Co., Ltd.). Briefly, diluted cDNA (1 μl) was added to the total reaction system (20 μl) and the PCR program with the following cycling parameters: An initial predenaturation step at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec and annealing at 60˚C for 34 sec, which was performed on a 7500 Real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Each sample was run in triplicate. Primers (Shanghai GenePharma Co., Ltd., Shanghai, China) were designed as follows: NF-κB, 5'-CTG GCA GCT CTT CTC AAA GC-3' (forward) and 5'-CCA GGT CAT AGA GAG GCT CAA-3' (reverse); β-actin, 5'-TCA GGT CAT CAC TAT CGG CAA T-3' (forward) and 5'-AAA GAA AGG GTG TAA AAC GCA-3' (reverse). NF-κB expression was calculated via the comparative threshold (Cq) using the $2^{-\Delta\Delta Cq}$ method (23). The relative expression levels were normalized to β-actin.

Western blot analysis. At 14 days after CCI, DRG was isolated immediately after decapitation. The tissues were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich; Merck KGaA). Following centrifugation at 12,000 x g for 30 min at 4˚C, the protein concentration was measured by the BCA Protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein (~20 μg) was subjected to 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), followed by blocking with 5% non-fat milk (Shuangcheng Nestlé Co., Ltd., Shuangcheng, China) for 1 h at room temperature. Membranes were then incubated overnight at 4˚C with the following primary antibodies: Anti-TLR-2 (cat. no. ab108998; rabbit; 1:1,000;

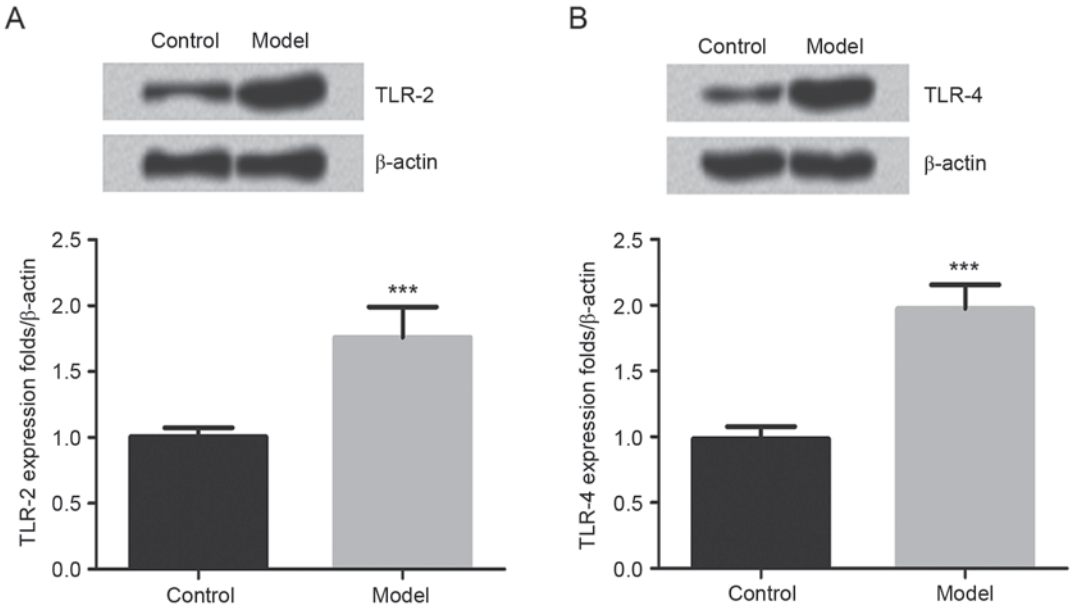

Figure 1. Protein expression levels of TLR-2 and TLR-4 were increased in the CCI model group. (A) TLR-2 and (B) TLR-4 protein expression levels were determined in control and CCI model rats by western blot analysis. Data are presented as the mean ± standard deviation. ***P<0.001 vs. control group. TLR, toll-like receptor; CCI, chronic constriction injury.

