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Inhibition of COX-2 alleviates lumbar spinal stenosis-induced chronic mechanical allodynia in rats

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ABSTRACT

Chronic low back pain due to lumbar spinal stenosis (LSS) is common, costly, mechanistically complex, and clinically challenging. However, the factors and mechanisms causing and mediating chronic pain induced by cauda equina compression remain unclear. Here, we examined the role of cyclooxygenase (COX)-2 in infiltrated macrophages, a key mediator of inflammation, in chronic neuropathic pain by LSS using an animal model. LSS was induced in adult male rats by cauda equina compression procedure using a silicone block within the epidural spaces of L5-L6 vertebrae. Locomotor deficit was observed after compression and mechanical allodynia was developed progressively for 4 weeks after injury. A number of macrophage were also infiltrated into the spinal parenchyma and cauda equina and COX-2 was expressed in infiltrated macrophages at 28 days after cauda equina compression. The administration of COX-2 inhibitors, celecoxib and MPO-0029, significantly alleviated LSS-induced chronic mechanical allodynia and inhibited the mRNA expression of inflammatory mediators such as *tnf-α, Il-1β, il-6,* and *inos.* Furthermore, COX-2 inhibitors significantly reduced prostaglandin E2 production. These results demonstrated the role of COX-2 in LSS-induced chronic neuropathic pain and suggest that the regulation of COX-2 can be considered as a therapeutic target to relive neuropathic pain.

1. Introduction

Lumbar spinal stenosis (LSS) is a medical condition in which the vertebrae canal becomes narrow by surrounding bone and soft tissues compromising neural structures, which leads to intermittent claudication, leg pain, and walking disability. This is due to the common occurrence of lumbar spondylolisthesis, slipped disk, ligamentous thickening and spinal degeneration that develops with aging [1]. Chronic LSS induces compression of cauda equina fibers, and hypersensitivity, sensitization of central nervous system (CNS) and peripheral nervous system (PNS), thereby causes generalized and severely debilitating neuropathic pain affecting millions of people worldwide. Surgical decompression has been the most sought surgical treatment option for LSS. However, the success rate of surgery is highly variable and decompressive laminectomy can result in the disruption of native anatomic support structures such as supraspinous ligament, and interspinous ligament, leading to muscular atrophy [2,3]. Opioid-based analgesics are prescribed for the medical management of chronic spinal pain disorders, including LSS. Unfortunately, it has recently been demonstrated that opioid analgesics offer little clinical benefit by way of pain reduction or functional improvement in patients with chronic musculoskeletal pain including LSS [4–6]. Hence, there exists a compelling need to find new treatment methods for relieving pain in LSS patients.

Among many causes, neuroinflammatory responses has been known to play a key role in spinal neuropathic pain development and maintenance. After tissue injury, immune cells migrate to the injury site and pro-inflammatory cytokines are produced, mediating inflammatory

Abbreviations: CNS, central nervous system; COX-2, cyclooxygenase-2; LSS, lumbar spinal stenosis; PWT, paw withdrawal threshold; PNS, peripheral nervous system; PGE2, prostaglandin E2; SCI, spinal cord injury

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reaction. Pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, and mediators including COX-2, iNOS, and prostaglandin E2 have been suggested to induce hypersensitization of pain transmitting neurons [7–9]. Several reports showed that blocking the expression of inflammatory cytokines and mediators in chronic neuropathic pain-induced rats by acupuncture, sodium channel blocker, 17β-estradiol, and minocycline inhibits chronic below-level neuropathic pain after spinal cord injury (SCI) [10–12]. In addition, immune cell infiltration was observed in spinal cord and cauda equina fiber in LSS animal model [13,14]. Shunmugavel et al. [13] and Khan et al. [15] reported that S-nitrosoglutathione and cytosolic phospholipase A2 administration inhibited inflammatory response and suppressed acute pain after cauda equina compression in rat, suggesting a link between inflammation and LSS-induced neuropathic pain. However, the association of neuroinflammation with LSS-induced chronic pain has not yet been elucidated.

Here, we determined the expressing cell type and the expression pattern of COX-2, a typical inflammatory mediator, and examined whether COX-2 inhibitors, celecoxib and a novel compound, MPO-0029, inhibit inflammation in cauda equina and thereby relieve LSS-induced chronic neuropathic pain in rat.

2. Materials and methods

2.1. Animals and ethics statement

A total of 167 Sprague-Dawley male rats (250–270 g; Samtako, Osan, Korea) were used in this study. The animals were maintained under a constant temperature (23 ± 1 °C) and humidity (60 ± 10%) under a 12 h light/ dark cycle (light on 07:30–19:30 h) with ad libitum access to drinking water and food. The rats were housed one per cage (410 × 282 × 153 mm, transparent poly carbonate) with aspen shaving bedding and were fed a commercial diet (5 L79, PMI Nutrition International, St Louis, MO) and commercial standard chow (Lab Diet 5L79l Purina Mills, Richmond, IN). All animal experiments were performed in accordance with the Guidelines of Animal Care Committee of the Kyung Hee University (permission number: KHUASP(SE)-15-006) and followed the Ethical issues of the International Association for the Study of Pain [16].

