Spinal Neural Cyclooxygenase-2 Mediates Pain Caused in a Rat Model of Lumbar Disk Herniation

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Abstract: Application of nucleus pulposus to nerve root generates radicular pain. We demonstrated that these animals showed allodynia for 2 weeks, and cyclooxygenase-2 (COX-2) immunoreactivities were up-regulated in the spinal dorsal horn. COX-2 immunoreactivities were shown in neurons; however, they were not in astrocytes. Intrathecal administration of an antibody to COX-2 decreased allodynia. Our results suggest that COX-2 in spinal cord might be a target for treatment of patients with nerve root pain caused by lumbar disk herniation.

Perspective: Neural COX-2 might mediate nerve root pain in the spinal cord caused by lumbar disk herniation in rats.

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Radicular pain is a common symptom of lumbar disk herniation, which leads to mechanical compression and inflammation. Prostaglandins (PGs) and cytokines generated at the inflammatory site produce associated pain. Nonsteroidal anti-inflammatory drugs are effective for patients suffering from lumbar disk herniation because these drugs decrease inflammation at the site of the herniation. PGs are known to be produced by cyclooxygenase (COX), which catalyzes their synthesis from arachidonic acid. Two forms of COX have been identified: COX-1, which is constitutively expressed in most tissues and organs, and COX-2, which is an inducible enzyme localized primarily in inflammatory cells and tissues. Inflammation causes the induction of COX-2, leading to the release of prostanoids, which sensitize peripheral nociceptor terminals and produce localized pain hypersensitivity. COX-2 expression is related to the pathogenesis of lumbar disk herniation through up-regulated production of PGs. Indeed, Kawakami et al reported that the introduction of a COX-2 inhibitor into inflammatory sites attenuated pain behavior after application of nucleus pulposus to nerve roots in the rat. COX-2 is present in glial cells and neurons in the spinal cord. It has also been reported that intrathecal administration of a COX-2 inhibitor attenuated pain after nerve root ligation. However, the cell types that express COX-2 and the change in amount of COX-2 induction in the spinal cord after application of nucleus pulposus have not been clarified.

In this report, we describe changes in COX-2 immunoreactivity in neural and glial cells of the spinal dorsal horn and behavioral effects of intrathecal injection of anti-COX-2 antibody in an attempt to reduce pain sensation in the rat lumbar disk herniation model.

Materials and Methods

All protocols for animal procedures in these experiments were approved by the ethics committees of our institutions following National Institute of Health Guidelines for the Care and Use of Laboratory Animals, revised 1996.

Rat Lumbar Disk Herniation Model

Fifty-seven–week–old male Sprague-Dawley rats (200 to 250 g) were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneal). Laminctomies were performed, exposing the left L5 nerve roots and associated dorsal root ganglia. The base of the rat tails was injected with 1% lidocaine (1 mL) for local anesthesia and ligated with 2-o silk surgical suture to avoid continuous bleeding during the experiment. Autologous nucleus pulposus was harvested from the amputated tails and applied to the left L5 nerve roots just proximal to the dorsal root ganglia (nucleus pulposus group, n = 25). Nucleus pulposus was harvested from between 2nd and 3rd vertebrae of each tail; the amount of nucleus pulposus was almost the same between rats. In a preliminary experiment, we checked after perfusion that most of the harvested nucleus pulposus remained where it had been placed. In the sham-operated group, nucleus pulposus was also harvested from tails, but a similar volume of muscle was harvested from the surgical area on the back and applied instead to the neural tissue (sham-operated group, n = 25).
Represent the mean ± SEM. The criterion for significance was $P < .05$. Figure 1. Mechanical allodynia measured by von Frey hair stimulation of the hind paw. Data from the paw of the nucleus pulposus group (solid circle), sham-operated animals (open circle), control group (open square) are expressed as 50% of threshold.

**Evaluation of Tactile Alldynia**

Eighteen rats were used (control group, $n = 6$; nuclear pulposus group, $n = 6$; sham-operated group, $n = 6$). There were no animal dropouts at each day. Mechanical thresholds were measured using von Frey filaments with logarithmically incremental rigidity (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.1 g; Stoelting, Wood Dale, IL) to calculate the 50% probability thresholds for mechanical paw withdrawal, as previously described.2 beginning with the 2.00-g probe, filaments were applied to the left plantar surface of the hind paw corresponding L5 dermatome for 6 to 8 seconds in a stepwise ascending or descending order following negative or positive withdrawal responses, respectively, until 6 consecutive responses were noted.26 Fifty percent withdrawal thresholds were calculated according to the method of Dixon.9 In the absence of foot withdrawal in response to the application of a 15.1-g von Frey filament, 15.1 g was then assigned as the mechanical threshold.