Abcam, Cambridge, UK); anti-TLR-4 (cat. no. ab8376; mouse; 1:1,000; Abcam); and anti-NF-κB (cat. no. ab32360; rabbit; 1:1,000; Abcam). Subsequently, the rinsed membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. ab6721, goat anti-rabbit, 1:5,000, Abcam; cat. no. ab6789, goat anti-mouse, 1:5,000, Abcam) for 1 h at room temperature. In order to visualize the immunoreactive proteins bands, Amersham ECL Western Blotting Detection kit (GE Healthcare Life Sciences, Little Chalfont, UK) was employed, according to the manufacturer's protocol. The intensity of the bands was determined by ImageJ software version 1.4.3.67 (National Institutes of Health, Bethesda, MA, USA). The blots were stripped utilizing Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Inc.) for 15 min at room temperature and the membrane was incubated with antibody against β-actin (cat. no. ab8227; rabbit; 1:1,000; Abcam) as a loading control.

Determination of proinflammatory cytokine concentration. At 14 days after modeling, the DRG was isolated from the sciatic nerve and homogenized in 5X volume of PBS. Homogenates were centrifuged at 12,000 x g for 30 min at 4˚C and the supernatant was used for ELISA. The concentration of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 were determined by ELISA kits (cat. no. RTA00 for TNF-α and cat. no. R6000B for IL-6; R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the final concentrations were adjusted for the protein concentration.

Statistical analysis. Data are presented as the mean ± standard deviation. One-way analysis of variance with Bonferroni's post hoc test was used for multiple comparisons. Differences between two groups were determined by two-tailed unpaired t-tests. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

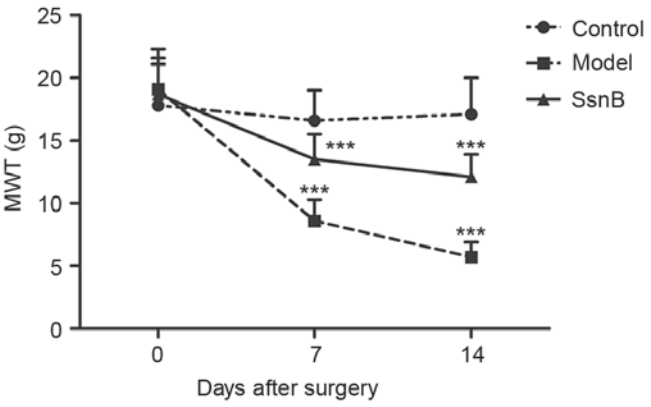

Figure 2. SsnB treatment reduced MWTs in rats with chronic constriction injury. Data are presented as the mean ± standard deviation. ***P<0.001 vs. control group. SsnB, sparstolonin B; MWT, mechanical withdrawal threshold.

Results

CCI increases the protein expression of TLR-2 and TLR-4. At 14 days after surgery, the protein expression levels of TLR-2 and TLR-4 in the dorsal DRG tissues of rats were measured by western blot analysis. Fig. 1 demonstrates that the expression of TLR-2 and TLR-4 was significantly increased in rats with CCI compared with the control group (P<0.001). These results indicated that the CCI model was constructed successfully.

SsnB alleviates NP in SD rats. The pain sensitivity of rats was determined by WMTs. As indicated in Fig. 2, rats in each of the three groups exhibited similar WMTs prior to surgery.