2.2. Surgery and cauda equine compression

Cauda equina compression was induced based on the previous report by Ma et al. [17] with minor modification. Rats were anesthetized with chloral hydrate (500 mg/kg) by intraperitoneal (i.p.) injection and the back regions were shaved and the vertebral plate was exposed at the L4-S2 level. The ligamentum flavum between L4 and L5 was removed. Subsequently a piece of trapezoid-shaped silicon block (1.00 mm Length x 1.3–1.2 mm Width x 1.0 mm Height) was inserted into the epidural space under the L5 and L6 vertebral plate without disrupting the dural sac (Fig. 1). For sham-operated group, animals were only posterior open and drilled, without inserting silicon block. Throughout the surgical procedure, body temperature was maintained at 37 ± 0.5 °C with a heating pad (Biomed S.L., Alicante, Spain). After injury, muscles and skin were closed in layers, and rats were placed in a temperature and humidity-controlled chamber overnight. Post-operatively, rats were received subcutaneously supplemental fluids (5 ml, lactated ringer) and antibiotics (gentamicin, 5 mg/kg, intramuscular injection) once daily for 5 days (d) after surgery. Rats were housed one per cage with water and food easily accessible. Body weights and the remaining chow and water weight were recorded each morning for all animals.

2.3. Behavioral assessment

Locomotor activity was assessed using a rotarod system (Rota Rod-R V2.0, B. S. Technolab. Inc) according to the previous report [15]. Rats were conditioned on the rod with an increasing speed from 4 rpm to 40 rpm (accelerated by 1 rpm per 5 s). Walking time until the rat fell off the rotating rod was measured three times for each animal. Before the measurement, rats were familiarized to the rod with a constant speed of 4 rpm for 3 min. The inter-experimental gap was 20 min. The mean of three trials was calculated for statistical analyses. Mechanical allodynia was assessed by the paw withdrawal threshold (PWT) in response to probing with a series of calibrated von Frey filaments as in our previous report [10]. Pain behavioral testing was performed by trained investigators who were blind as to the experimental conditions.

2.4. Drug administration

At 28 d after cauda equina compression, we selected only those rats that weighed tween 350–380 g and developed with chronic neuropathic pain (mechanical allodynia, PWT; 2.5–4.0 g), and then divided randomly into each experimental group including vehicle, celecoxib, MPO-0029 treatments (Fig. 1D). Celecoxib (Sigma, St. Louis, MO) or MPO-0029 [18] was dissolved in methyl pyrrolidone:Tween-80:saline (1:1:8, 100 μl) and administrated i.p. at a dose of 2, 5, or 10 mg/kg. Vehicle group received equivolumetric injection of methyl pyrrolidone:Tween-80:saline (1:1:8) at the corresponding time points.

2.5. Tissue preparation

At the time of peak effect (30 min after drug injection), animals were anesthetized with chloral hydrate (500 mg/kg) and perfused transcardially with 0.1 M PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. The a 40 mm cauda equina segment (0 to 40 mm rostral to the compression site) was embedded in OCT for frozen sections and cross sections were then cut at 10 μm on a cryostat (CM1850; Leica, Wetzlar, Germany). For molecular work, rats were perfused with 0.1 M PBS and a 20 mm cauda equina segment, centered at the lesion site, was isolated and frozen at −80 °C until use as previously described [10].

2.6. Immunohistochemistry

Frozen sections were processed for immunohistochemistry with antibodies against ED-1 (CD68, 1:200; Serotec, Raleigh, NC) and COX-2 (1:100; Abcam, Cambridge, MA) as previously described [11]. Fluorescence signal was detected by a fluorescence microscope (BX51, Olympus, Japan), and the capture of images and measurement of signal co-localization was performed with MetaMorph software (Molecular devices, Sunnyvale, CA).

2.7. Western blot

Total protein from cauda equina segments including compression site was prepared and Western blot analysis was performed as previously described [19]. The primary antibodies used in Western blot are as follows; COX-2 (1:1000; Abcam) and β-tubulin (1:30000, Sigma). Quantification of bands was performed by AlphaImager software (Alpha Innotech Corporation, San Leandro, CA).

