

Upregulation of Spinal Cyclooxygenase-2 in Rats after Surgical Incision

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Background: Although upregulation of cyclooxygenase (COX)-2 in spinal cord after peripheral inflammation has been well documented, the effect of surgery on spinal COX-2 has not been examined in detail. The present study uses a bilateral foot incision in rats to examine the magnitude and duration of surgically induced changes in spinal COX-2 protein.

Methods: A longitudinal incision was made in both plantar hind paws of isoflurane-anesthetized rats. Spinal cords were removed at various postoperative times (1–48 h), and spinal COX-2 protein levels were compared with the results of Western blot analysis. Ropivacaine-induced blockade of sciatic nerve function was used to determine the importance of afferent nerve activity on spinal COX-2 after incision. Dexamethasone and the COX-2-selective inhibitor L-745,337 were administered intrathecally to modulate spinal COX-2 after incision.

Results: COX-2 protein levels increased in the lumbar spinal cord at 3 (1.32-fold) and 6 (1.26-fold) h after bilateral foot incision. At later times, lumbar COX-2 levels were no different than in control animals not undergoing surgery. Cervical COX-2 protein levels remained unchanged. Sciatic nerve blockade with ropivacaine did not prevent the increase in lumbar spinal COX-2 protein levels after incision. Intrathecal dexamethasone decreased lumbar spinal COX-2 levels after incision, and an intrathecal COX-2-selective inhibitor did not reduce the COX-2 upregulation.

Conclusions: After bilateral foot incision in rats, lumbar spinal COX-2 protein levels increase, although the magnitude and duration are less than reported in models of peripheral inflammation. This COX-2 upregulation does not seem to be mediated by afferent nerve activity.

FOLLOWING peripheral inflammation, there is a cascade of events leading to hypersensitivity of sensory nerve endings.¹ There is also a similar upregulation of cytokines, prostaglandins, and cyclooxygenase (COX)-2 in the spinal cord.^{2–5} Injection of inflammatory agents such as complete Freund's adjuvant (CFA) in the hind paw of the rat causes an increase in lumbar spinal COX-2 mRNA but not COX-1.^{2,3} Correspondingly, peripheral inflammation induces an increase in lumbar COX-2 protein, with COX-1 protein levels remaining unchanged.^{4,5} This selective upregulation of spinal COX-2 has also been reported after peripheral nerve injury.⁶

The causative factors producing spinal cord changes after prolonged noxious exposure of peripheral nerve terminals remain incompletely defined.^{5,7,8} Peripheral

inflammation stimulates increases in blood cytokines, some of which can cross the blood-brain barrier and diffuse into the spinal cord to stimulate a cascade of COX-2 and prostaglandin production. However, it is also possible that increased neural activity arising from sensitized peripheral nerve terminals may be a causative factor in spinal COX-2 upregulation.⁵

The increase in spinal COX-2 and resulting prostaglandins may be an important aspect of the pain resulting from peripheral injury.^{5,9} This mechanism is consistent with observations from inflammatory pain models, in which intrathecal administration of small doses of COX-2 but not COX-1 inhibitors reduces hyperalgesia.^{5,9–11} Systemic nonsteroidal antiinflammatory drugs, including COX-2-specific inhibitors, also reduce hypersensitivity from peripheral inflammation, but whether they act primarily at the sensitized peripheral nerve terminals or in the central nervous system cannot be discerned. Interestingly, systemic steroid can reduce spinal COX-2 mRNA production after peripheral inflammation, although systemic nonsteroidal antiinflammatory drugs or COX-2-specific inhibitors are ineffective.^{3,12}