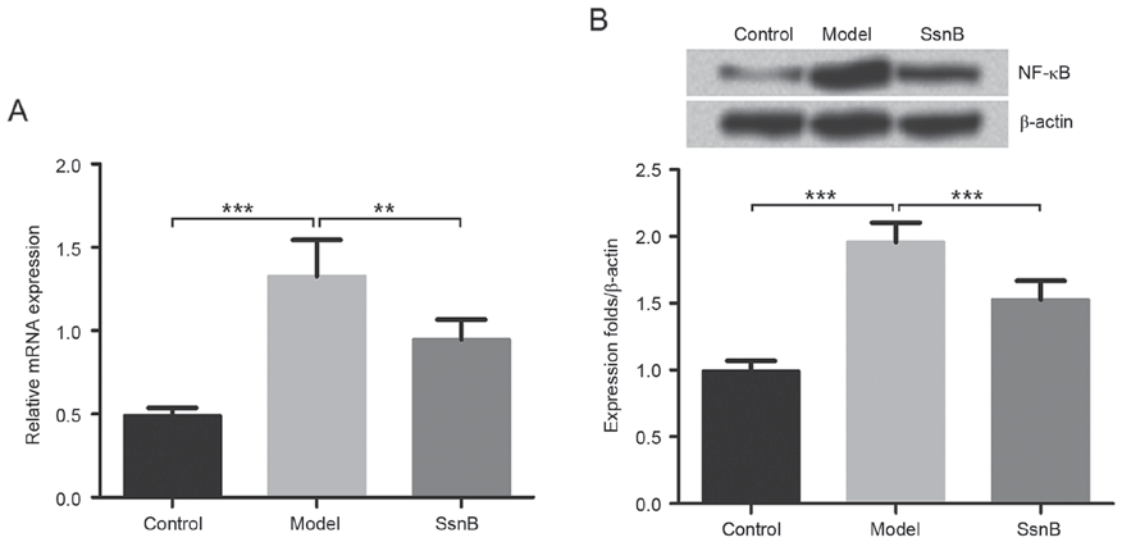

Figure 3. mRNA and protein expression levels of NF-κB were reduced in the SsnB group compared with the model group. (A) mRNA and (B) protein expression levels of NF-κB were determined by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. Data are presented as the mean ± standard deviation. **P<0.01 and ***P<0.001, as indicated. NF-κB, nuclear factor-κB; SsnB, sparstolonin B.

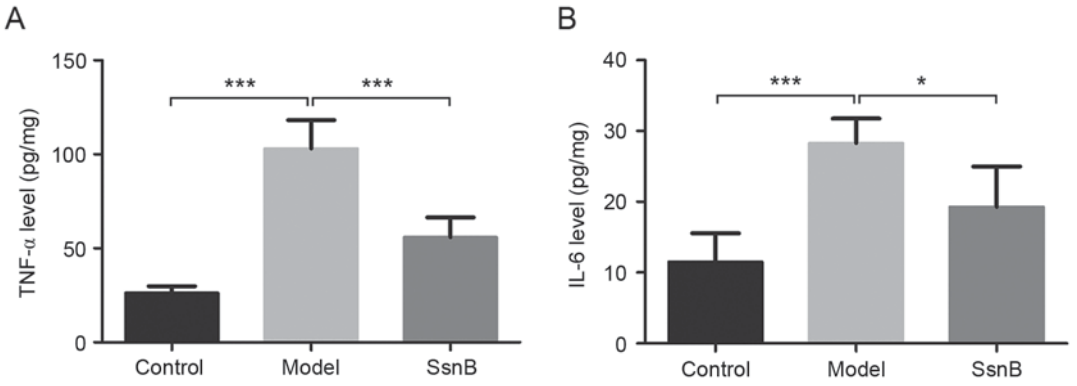

Figure 4. Concentrations of TNF-α and IL-6 were reduced in the SsnB group compared with the model group. (A) TNF-α and (B) IL-6 protein concentrations were determined by ELISA. Data are presented as the mean ± standard deviation. *P<0.05 and ***P<0.001, as indicated. TNF, tumor necrosis factor; IL, interleukin; SsnB, sparstolonin B.

Following surgery, the WMT of rats in the control group was similar to the WMT prior to surgery. However, the WMT of rats in the model group was significantly reduced compared with the control control group at 7 and 14 days after surgery (P<0.001). This demonstrates that the complete CCI surgery successfully reduced the WMT of rats. Rats that received SsnB injection exhibited a significantly increased WMT compared with the model group (P<0.001) at days 7 and 14 after surgery. These results indicate that SsnB markedly alleviated the NP in rats.

SsnB decreases the mRNA and protein expression of NF-κB. At 14 days after surgery, the mRNA and protein expression levels of NF-κB in dorsal DRG tissues of rats were determined by RT-qPCR and western blot analysis, respectively. Fig. 3 demonstrates that the mRNA and protein expression levels of NF-κB in the model group were increased significantly compared with the control group (P<0.001). However, with administration of SsnB, the mRNA and protein expression levels of NF-κB were significantly decreased in the SsnB group compared with the model group (P<0.01 and P<0.001, respectively). These results indicate that SsnB suppresses the expression of NF-κB.