2.8. RNA isolation and RT-PCR

Total RNA from cauda equina segments including compression site was prepared and RT-PCR for *tnf-α*, *il-1β*, *il-6*, *cox-2*, *inos* and *gapdh* were performed as previously described [10]. The sequences of the primers are as follows (5′-3′): *tnf-α* forward, 5′- CCC AGA CCC TCA CAC TCA GAT-3′; reverse, 5′- TTG TCC CTT GAA GAG AAC CTG-3′; *il-1β* forward, 5′- GCA GCT ACC TAT GTC TTG CCC GTG-3′, reverse, 5′- GTC GTT GCT TGT CTC TCC TTG TA-3′; *il-6* forward, 5′- AAG TTT CTC TCC GCA AGA TAC TTC CAG CCA-3′; reverse, 5′- AGG CAA ATT TCC TGG TTA TAT CCA GTT-3′; *cox-2* forward, 5′- CCA TGT CAA AAC CGT GGT GAA TG-3′; reverse, 5′- ATG GGA GTT GGG CAG TCA TCA G-3′; inos

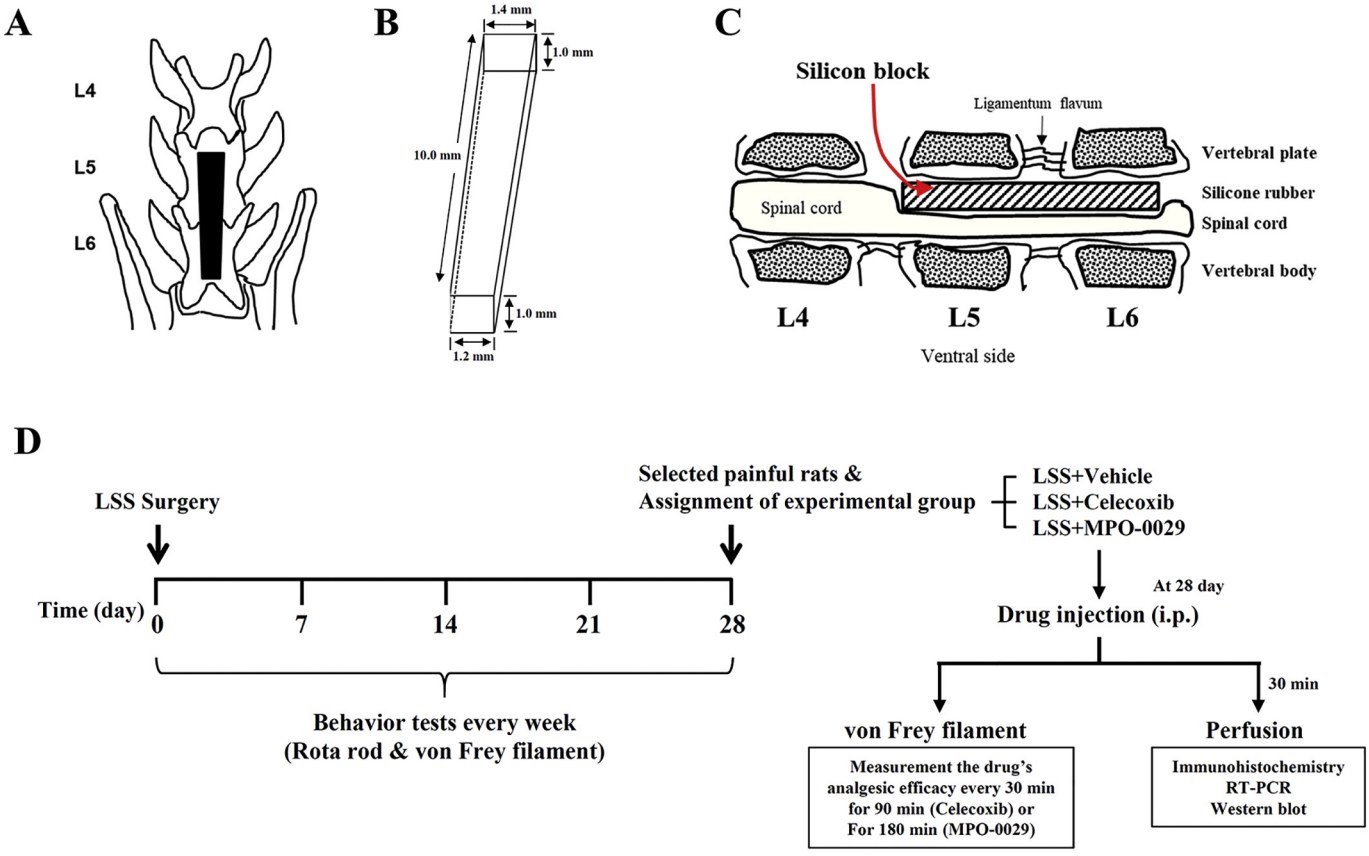

Fig. 1. Schematic of cauda equina compression animal model. (A) The location of silicone block (B) The dimension of silicone block. (C) Schematic of surgical procedure placing silicone blocks in epidural spaces. (D) Scheme of experimental design of the study.