**COX-2 Immunohistochemistry**

Twenty-seven rats were used for evaluation in this study (control group, $n = 3$; nucleus pulposus group, $n = 12$; sham-operated group, $n = 12$). At 2, 4, 8, and 16 days after surgery, rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneal) and perfused transcardially with 500 mL of 4% paraformaldehyde in phosphate buffer (0.1 mol/L, pH 7.4). The spinal cord was resected at the level of L5 and cut into 20-μm sections on a cryostat (nucleus pulposus group, $n = 3$; sham-operated group, $n = 3$ on each day).

Sections were incubated in a blocking solution containing 0.3% Triton X-100 and 5% skim milk in 0.01mol/L phosphate-buffered saline for 90 minutes at room temperature. Sections were processed for COX-2 immunohistochemistry by using a free-floating avidin biotin complex technique by incubation with goat antibody to COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1000 in blocking solution) for 20 hours at 4°C followed by incubation with biotinylated rabbit anti-goat immunoglobulin G (Vector Labs, Burlingame, CA; 1:100 in blocking solution). COX-2-immunoreactivity was visualized with fluorescein isothiocyanate (FITC) conjugated to avidin D (Vector Labs; 1:100 in blocking solution). The sections were examined by using a fluorescent microscope (Nikon, Tokyo, Japan) with an FITC ($\lambda_{465\,nm}$ excitation and $\lambda_{505\,nm}$ emission). We counted 10 sections of spinal cord. Spinal neurons were counted at 400× magnification by using a counting grid. The number of COX-2 immunoreactive cells per 0.0225 mm² was counted in ipsilateral laminae I and II (superficial layers of dorsal horn) and lamina VII (deep layer of ventral horn) and averaged for each animal.

**Double-Labeling Immunohistochemistry Studies**

To determine cell types expressing COX-2, double-labeling methods were performed. Samples were collected from the same nucleus pulposus group described above (at day 4 after surgery, $n = 3$). After incubation with goat antibody to COX-2 for 20 hours at 4°C, sections were incubated with rabbit anti-goat Alexa 488 (FITC, 1:400; Molecular Probes Inc, Eugene, OR). After incubation with mouse antibody to NeuN (marker for neuron; 1:1000; Chemicon, Temecula, CA) or mouse antibody to glial fibrillary acidic protein (GFAP) (marker for astrocyte; 1:1000; Dako, Carpentaria, CA) for 20 hours at 4°C, the sections were incubated with rabbit anti-mouse Alexa 594 (Texas red; 1:400; Molecular Probes Inc).

Double-positive neurons were evaluated by using a G-1A filter ($\lambda_{466\,nm}$ excitation and $\lambda_{575\,nm}$ emission) and FITC filter. We examined 10 sections of spinal cord. Spinal neurons and astrocytes were observed at 400× magnification through a counting grid. COX-2 and GFAP immunoreactive cells were observed in all laminae of ipsilateral spinal cord.

**Effects of Intrathecally Administered COX-2 Antibody**

We examined the effects of intrathecally administered COX-2 antibody in the animal pain model. Chronic indwelling intrathecal catheters were placed by passing a PE-10 catheter through an incision in the atlanto-occipital membrane to a position 9 cm caudal to the cistern at the level of the lumbar enlargement. This procedure was performed 7 days before the spinal surgery. In a preliminary study we found that the amount of COX-2 reached a maximum on
day 4. Thus, 4 days after application of nucleus pulposus to nerve roots, the effects of COX-2 antibody (5 or 15 μg in saline, 200 μg per mL; Santa Cruz Biotechnology) or the same volume of saline (25 or 75 μL) on tactile allodynia were evaluated (nucleus pulposus + 5 μg COX-2 antibody group, n = 4; nucleus pulposus + 15 μg COX-2 antibody group, n = 4; nucleus pulposus + saline group, n = 4). These rats were different from rats used as described above. Mechanical thresholds were measured by using von Frey filaments as described above: before and at 20, 40, and 60 minutes after administration of COX-2 antibody or saline.