SsnB exerts an anti-inflammatory effect on CCI. Levels of proinflammatory cytokines (TNF-α and IL-6) were evaluated by ELISA. As presented in Fig. 4, the model group exhibited significantly increased levels of TNF-α and IL-6 compared with the control group (P<0.001). However, the levels of TNF-α and IL-6 were significantly decreased in the SsnB group compared with the model group (P<0.001 and P<0.05, respectively). These results indicated that SsnB has an anti-inflammatory effect during CCI.

Discussion

NP is a common public health problem that cannot be effectively reduced by analgesics (24). Therefore, the present study constructed a CCI model in SD rats and focused on TLR-2 and TLR-4 to investigate a novel reagent for alleviating NP. The current study demonstrated that the protein expression of TLR-2 and TLR-4 were increased in rats with CCI compared

with controls. In addition, it was observed that SsnB significantly increased the MWT in CCI rats. As a downstream factor of TLRs, the mRNA and protein expression levels of NF-κB were investigated. The results indicated that SsnB reduced the mRNA and protein expression of NF-κB. Further experiments by ELISA indicated that SsnB also downregulated the secretion of the proinflammatory factors TNF-α and IL-6.

Previous studies have fully validated the critical role of proinflammatory cytokines in pathological pain (11). Firstly, a variety of cytokines interact with astrocytes or microglia and elevate the conductivity of α-amino-3-hydroxy-5 methyl-4-isoxazole-propionic acid and N-methyl-D-aspartate receptors. The enhanced conductivity and the increased expression of these receptor types on the neuronal surface facilitates neuronal excitability and synaptic strength (25). Furthermore, proinflammatory cytokines lead to increases in the concentration of sodium, which subsequently increases the neuronal excitability via reduced potassium channel activity (26). In addition, proinflammatory cytokines also affect the inhibitory neurotransmitter γ-aminobutyric acid receptors (27). As neuronal excitability is a hallmark of NP, the proinflammatory cytokines TNF-α and IL-6 may enhance pain sensitivity. Therefore, the increased protein levels of TNF-α and IL-6 observed in the present study may be a reason for the decreased MWT in rats with CCI.

Various previous studies have supported the hypothesis that the expression levels of TLR-2 and TLR-4 may be upregulated in rats with nerve damage. One study indicated that the expression of TLR-2 in mouse sciatic nerves was significantly upregulated at 14 days after injury (28), whilst another study reported that the expression of TLR-4 at the spinal levels in SD rats was increased on day 7 after injury (29). In addition, Jurga *et al* (30) also demonstrated that the expression of TLR-2 and TLR-4 was increased in DGR at day 14 after CCI. TLRs have a pivotal role in the innate immune response (31). When TLRs are activated, the adaptor molecules to TLRs are attracted. In terms of TLR-2 and TLR-4, the relevant adaptor molecule is myeloid differentiation primary response gene 88 (MyD88) (32). In addition, the toll-receptor-associated activator of interferon (TRIF) is another adaptor molecule for TLR-4 (33). Activation of MyD88 and TRIF pathways subsequently induce activation of NF-κB (34). Thereafter, a variety of proinflammatory cytokines are produced, including TNF-α and IL-6. Endogenous signals that are termed damage-associated molecular patterns activate TLR receptors and cause further activation of a variety of cell types (35). These cell types include glial cells, which have been proven to be essential mediators in the pathological pain process (36). Therefore, in terms of the results in the present study, we concluded that TLR-2 and TLR-4 were activated following CCI in rats, which led to the activation of the NF-κB signaling pathway and subsequently increased the production of proinflammatory cytokines, leading to increased pain sensitivity. As SsnB was reported to be an antagonist of TLR-2 and TLR-4, the alleviation of pain sensitivity by SsnB reported in the present study may occur via suppression of the NF-κB signaling pathway and reduced expression of proinflammatory cytokines.

In conclusion, the results of the present study demonstrated that SsnB was an effective reagent for the inhibition of NP via suppression of TLR-2 and TLR-4. The present study may provide a novel theoretical basis for the application of SsnB in the therapeutic treatment of NP. Further studies are required to understand the mechanism in more depth and to validate the toxicity of SsnB in NP.

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