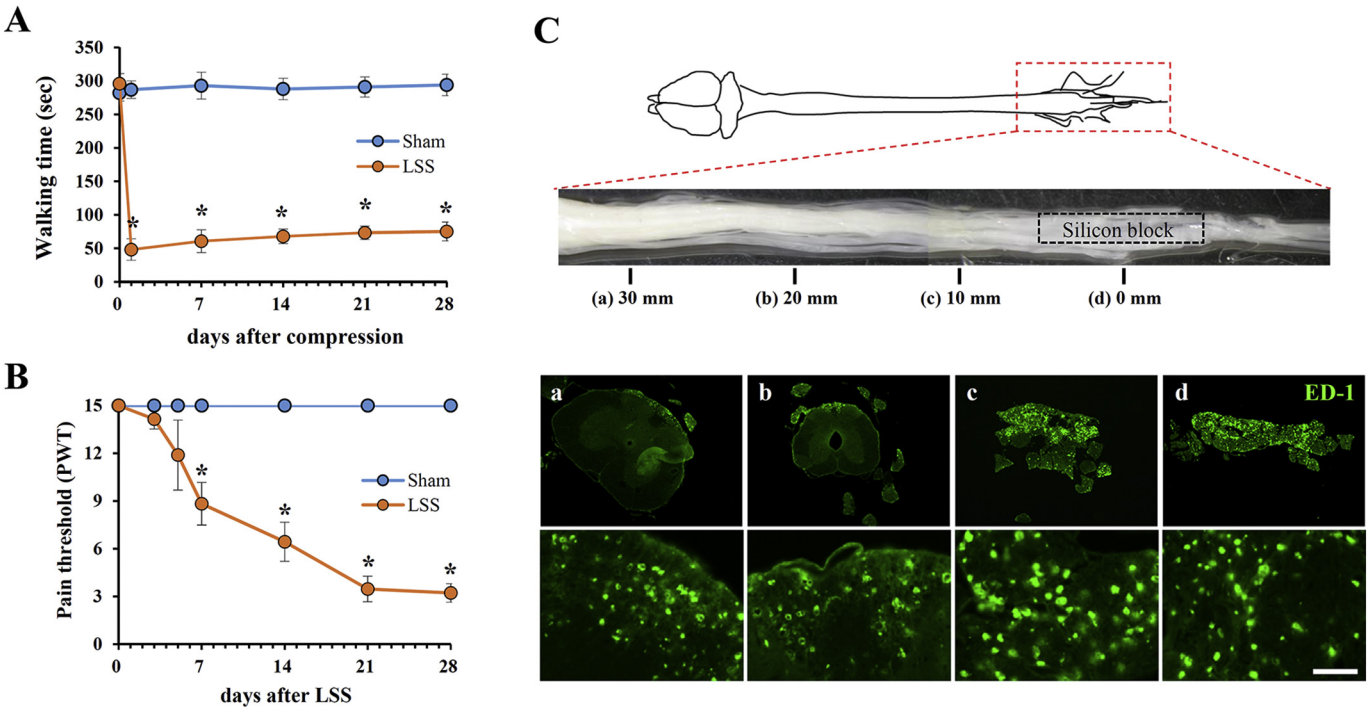

Fig. 2. Macrophage recruitment in cauda equina in chronic pain-developed rats. (A) Time course of change in walking duration on the rotarod (n = 20/group). (B) Pain responses to mechanical stimuli. Mechanical allodynia was assessed by the paw withdrawal threshold (PWT) in response to von Frey filaments (n = 20/group). Data are presented as the mean ± SEM. *p < 0.05 vs. sham. (C) Representative images showing immunofluorescence staining for ED-1 (macrophage marker) in various lesions. The upper panel shows the position of representative immunostaining images in bottom panel.