Figure 2. The number of COX-2 immunoreactive cells in spinal dorsal horn was less in nonoperated animals (A) than that in sham-operated animals (B) and in the nucleus pulposus group (C) on day 4. The number of COX immunoreactive cells is higher in nucleus pulposus group when compared with sham-operated animals on day 4 (D, nonoperated animals; E, sham-operated; and F, nucleus pulposus group).

Figure 3. Time course of change in COX-2 expression in the spinal cord. The open circles indicate the number of COX-2 immunoreactive cells in the spinal dorsal horn (A) and ventral horn (B) of the sham-operated group. The solid circles correspond to the nucleus pulposus group (A, dorsal horn; B, ventral horn). Results are expressed as mean ± SEM. Significant differences at each point were evaluated by using ANOVA where the criterion for significance was P < .05. Significant differences between group values at the same time point.
Statistical Analysis

The paw withdrawal latencies of hind paws in each group before surgery (day 0) and on days 2, 4, 6, 8, 10, 12, 14, and 16 were compared by one-way analysis of variance (ANOVA) for repeated measurements. For multiple comparisons, we used Dunnett’s test. Comparison of the number of COX-2 immunoreactive neurons between groups was made by one-way ANOVA with Dunnett’s test.

Results

Mechanical Allodynia Caused by Disk Herniation

In the nucleus pulposus group significant mechanical allodynia was observed, compared with the control and sham-operated groups ($P < .05$) (Fig 1). This mechanical allodynia continued through day 12. However, in the sham-operated group significant mechanical allodynia was not observed, compared with the control group ($P < .05$).

COX-2 Expression in Spinal Cord

COX-2 protein was expressed in the spinal cord in control, sham-operated, and nucleus pulposus groups (Fig 2). COX-2 immunoreactive cells were observed in the spinal dorsal horn and ventral horn. The average numbers of COX-2 immunoreactive cells in control group were $10 \pm 2$ (mean $\pm$ standard error of mean [SEM]) in superficial layers and $5 \pm 1$ (mean $\pm$ SEM) in deep layers. The intensity and number of COX-2 immunoreactive cells in both superficial laminae and deep laminae in the nucleus pulposus group from day 2 to day 16 and those in the sham-operated group on day 2 were higher than those of control groups ($P < .05$). The number of COX-2 immunoreactive cells in both the superficial and deep laminae in the nucleus pulposus group was significantly higher on days 2, 4, and 8 than in the sham-operated group ($P < .05$) (Fig 3).

Figure 4. These photomicrographs show COX-2 expression, GFAP immunoreactive astrocytes, and NeuN immunoreactive neurons in the nucleus pulposus group on day 4. GFAP immunoreactivity (B) was seen around COX-2 immunoreactive cells (A), but GFAP immunoreactivity was not colabeled with COX-2 expression (A and B are the same sections). On the other hand, most COX-2 immunoreactive cells (C) were colocalized with NeuN immunoreactive neurons (D). COX-2 expression can be located in the nucleus of these neurons (C and D are the same sections).
**COX-2 and NeuN or GFAP Immunoreactive Cells**

GFAP immunoreactive astrocytes were observed in superficial and deep laminae in control, sham-operated, and nucleus pulposus groups. The distributional pattern of GFAP immunoreactive astrocytes was not different between superficial and deep laminae of the spinal cord. Most COX-2 immunoreactive cells were colabeled with NeuN. However, COX-2 immunoreactivity was not colocalized with GFAP immunoreactive astrocytes in either group. Fig 4 shows double-labeled cells in the deep laminae of the spinal cord in the nucleus pulposus groups at day 4. COX-2 was expressed in the nucleus of NeuN immunoreactive neurons.

**Effect of Administration of Anti-COX-2 Antibody**

Intrathecal administration of anti-COX-2 antibody on day 4 reduced the level of mechanical allodynia in a dose-dependent manner (5 and 15 μg) in the nucleus pulposus group (P < .05) (Fig 5). A significant reduction in pain behavior was observed 20, 40, and 60 minutes after administration of COX-2 antibody (P < .05) (Fig 5).

