Role for Cyclooxygenase 2 in the Development and Maintenance of Neuropathic Pain and Spinal Glial Activation

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Background: Lines of evidence have indicated that cyclooxygenase 2 plays a role in the pathophysiology of neuropathic pain. However, the site and mechanism of its action are still unclear. Spinal glia has also been reported to mediate pathologic pain states. The authors evaluated the effect of continuous intrathecal or systemic cyclooxygenase-2 inhibitor on the development and maintenance of neuropathic pain and glial activation in a spinal nerve ligation model of rats.

Methods: Continuous intrathecal infusion of meloxicam (32 or 320 μg·kg⁻¹·day⁻¹) or saline was started immediately after L5–L6 spinal nerve ligation. Mechanical allodynia and thermal hyperalgesia were evaluated on days 4 and 7 postoperatively. Spinal astrocytic activation was evaluated with glial fibrillary acidic protein immunoreactivity on day 7. In other groups of rats, continuous intrathecal meloxicam was started 7 days after spinal nerve ligation, and effects on established neuropathic pain and glial activation were evaluated. Last, effects of continuous systemic meloxicam (16 mg·kg⁻¹·day⁻¹) on existing neuropathic pain and glial activation were examined.

Results: Intrathecal meloxicam prevented the development of mechanical allodynia and thermal hyperalgesia induced by spinal nerve ligation. It also inhibited spinal glial activation responses. In contrast, when started 7 days after the nerve ligation, intrathecal meloxicam did not reverse established neuropathic pain and glial activation. Systemic meloxicam started 7 days after ligation partially reversed neuropathic behaviors but not glial activation.

Conclusions: Spinal cyclooxygenase 2 mediates the development but not the maintenance of neuropathic pain and glial activation in rats. Peripheral cyclooxygenase 2 plays a part in the maintenance of neuropathic pain.

THE role of spinal cyclooxygenase (COX)-2 in the pathophysiology of neuropathic pain is still controversial. Previous studies have indicated that spinal COX-2 messenger RNA or protein production is enhanced by nerve injury as well as peripheral inflammation. Enhanced COX-2 production may lead to release of spinal prostaglandins, which can produce increased neuronal excitability in the spinal cord (central sensitization). Intrathecal COX-2 inhibitor has been shown to prevent inflammatory or nerve injury-induced hyperalgesia or allodynia, indicating that spinal COX-2 mediates the development of neuropathic pain states. However, other studies have shown that COX-2 expression is not enhanced remarkably by nerve injury. Moreover, intrathecal COX-2 inhibitor was ineffective, or rather, intrathecal COX-1 inhibitor was effective, in preventing neuropathic hypersensitive states.

There has been strong line of evidence showing that spinal glia mediates neuropathic pain. Spinal glial activation is observed in various types of nerve injury, and pharmacologic modulation of glial activation can affect the development of neuropathic pain. We previously showed that continuous intrathecal methylprednisolone inhibited spinal glial activation and the development and maintenance of neuropathic pain in a rat spinal nerve ligation model. Intrathecal glucocorticoid can possibly inhibit the spinal production of prostaglandins and inflammatory cytokines, which may lead to blockade of glial activation and neuropathic pain states. Moreover, a continuous mode of administration may have a greater advantage as compared with an intermittent one because interruption of drug effect can be avoided.

To explore the involvement of COX-2 in the development and maintenance of neuropathic pain, we examined the effect of continuous intrathecal COX-2 inhibitor on pain behaviors and spinal glial activation in a rat spinal nerve ligation model. Furthermore, because intrathecal COX-2 inhibitor was ineffective on established allodynia and hyperalgesia, we tested the effect of systemic COX-2 inhibitor to explore the role of peripheral COX-2 in the maintenance of neuropathic pain.

Materials and Methods

Animals

All experiments were performed using male Sprague-Dawley rats, each weighing 150 g on the day of surgery. Rats were housed individually in plastic cages with soft bedding at room temperature and maintained on a 12-h light–12-h dark cycle with free access to food and water. The following studies were performed under a protocol approved by the Institutional Animal Care Committee of the University of Tokyo (Tokyo, Japan).