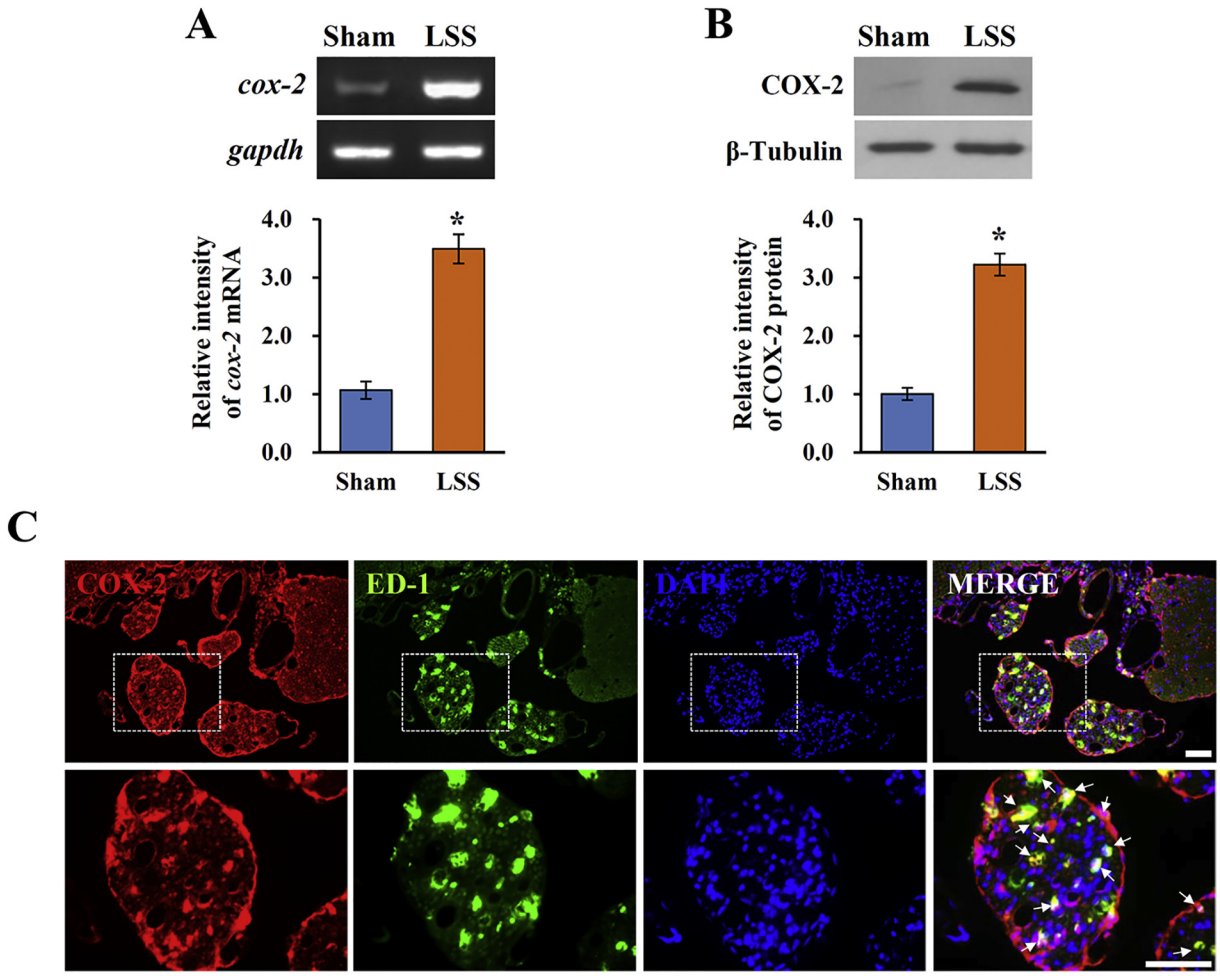

Fig. 3. The expression of COX-2 in infiltrated macrophages of injured cauda equina. At 28 d after cauda equina compression, cauda equina segment were prepared for RT-PCR, Western blot, and immunohistochemistry. (A) RT-PCR and quantification analysis for *cox-2* (n = 3/group). (B) Western blot and quantification analysis for COX-2 (n = 3/group). Data are presented as the mean ± SD. *$p < 0.05$ vs. sham. (C) Representative images showing double immunofluorescence staining for COX-2 and ED-1 at lesion epicenter of cauda equina at 28 d after compression injury. The bottom panels are high magnification images of the box area in the upper panels. Arrows indicate COX-2/ED-1-positive cells. Scale bar, 100 μm.

forward, 5'-CTC CAT GAC TCT CAG CAC AGA G-3′, reverse, 5'-GCA CCG AAG ATA TCC TCA TGA T-3; *gapdh* forward, 5′- AAC TTT GGC ATT GTG GAA GG-3′; reverse, 5′- GGA GAC AAC CTG GTC CTC AG-3′.

2.9. Measurement of prostaglandin E2 (PGE2) level

The level of PGE2 in the cauda equina fibers was assayed using PGE2 ELISA kit (Monoclonal; Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instruction as previously described [10].

2.10. Statistical analysis

Data are presented as mean ± SD or SEM. Comparison in between experimental groups was evaluated for statistical significance using either the unpaired Student's *t*-test. Multiple comparisons between groups were performed one-way ANOVA. Some behavioral scores were analyzed by repeated measures ANOVA. Dunnett's case-comparison was used as Post hoc analysis. The size of groups was expressed by the number of animals in each group. Statistical significance was accepted with $p < 0.05$. All statistical analyses were performed by using SPSS 15.0 (SPSS Science, Chicago, IL).