**Discussion**

In this study we demonstrated that lumbar disk herniation to spinal nerve root produces hyperalgesia in rats, leading to the expression of COX-2 in the spinal dorsal and ventral horns. COX-2 expression was seen in neurons, but not in astrocytes. Hyperalgesia was decreased by intrathecal administration of anti-COX-2 antibody.

Previous studies of animal models involving lumbar disk herniation reported thermal hyperalgesia or mechanical allodynia. Application of nucleus pulposus onto nerve roots has been shown to induce edema of nerve roots and dorsal root ganglia and alter conduction velocity.

Inflammatory mediators such as phospholipase A2 and PGs are abundantly produced and released by inflammatory cells. It has been suggested that PGs in nerve roots produce sciatic pain in the herniation of the lumbar disk model. Furthermore, this pain was reported to be abolished by phospholipase A2 or COX-2 inhibitor.

Injection of carrageenan in the knee joint induces inflammation involving up-regulation and release of PGs in the spinal dorsal horn accompanied by glutamate and aspartate increases related to pain transmission. These increases are accompanied by elevation of COX-2, glutamate, and aspartate in the spinal cord, resulting in hyperalgesia and mechanical allodynia.

Proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor (TNF), are known mediators of the peripheral inflammatory response. These compounds are also synthesized and released in several kinds of nerve injury. Recently, TNF has been strongly linked to the nucleus pulposus effects on nerve roots. Indeed, TNF is abundantly expressed in the herniated nucleus pulposus model. Inhibition of TNF prevents nucleus pulposus induced thrombus formation, intraneural edema, and reduction of nerve conduction velocity. These results support the possibility that TNF is involved in mechanisms of inflammatory and neuropathic pain after application of nucleus pulposus onto nerve roots.

TNF produced at spinal nerve sites is transported along axons to the spinal dorsal horn, where it is correlated with expression of TNF receptors, and might activate central cytokines in the pathogenesis of painful states. Expression of IL-6 and IL-1β increased in the nerve root and dorsal root ganglia after exposure to nucleus pulposus. Direct mechanical and/or chemical injury to lumbar roots gives rise to pain behavior. In this lumbar root injury model enhanced IL-1β expression was also observed in the entry zone of spinal dorsal horn. Intraspinal injection of IL-1 increases COX-2 mRNA, resulting in increased release of PGs, TNF and IL-6 stimulate spinal neurons and glial cells, which produce PGs through the COX-2 pathway. TNF can induce proliferation of glial cells, which, in turn, express more TNF, IL-6, and PGs, suggesting positive feedback via these receptors and the COX-2 pathway in glial cells. However, in the current model, COX-2 was not induced in glial cells.

In the current study, application of muscle to nerve root (sham-operated group) did not produce hyperalgesia; however, it did up-regulate COX-2 induction at day 2 compared with the control group. Surgery close to the nerve root might produce COX-2 induction in the spinal cord.

**Figure 5.** Effects of intrathecal administration of 5 or 15 μg of COX-2 antibody on the 50% threshold for paw withdrawal in the nucleus pulposus model. Intrathecally administered COX-2 antibody increased the threshold by 50%. Solid triangles represent the nucleus pulposus + COX-2 antibody (5 μg) group, solid circles represent the nucleus pulposus + COX-2 antibody (15 μg) group, and solid squares represent the nucleus pulposus + saline group. Ordinate: 50% threshold for paw withdrawal from von Frey hair stimulus (g). Abscissa: time in minutes after antibody administration. Each line represents the mean ± SEM. The criterion for significance was P < .05. Significant differences between group values at the same time point.
the other hand, muscle itself contains calcitonin gene-related peptide, which is one of the neurotransmitters associated with pain.23 Cytokine levels, such as those of IL-6, increase in close association with muscle damage.24 Neuropeptides and cytokines in muscles might also induce COX-2 in the spinal cord.

We believe that the level of PGs, proinflammatory cytokines, and some neurotransmitters increased in the inflammatory site. Proinflammatory cytokines and neurotransmitters were most likely transported into the spinal dorsal horn and activated neurons and glial cells. Of these cells, spinal neurons produced more proinflammatory cytokines and PGs through cytokine receptors and the COX-2 pathway.

In humans, COX-2 inhibitors are effective in reducing patient suffering from lumbar disk herniation. The mechanism of pain relief is through partial blockade of PG production in the spinal nerve root and perhaps also by blocking production in the dorsal root ganglia and spinal dorsal horn.

References

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