Surgical Procedure

All of the surgical procedures were performed under inhalational anesthesia with isoflurane in 100% oxygen, induced at 5% and maintained at 2%. Animals showing...
neurologic deficits were excluded from the following experiments.

**Spinal Nerve Ligation.** Neuropathic pain was induced following the methods of Kim and Chung. L5 and L6 nerves were exposed and tightly ligated with 6-0 silk threads as we previously reported.

**Intrathecal Catheterization.** A chronic intrathecal catheter was introduced under isoflurane anesthesia. As we previously described, a polyethylene PE-10 catheter was inserted intrathecally at the L4–L5 interspace for 1.5 cm in the cervical direction. The catheter was sutured to the overlying fascia for fixation.

**Implantation of an Infusion Osmotic Pump.** Infusion osmotic pumps with a flow moderator (ALZET, Cupertino, CA) were used for continuous intrathecal (1 μl/h) or systemic (10 μl/h) drug administration.

**Drugs**
Meloxicam sodium (4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxyamide1,1-dioxide sodium; Sigma-Aldrich, St. Louis, MO) was dissolved in normal saline. The doses of meloxicam were selected according to our pilot study.

**Behavioral Assessment**
All of the behavioral tests were performed between 10 AM and 3 PM by an examiner blinded as to the treatment groups. Mechanical and thermal thresholds were determined as we previously reported.

**Mechanical Threshold.** To quantify mechanical sensitivity of the foot, the threshold of foot withdrawal in response to normally innocuous mechanical stimuli was determined by using the von Frey filaments and the up-down method.

**Thermal Threshold.** The latency of foot withdrawal to noxious heat stimuli was measured using the paw withdrawal apparatus. Light intensity was preset to obtain a baseline latency of approximately 10 s. Ten withdrawal latencies were collected with at least 5-min intervals, and the middle 6 of the 10 latencies were averaged.

**Immunohistochemistry**
Immunostaining was performed on the free-floating sections of the spinal cord as previously reported. Briefly, animals were deeply anesthetized with sodium pentobarbital (100 mg/kg) and were perfused with heparinized normal saline, followed by ice-cold 4% paraformaldehyde. The spinal cord around L5 and L6 was removed and postfixed. Tissues were then stored in 30% sucrose solution for cryoprotection. A thin slice was placed on the ventral horn contralateral to the spinal nerve ligation. Every fifth section (40 μm thick) of the spinal cord was first incubated in 0.3% hydrogen peroxide for endogenous peroxidase blocking. The sections were incubated for 60 min in blocking solution (0.1 M phosphate-buffered saline containing 0.3% Triton X-100 and 5.0% normal rabbit serum) and then in goat primary antibody to glial fibrillary acidic protein (GFAP, 1:2,000; Santa-Cruz Biotechnology, Santa Cruz, CA) overnight. The sections were incubated in the biotinylated rabbit antibody to goat immunoglobulin G (1:5,000; Vector Laboratories, Burlingame, CA) and then in avidin-biotin-peroxidase complex (Vectorstain ABC-Elite kit; Vector Laboratories). Visualization of the reaction product was achieved by incubation for 4 min with diaminobenzidine and nickel-ammonium sulfate to which hydrogen peroxide was added (DAB kit; Vector Laboratories). The sections were placed on a slide glass and dried overnight. Care was taken to process samples from different groups simultaneously in order to minimize the effect of fluctuation in staining.

**Astrocytic Activation Responses**
Assessments of astrocytic responses were performed in three sections chosen at random from each animal as described previously.

**Number of Positive Cells.** Astrocytes with positive GFAP immunoreactivity in the left dorsal horn of the spinal cord were counted under ×200 magnification and totaled for the three sections.

**Number of Pixels.** The area of GFAP immunostaining was measured in the dorsal horn of the spinal cord with a computer-assisted image analysis system (NIH Image; U.S. National Institutes of Health, Bethesda, MD).