The effects of surgical incision on spinal COX-2 production and central hypersensitivity currently remain speculative. In a recent pharmacologic study using the incisional pain model of Brennan *et al.*,¹³ we demonstrated that unlike the inflammatory hind paw injection models, neither intrathecal nor systemic administration of a COX-2-specific inhibitor alone reduces hyperalgesia.¹⁴ However, intrathecal coadministration of a COX-2 inhibitor and morphine is more effective in reducing mechanical hypersensitivity than morphine alone,¹⁴ suggesting that COX-2 increases in the spinal cord may be occurring to some extent after surgical incision. After an abdominal incision in sheep, no overall change in spinal COX-2 levels is observed on the day after surgery,¹⁵ which is contrary to events reported in rat inflammatory models.^{4,5} The current study was designed to quantitate COX-2 protein concentration in the rat spinal cord during the period immediately after surgical incision. A secondary goal was to evaluate the role of peripheral nerve activity and the effect of an intrathecal steroid or COX-2 inhibitor on spinal concentrations of COX-2 measured after surgical incision.

Materials and Methods

Incisional Model

Experiments were performed on 250–300-g male Sprague-Dawley rats (Sasco, Wilmington, MA) and were

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approved by the Institutional Animal Care and Use Committee. Animals were briefly (3 min) anesthetized with 1.5% isoflurane in oxygen, and an incision was made in both the left and right plantar hind paws. Using sterile technique, a 1-cm long longitudinal incision was made into the plantar skin with a no. 11 scalpel blade, starting 0.5 cm from the edge of the heel.¹³ The incision was slightly lateral of midline to include the territory of the sciatic nerve and minimize involvement of the saphenous nerve. The plantaris muscle was elevated and incised longitudinally (0.5 cm) with the no. 11 blade. The skin was closed with 4-0 nylon sutures using an everted mattress pattern, and a topical triple antibiotic ointment was applied to the plantar hind paw. In one experiment, a hind paw incision was performed on only one leg.

Spinal Cord Removal

At 1, 3, 6, 12, 24, and 48 h after bilateral plantar foot incision and 3 h after unilateral foot incision, the spinal cord was rapidly removed by ejection.¹⁶ Briefly, the animals were lightly anesthetized with isoflurane, and the spinal cord was severed above the C1 vertebra. The spinal column was removed intact starting at the bottom of the L6 vertebra. A 16-gauge needle, with attached 20-ml syringe filled with cold saline, was inserted into the caudal spinal column for a distance of 1 cm. The syringe plunger was rapidly pressed, and the intact spinal cord was ejected through the C1 vertebral opening into a Petri dish filled with cold saline. The spinal cord was then transferred to a glass plate over ice, and a 12-mm section of lumbar cord (L4–L6 spinal level) was removed with a no. 10 scalpel blade, frozen in cold 2-methylbutane, and placed in a cryogenic vial over dry ice. The time from the spinal cord severing to the placing of the sample vial on dry ice was 3 to 4 min. A 10-mm section of cervical cord (C3–C5) was also removed and frozen. All spinal cord samples were maintained at -80°C until assay. For the bilateral incision, there were five spinal cords sampled at each postincision time point and eight controls without surgical incision but with the same exposure time to isoflurane. For the unilateral incision, there were six spinal cords sampled at 3 h after incision and six controls without incision.

Western Blot Analysis

Spinal cord sections (10–12 mm long) were thawed to about -10°C and placed on a surface cooled by dry ice. Using a cold no. 15 scalpel blade, each section was minced into eight pieces, which were transferred to a 4-ml polypropylene centrifuge tube filled with 350 μl lysis buffer containing 0.1 M Tris buffer (pH 8.1), 0.9% sodium chloride, 1 mM EDTA, and a protease inhibitor cocktail (P8340; Sigma, St. Louis, MO). The contents of the tubes were homogenized with a fine-tipped ultrasonic probe and transferred with a Pasteur pipette to an empty 4-ml tube. The probe tip was then washed in a

4-ml tube containing 350 μl lysis buffer with 0.5% of solubilizer NP-40, and the contents of that tube were added to the first tube (total volume now about 700 μl). Each combined tube was placed on an aliquot mixer at 4°C for 30 min of mixing and then centrifuged (20,000 rpm at 4°C for 10 min). The clear supernatant was transferred to a 1.5-ml siliconized polypropylene centrifuge tube. The protein content of the supernatant was determined using a commercial kit (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL), and the concentration of all samples was diluted down to 2.0 mg/ml with lysis buffer. Diluted supernatants were then frozen at -80°C until Western blot assay.