3. Results

3.1. Silicone-block induced cauda equina compression induced chronic mechanical allodynia

We first examined whether chronic neuropathic pain is developed after cauda equina compression. Motor and sensory testing were performed before surgery and in specific time points between 1 and 28 d after surgery. All animals were able to walk on rotarod for 289 ± 15 s before surgery. At 1 d after cauda equina compression, the latency on rotarod significantly lowered to 48.2 ± 16 s. With the progression of spontaneous recovery, the walking efficiency increased a little further to 75.2 ± 14 s at 28 d (Fig. 2A). On responses to innoxious, mechanical stimuli, mechanical PWT decreased gradually beginning 3 d after compression and significant tactile allodynia was developed through 28 d (PWT, 3.22 ± 0.6) (Fig. 2B). Sham operated animals did not show any significant change in both the walking time on rotarod and tactile withdrawal threshold on any day tested.

3.2. Compression caused macrophage infiltration in spinal cord and cauda equina

Infiltration of inflammatory cells, as well as activation of resident immune cells in response to nervous system damage, leads to

subsequent production and secretion of various inflammatory mediators. These mediators promote neuroimmune activation and can sensitize primary afferent neurons, leading to pain hypersensitivity [20,21]. Recently macrophages infiltration was observed in spinal cord tissue and cauda equina fiber at 14 d after cauda equina compression [13,14]. Therefore, we examined the spatial pattern of macrophage infiltrated in different regions of the spinal cord and cauda equina by immunostaining against ED-1 antibody at 28 d after compression. ED-1 positive macrophages were observed in compressed cauda equina (Fig. 2C, 0 mm). In addition, infiltrated macrophages were also observed in the uncompressed regions and in the dorsal funiculus of the spinal cord 30 mm away from the lesion epicenter (Fig. 2C 10, 20, 30 mm).

3.3. COX-2 is expressed in infiltrated macrophage in injured cauda equina

Macrophages play important roles in various inflammatory responses. Particularly, COX-2 is highly inducible by inflammatory stimuli; thus, it has been considered as the most appropriate target for anti-inflammatory drugs. Both COX-2 expression and the subsequent release of PGE2 after injury have been known to play a major role in peripheral and/or central sensitization, acting to alter the threshold and excitability of the nociceptor peripheral terminal [22,23]. To determine the role of COX-2 on LSS-induced chronic neuropathic pain, we first examined whether COX-2 expression is induced by cauda equina compression. As shown in Fig. 3A and B, both COX-2 mRNA and protein expression were induced in cauda equina at 28 d after injury. COX-2-positive cells were also colocalized with a macrophage marker, ED-1, in the compression lesion at 28 d after LSS injury (Fig. 3C).

3.4. The inhibition of COX-2 activity alleviated LSS-induced chronic mechanical allodynia

Data showing that COX-2 expression was increased in macrophages after injury led us to postulate that COX-2 would involve in LSS-induced chronic neuropathic pain by mediating inflammation in cauda equina. To investigate the role of COX-2 in chronic mechanical allodynia, a COX-2 specific inhibitor, celecoxib (2, 5, 10 mg/kg, i.p.), was administered into the neuropathic pain-induced rats at 28 d after injury. At 30 min and 60 min after injection, celecoxib (10 mg/kg) treatment exhibited significant increases in the mechanical pain threshold as compared to vehicle control (Fig. 4A). In addition, when a novel COX-2 inhibitor, MPO-0029 (2, 5, 10 mg/kg) [18] was treated via i.p., the threshold of the mechanical PWT was significantly increased from 30 min after injection as compared to vehicle control in a dose dependent manner (Fig. 4B). Furthermore, the analgesic effect of high dose MPO-0029 was maintained up to 3 h after injection. These results indicate that COX-2 may mediate chronic pain after cauda equina compression.

3.5. Celecoxib and MPO-0029 inhibit the expression of inflammatory mediators in cauda equina after injury

Next, we determined whether COX-2 inhibitors suppress the production of inflammatory mediators by RT-PCR and PGE2 ELISA using sample treated with drugs on 28 d. RT-PCR data shows that the expression of *tnf-α*, *il-1β*, *il-6* and *inos* mRNA was significantly decreased by celecoxib or MPO-0029 at 30 min after treatment (Fig. 4C, D). In addition, PGE2 level in the cauda equina was markedly increased by cauda equina compression as compared to sham control and the administration of COX-2 inhibitors (celecoxib and MPO-0029) significantly alleviated the production of PGE2 when compared with vehicle (Fig. 4E).