**Morphologic Classification.** The sections were surveyed under ×400 magnification and scored according to classification by Colburn et al. Criteria for each class were as follows: baseline staining (−): astrocytes exhibit extensive fine projections, cells were well spaced and neatly arranged; mild response (+): astrocytes still exhibit numerous long but thickening projections, less area between individual astrocytes, GFAP immunoreactivity more apparent; moderate response (++): astrocytes were less ramified/exhibit bold projections, increased density of astrocytic cells now overlapping, prominent GFAP immunoreactivity; intense response (+++): astrocytes becoming rounded with few projections, densely arranged/overlapping, intense GFAP immunoreactivity.

**Protocols**
**Experiment 1: Intrathecal Meloxicam and Development of Neuropathic Pain.** We first examined the effect of continuous intrathecal administration of meloxicam on the development of neuropathic pain and spinal astrocytic activation responses. Rats were anesthetized with isoflurane, and the left L5 and L6 spinal nerves were tightly ligated. Then, a catheter was inserted intrathecally through the L4–L5 interspace, and meloxicam (52 or 320 μg · kg⁻¹ · day⁻¹) or saline was delivered
continuously with an osmotic pump (n = 6 for each group). At 3, 5, and 7 days postoperatively, mechanical allodynia and thermal hyperalgesia were assessed with tactile sensitivity to von Frey hairs and paw withdrawal latencies to heat stimulus, respectively. After the behavioral tests on day 7, rats were perfused with 4% paraformaldehyde, and the lumbar spinal cord was removed for immunohistochemical processing with GFAP antibody.

**Experiment 2: Intrathecal Meloxicam and Existing Neuropathic Pain.** The effect of continuous intrathecal administration of meloxicam on existing neuropathic pain and spinal astrocytic activation responses were examined. Rats were anesthetized, and left L5 and L6 spinal nerves were tightly ligated. Seven days after spinal nerve ligation, development of neuropathic pain was confirmed with behavioral tests, and a second surgical procedure was performed to place an intrathecal catheter. Then, meloxicam (320 or 32 μg·kg⁻¹·day⁻¹) or saline was delivered continuously with an osmotic pump (n = 6 for each group). Mechanical allodynia and thermal hyperalgesia were assessed on days 4, 7, 11, and 14 after the spinal nerve ligation. After the behavioral tests on day 14, rats were perfused with fixative, and the lumbar spinal cord was removed for immunohistochemical processing with GFAP antibody.

**Experiment 3: Systemic Meloxicam and Existing Neuropathic Pain.** The effect of continuous systemic administration of meloxicam on existing neuropathic pain and spinal astrocytic activation responses were examined. As described previously, rats were anesthetized, and left L5 and L6 spinal nerves were tightly ligated. Seven days after spinal nerve ligation, development of neuropathic pain was confirmed with behavioral tests, and an osmotic infusion pump was implanted subcutaneously. Then, systemic meloxicam (16 mg·kg⁻¹·day⁻¹) or saline was delivered continuously with an osmotic infusion pump (n = 6 for each group). Mechanical allodynia and thermal hyperalgesia were assessed on days 4, 7, 11, and 14 after the spinal nerve ligation. After the behavioral tests on day 14, rats were perfused with fixative, and the lumbar spinal cord was removed for immunohistochemical processing with GFAP antibody.

**Statistical Analysis**

Data were expressed as mean ± SD. All the behavioral data were analyzed by one-way analysis of variance at each time point followed by Bonferroni multiple comparison test. Temporal change in each group was analyzed with repeated-measures analysis of variance and Dunnett test. Image analysis data were compared with one-way analysis of variance and the Bonferroni test. Data of morphologic classification were analyzed with the Mann–Whitney test. P values less than 0.05 were considered significant in each test.

![Fig. 1](image-url)  
**Fig. 1.** (A) Changes in the mechanical sensitivity to von Frey filaments after spinal nerve ligation and the effect of continuous intrathecal meloxicam. Meloxicam (32 and 320 μg·kg⁻¹·day⁻¹) started immediately after nerve ligation inhibited the development of mechanical allodynia. (B) Changes in the paw withdrawal latency to heat stimuli after spinal nerve ligation and effect of continuous intrathecal meloxicam. Meloxicam (32 and 320 μg·kg⁻¹·day⁻¹) inhibited the development of thermal hyperalgesia. *Bar* above the x-axis represents continuous intrathecal treatment with meloxicam or saline. *P < 0.0001 versus saline.

**Results**

All of the rats maintained good health and continued to gain weight throughout the experimental period. No infection or motor dysfunction was observed in any of the animals. There was no significant difference in weight between the groups. No abnormalities were observed on visual inspection of the spinal cords.

**Experiment 1: Intrathecal Meloxicam and Development of Neuropathic Pain**

Figure 1A shows the temporal changes in the mechanical sensitivity to von Frey filaments after spinal nerve ligation and the effect of continuous intrathecal meloxicam started immediately after ligation. A significant decrease in the tactile thresholds was observed on days 3–7 as compared with day 0 in the saline group, indicating the development of mechanical allodynia. Meloxicam started immediately after nerve ligation inhibited the decrease in the tactile thresholds as compared with the saline group on days 3–7, indicating that intrathecal meloxicam prevented the development of mechanical allodynia. Figure 1B shows the changes in paw withdrawal latencies to heat stimuli. A significant decrease in the latencies was observed on days 3–7 as compared with day 0 in the saline group, indicating the develop-
ment of thermal hyperalgesia. Meloxicam (32 and 320 μg · kg⁻¹ · day⁻¹) significantly inhibited the decrease in the tactile thresholds as compared with the saline group on days 3–7, indicating that intrathecal meloxicam prevented the development of thermal hyperalgesia. Figure 2 demonstrates the GFAP immunoreactivity in the spinal dorsal horn after 7-day treatment with continuous intrathecal meloxicam or saline started immediately after spinal nerve ligation. (A) Lower magnification image showing the area of the dorsal horn analyzed. (B) Astrocytic activation was not observed in normal rat. (C) Prominent astrocytic activation was observed in rats nerve-ligated and treated with saline. Astrocytic activation was remarkably suppressed by treatment with intrathecal meloxicam (320 μg · kg⁻¹ · day⁻¹ [D] and 32 μg · kg⁻¹ · day⁻¹ [E]). Bar = 100 μm.

Table 1. Effect of Intrathecal Meloxicam Started Immediately after Spinal Nerve Ligation on Spinal Astrocytic Activation

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<th>No. of Positive Cells</th>
<th>No. of Pixels</th>
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</tr>
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<td>Saline</td>
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<td>46,683 ± 1,970</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+++ 7</td>
</tr>
<tr>
<td>Meloxicam (32 μg · kg⁻¹ · day⁻¹)</td>
<td>16 ± 5*</td>
<td>5,174 ± 383*</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ 11</td>
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<tr>
<td></td>
<td></td>
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<td>+++ 0</td>
</tr>
<tr>
<td>Meloxicam (320 μg · kg⁻¹ · day⁻¹)</td>
<td>13 ± 4*</td>
<td>4,028 ± 303*</td>
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<td></td>
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<td>+ 8</td>
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<td>+++ 0</td>
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</table>

Data are presented as mean ± SD. See text for details of morphologic classification.

* P < 0.0001 vs. saline.
− = baseline staining; + = mild response; ++ = moderate response; +++ = intense response.
Effect of Intrathecal Meloxicam and Established Neuropathic Pain

Figure 3A illustrates the temporal changes in the mechanical sensitivity after spinal nerve ligation and the effect of continuous intrathecal meloxicam started 7 days after ligation. A significant decrease in the tactile thresholds was observed on days 4–14 as compared with day 0 in the saline group, indicating the development and maintenance of mechanical allodynia. Intrathecal meloxicam (32 and 320 μg · kg⁻¹ · day⁻¹) did not significantly alter the thresholds as compared with saline, indicating that intrathecal meloxicam was ineffective on established thermal hyperalgesia. Figure 3B shows the changes in the paw withdrawal latencies to heat stimuli. A significant decrease in the latencies was observed on days 4–14 as compared with day 0 in the saline group, indicating the development and maintenance of thermal hyperalgesia. Intrathecal meloxicam (32 and 320 μg · kg⁻¹ · day⁻¹) did not significantly alter the latencies as compared with the saline group, indicating that intrathecal meloxicam was ineffective on established thermal hyperalgesia. Figure 4 demonstrates the GFAP immunoreactivity in the spinal dorsal horn. Astrocytic activation were not significantly altered by treatment with continuous intrathecal meloxicam (32 and 320 μg · kg⁻¹ · day⁻¹) as compared with saline (table 2).