4. Discussion

Lumbar spinal stenosis (LSS) classically presents as bilateral neurogenic claudication. In fact, chronic neuropathic pain is an important problem for LSS patients, with leg pain occurring in > 90% of patients. However, the therapy strategy for LSS pain is very limited because the understanding of the pathogenesis and molecular mechanism of this disease is poor. Several studies have shown the decrease of pain threshold of hindlimb in LSS animal models [13,15,17], but there were no studies on mechanisms of neuropathic pain using chronic pain-induced rats. In the present study, we used cauda equina compression rat model described by Ma et al. [17] with some modification, and we processed all experiment using the selected animals showing chronic mechanical allodynia at 28 d after cauda equina compression. Our data showed that infiltrated macrophages were observed in injured cauda equina at 28 d after injury and COX-2 expression was induced in the infiltrated macrophages. In addition, celecoxib, well known COX-2 inhibitor, has analgesic effect on LSS-induced chronic mechanical allodynia, suggesting that COX-2 may mediate to chronic neuropathic pain in LSS model. Notably, we found that the degree and duration of the analgesic effects of MPO-0029, a novel COX-2 inhibitor, were significantly higher than those of celecoxib at same dose in mechanical allodynia (see Fig. 4). MPO-0029, one of 1-methyl-1H-pyrrole-2,5-dione derivatives (compound 9d), was identified as more potent and selective COX-2 inhibitor [PGE2 IC_{50} = 8.7 -nM, COX-2 IC_{50} = 6.0 nM; COX-2 selectivity index (SI) ≥168] than celecoxib as reported [18]. Furthermore, COX-2 inhibitors efficiently inhibited the production of inflammatory cytokines and mediators in the cauda equina.

Cyclooxygenase is an enzyme that catalyzes the conversion of arachidonic acid to prostaglandins, which are the key mediator in inflammation and pain hypersensitivity. Several studies have reported that the induction of COX-2 is associated with neuropathic pain. COX-2 inhibitors, SC-58125 and NS-398, attenuated streptozotocin-induced mechanical hyperalgesia [24]. Celecoxib inhibited chronic construction injury-induced hyperalgesia by inhibiting the expression of P2X(3) receptors in the dorsal root ganglion (DRG) [25]. Recently, Jiang et al. [26] showed that celecoxib alleviated oxaliplatin-induced neuropathic pain through inhibiting PI3K/Akt2 pathway in the mouse DRG. However, the change of COX-2 expression and the role of COX-2 in LSS-induced chronic neuropathic pain has not been examined. Herein, for the first time, our study elucidated the role of COX-2 in neuropathic pain induced after cauda equina compression. Our data showed that COX-2 is upregulated at 28 d after injury and mainly observed in infiltrated macrophages in injured cauda equina, which mediates chronic mechanical allodynia of hindlimb. Administration of COX-2 inhibitors, celecoxib and MPO-0029, significantly increased pain threshold compared with vehicle-treated rats and inhibited PGE2 production (Fig. 4). It has been known that PGE2, acting through the EP2 receptor located on dorsal horn neurons, directly depolarizes spinal neurons and thereby is sufficient to induce changes in their excitability state, which poises them to inappropriately amplify innocuous and noxious sensory stimuli [9]. Our data suggest that the analgesic effects by COX-2 inhibitors may be mediated by attenuating action of PGE2 through EP2, which contributes to the sensitization of spinal neurons after injury. Future study will be needed to elucidate the precise mechanism underlying COX-2/PGE2-mediated spinal neuron pain signaling pathway in LSS model.

Chronic pain may result from aberrant neuronal activity including peripheral and/or central sensitization of primary sensory neurons and neurons in the CNS. In addition, neuroinflammation in the PNS and CNS is also associated with persistent chronic pain development and plays an important role in chronic pain maintenance [27,28]. The neuroinflammation has been known to mediated by the activation of infiltrated leukocytes and glial cells followed increased production of inflammatory mediators, which reduce the nociceptive threshold, resulting in the peripheral and/or central sensitization [8,20]. In this study, we found that COX-2 is expressed in infiltrated macrophages