Table 2. Effect of Intrathecal Meloxicam on Spinal Astrocytic Activation in Rats with Established Neuropathic Pain

<table>
<thead>
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<th>Group</th>
<th>No. of Positive Cells</th>
<th>No. of Pixels</th>
<th>Morphologic Classification</th>
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<td>95 ± 7</td>
<td>39,652 ± 511</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>+++ 4</td>
</tr>
<tr>
<td>Meloxicam (32 μg · kg⁻¹ · day⁻¹)</td>
<td>98 ± 6</td>
<td>40,147 ± 903</td>
<td>– 0</td>
</tr>
<tr>
<td></td>
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<td>+ 3</td>
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<td></td>
<td></td>
<td></td>
<td>++ 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+++ 4</td>
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<tr>
<td>Meloxicam (320 μg · kg⁻¹ · day⁻¹)</td>
<td>93 ± 9</td>
<td>39,486 ± 1,048</td>
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<td></td>
<td></td>
<td></td>
<td>+ 2</td>
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<td></td>
<td></td>
<td></td>
<td>++ 13</td>
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<td></td>
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<td>+++ 3</td>
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Data are presented as mean ± SD. See text for details of morphologic classification.

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Astrocytic activation was observed in both the saline (saline started 7 days after spinal nerve ligation. Prominent after 7-day treatment with continuous systemic meloxicam or saline (fig. 6A) and the meloxicam (fig. 6B) groups. GFAP immunoreactivity in the spinal dorsal horn. Astrocytic activation responses were prominent in both the existing thermal hyperalgesia. Figure 6 demonstrates the systemic meloxicam partially reversed existing allodynia and hyperalgesia, indicating that peripheral COX-2 may play a role in the maintenance of neuropathic pain.

Several lines of evidence have suggested that spinal COX, especially COX-2, is involved in the development or maintenance of pathologic pain states or both. Peripheral inflammation in rats induces spinal release of prostaglandin E2 and up-regulation of spinal prostaglandin COX-2 but not COX-1. Acute peripheral inflammation induces cytokine (interleukin-1β) up-regulation, which may mediate COX-2 induction and inflammatory pain hypersensitivity. Regarding nerve injury, spinal COX-2 protein concentration is increased 1 day after spinal nerve ligation. Prostaglandin release induced by hind paw brushing was enhanced in spinal nerve-ligated rats, indicating increased synthesis of prostaglandin E2.

Spinal administration of prostaglandin E2 induces hyperalgesia and allodynia possibly through EP1 and EP2 receptors. Furthermore, spinal administration of COX-2 inhibitor affects the development or maintenance of pathologic pain states or both. Preemptive effect of intrathecal COX inhibitor on neuropathic pain was reported. Intrathecal indomethacin immediately or 1 day after spinal nerve ligation prevented the development of allodynia. Neuropathic pain did not develop in prostaglandin synthase-deficient mice. Intrathecal COX-2 inhibitor reversed allodynia caused by spinal nerve ligation and attenuated hyperalgesia induced by paw carrageenan injection.

On the other hand, several reports have suggested the involvement of spinal COX-1 but not COX-2 in neuropathic pain. Spinal COX-2 expression was only slightly enhanced by spinal nerve ligation, and systemic COX-2 compared with the saline group on days 11 and 14, indicating that systemic meloxicam partially reversed existing thermal hyperalgesia. Figure 6 demonstrates the GFAP immunoreactivity in the spinal dorsal horn. Astrocytic activation responses were prominent in both the saline (fig. 6A) and the meloxicam (fig. 6B) groups. Indices of spinal astrocytic activation were not significantly altered by systemic meloxicam as compared with saline (table 3).

**Discussion**

Continuous intrathecal meloxicam, a selective COX-2 inhibitor, started immediately after spinal nerve ligation, prevented the development of mechanical allodynia and thermal hyperalgesia. Spinal prostaglandin production by COX-2 may play a pivotal role in the development of neuropathic pain states. On the other hand, intrathecal meloxicam did not reverse established mechanical allodynia and thermal hyperalgesia. Spinal COX-2 is not involved in the maintenance of neuropathic pain. Systemic meloxicam partially reversed existing allodynia and hyperalgesia, indicating that peripheral COX-2 may play a role in the maintenance of neuropathic pain.

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**Table 3. Effect of Systemic Meloxicam on Spinal Astrocytic Activation in Rats with Established Neuropathic Pain**

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<th>Morphologic Classification</th>
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<tr>
<td>Saline</td>
<td>132 ± 15</td>
<td>37,203 ± 1,292</td>
<td>-</td>
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<tr>
<td>Meloxicam (16 mg . kg⁻¹ . day⁻¹)</td>
<td>125 ± 20</td>
<td>32,041 ± 2,085</td>
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Data are presented as mean ± SD. See text for details of morphologic classification.

- = baseline staining; + = mild response; ++ = moderate response; +++ = intense response.
inhibitor did not modify the development of allodynia and hyperalgesia. Spinal COX-1 expression was up-regulated by spinal nerve ligation. Prostaglandin E2 mediated by COX-1 excited dorsal root ganglion and dorsal horn neurons of neuropathic rats. Intra-thecal COX-1 but not COX-2 inhibitor started immediately after spinal nerve ligation prevented the development of alldynia. These contradictory results may come from differences of experimental settings, mode and dosage of drug administration, and selectivity of COX inhibitors. Regarding the mode of drug administration, we speculate that continuous administration is more advantageous compared with an intermittent one because interruption of drug effects can be avoided. Heffner et al. reported that intra-thecal COX-2 inhibitor started 2 days after nerve ligation reversed alldynia. Although these findings seem to contradict our results showing that established neuropathic pain was not reversed by intra-thecal meloxicam, timings of intra-thecal administration are rather different (2 vs. 7 days after nerve ligation). We speculate that processes leading from COX-2-mediated spinal inflammation to neuropathic pain states may be completed during these days, and established neuropathic pain states cannot be reversed by spinal COX-2 inhibitor.

There is growing evidence indicating that activation of spinal glia may contribute to pathologic pain states. Various types of nerve lesions induce spinal glial activation. Prostaglandin E2 mediates glutamatergic cross-talk between neurons and astrocytes, which may play critical roles in synaptic plasticity. Intrathecal pentoxifylline, a glial modulating agent, inhibited glial activation in nerve-transected rats and prevented and reversed existing tactile alldynia. Local injection of ketorolac, a nonselective COX inhibitor, effectively reversed existing allodynia and hyperalgesia. This is consistent with the idea that in established neuropathic pain, COX-2 acts in the periphery but not in the spinal cord. Because of the high COX-2/COX-1 selectivity of meloxicam, it seems rational to consider that the effects of meloxicam in our study were caused by COX-2 blockade. However, we cannot completely exclude the possibility that COX-1-blocking effect of meloxicam influenced our results. Although we selected meloxicam because it is widely used in clinical practice, use of a more selective COX-2 inhibitor could have been more advantageous.

Although there was no sham-operated control in this experiment, we showed in our previous study that sham operation (surgery without nerve ligation) did not induce glial activation or hypersensitivity. Therefore, we consider that glial activation and hypersensitivity shown in our experimental settings were caused by nerve injury rather than by inflammation associated with surgery.

In summary, in a rat spinal nerve ligation model, continuous intra-thecal COX-2 inhibitor prevented the development of neuropathic pain and spinal glial activation, whereas it was ineffective on existing neuropathic pain and glial activation. Systemic COX-2 inhibitor partially reversed existing alldynia and hyperalgesia. Spinal COX-2 mediates the development and peripheral COX-2 mediates the maintenance of neuropathic pain.
References


Dissociation of microglial activation and neuropathic pain behaviors following peripheral nerve injury in the rat. J Neuroimmunol 1997; 99:165–75

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