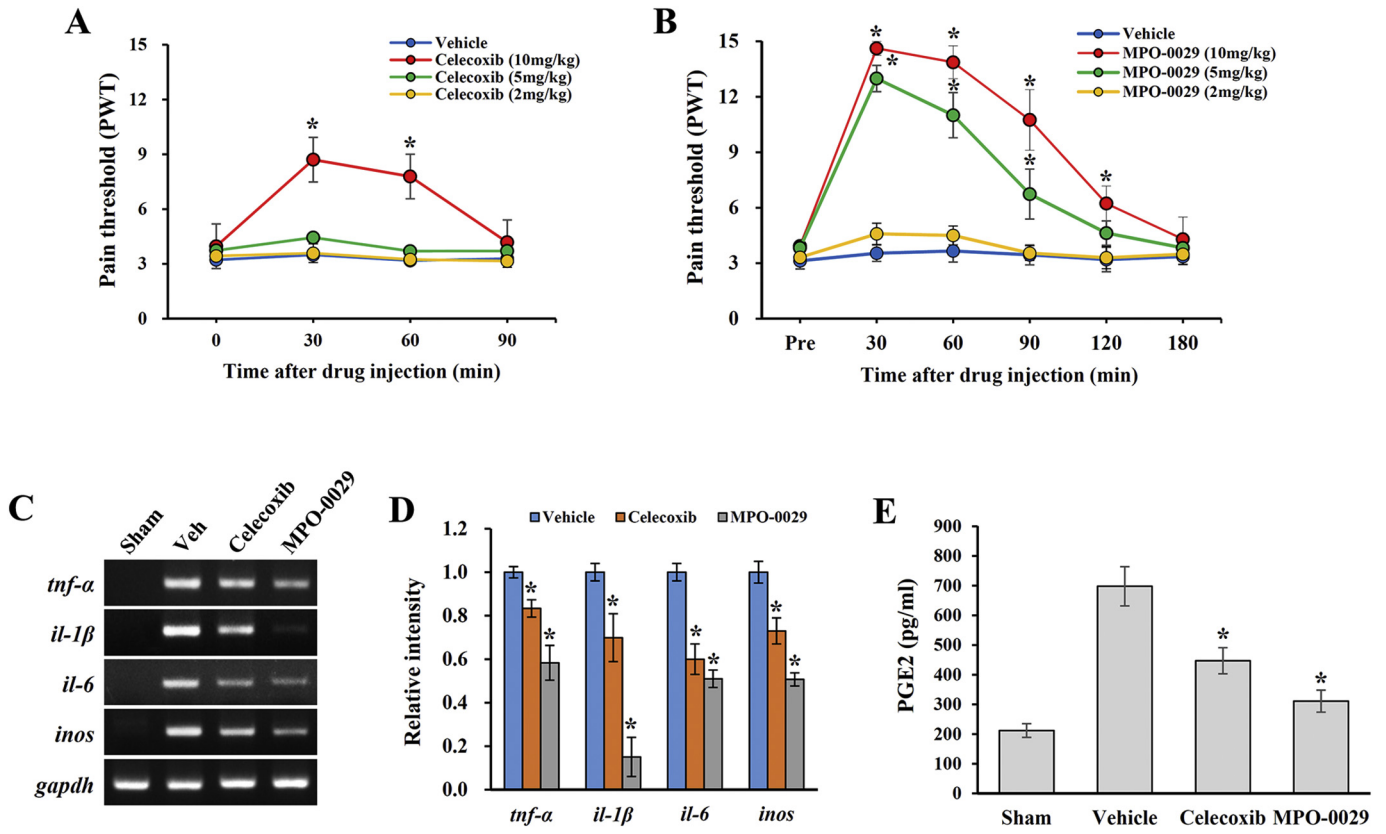

Fig. 4. COX-2 inhibitors alleviate cauda equina compression-induced mechanical allodynia and inflammation. At 28 d after compression, rats underwent a pre-test (Pre) and then injected with celecoxib (2, 5, 10 mg/kg, i.p.) or MPO-0029 (2, 5, 10 mg/kg, i.p.). (A, B) Mechanical allodynia by von Frey filaments (n = 10/group). Data are presented as the mean ± SEM. *p < 0.05 vs. vehicle. (C, D) RT-PCR and quantification analysis of tnf-α, *IL-1β, IL-6, and inos*. (n = 5/group) (E) PGE2 levels in the cauda equina at 30 min after celecoxib or MPO-0029 (10 mg/kg) treatment (n = 5/group). Data are presented as the mean ± SD. *p < 0.05 vs. vehicle.

after compression and the administration of COX-2 inhibitors significantly alleviated the mRNA expression of *tnf-α Il-1β, Il-6* and *inos* in cauda equina of chronic neuropathic pain-induced rats (Fig. 4), although the cell types expressing these inflammatory mediators were not examined here. Thus, our data suggest that the analgesic effects by celecoxib and MPO-0029 were mediated in part by inhibiting macrophage activation, thereby inhibiting inflammatory mediator production. However, we cannot rule out the possibility that COX-2 may affect glial cell activation in neuroinflammation after cauda equina compression.

Overall, our results provide that COX-2, produced in infiltrated macrophages in cauda equina, may play a pivotal role in chronic neuropathic pain after cauda equina compression and suggest that the inhibition of COX-2 can be considered as an important therapeutic strategy to alleviate chronic pain by blocking neuroinflammation in LSS patients.

Acknowledgments

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