Western blot analyses were performed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (3% stacking and 10% separating gel concentrations) and a dual-slab electrophoresis cell (Mini-Protean II; Bio-Rad, Hercules, CA). All samples were denatured with sodium dodecyl sulfate and reduced with 5% 2-mercaptoethanol (2 min at 95°C). Samples were applied to the stacking gel in a 35- μl volume, with the outer lane containing 88 ng COX-2 protein electrophoresis standard (no. 360120; Cayman, Ann Arbor, MI). Electrophoresis was performed at room temperature over 3 h, and the proteins were then transferred to nitrocellulose paper (BA85; Schleicher & Schuell, Keene, NH) overnight at 4°C . The nitrocellulose was incubated in a blocking buffer of 2% nonfat milk in Tris-buffered saline–Tween buffer for 1 h. The paper was washed three times with Tris-buffered saline–Tween buffer and transferred to an incubation buffer with primary antibody (COX-2 murine polyclonal antibody raised in rabbit; Cayman) at a 1:1,000 dilution for incubating and mixing for 1 h. Finally, after additional Tris-buffered saline–Tween buffer washes, the nitrocellulose was transferred to an incubation buffer with secondary antibody (anti-rabbit IgG peroxidase conjugate, A6667; Sigma) at a 1:200 dilution, incubated with mixing for 1 h, and again washed three times. In a darkroom, the nitrocellulose was incubated with a chemiluminescence reagent (Perkin Elmer, Boston, MA) and exposed on x-ray film (X-OMAT; Kodak, Rochester, NY) for 5–10 s. Band optical density was analyzed on the Fluor-S MultiImager system (Bio-Rad).

Nerve Block Experiment

Ten minutes before bilateral foot incision, animals were injected bilaterally with local anesthetic using a 25-gauge needle placed into the sciatic notch. The proper location was confirmed by a vigorous hindleg kick with monopolar stimulation *via* the needle (0.3 V, 2 ms, 2 Hz). Animals were injected with ropivacaine hydrochloride 0.5% ($n = 9$) or normal saline ($n = 9$), applying 0.5 ml into each sciatic notch. A second injection of each solution was made at 90 min after the foot incisions to maintain effective sensory block for 3 h after surgery. Sensory nerve block was confirmed by von Frey

filament testing of mechanical hyperalgesia,¹³ which demonstrated an elevation of withdrawal force threshold to 144 mN or greater in rats receiving local anesthetic block of the sciatic nerve (*vs.* a hypersensitive 20-mN threshold in control incision animals). At 3 h after foot incision, the spinal cords were rapidly removed and processed for Western blot analyses.

Intrathecal COX-2 Inhibitor or Steroid

Animals were implanted with intrathecal catheters for subsequent bolus drug injections using the cisterna magna insertion method of Yaksh and Rudy.¹⁷ The inserted catheter was 8.5 cm long, with the tip reaching the lumbar enlargement. All animals showing neurologic impairment after surgery were euthanized. Seven days after intrathecal catheter implantation, animals received an intrathecal injection of 8 μ l of one of three study compounds plus 8 μ l saline flush to clear total catheter dead space. The COX-2-specific inhibitor L-745,337 (Merck Frosst Canada, Kirkland, Quebec, Ontario, Canada) is water soluble and was given at a dose of 40 μ g, because that is the minimum dose that potentiates the antihyperalgesic effects of intrathecal morphine.¹⁴ The steroid dexamethasone sodium phosphate (Elkins-Sinn, Cherry Hill, NJ) at a dose of 8 μ g is also water soluble, and that dose produces a cerebrospinal fluid free-dexamethasone concentration of 0.5 μ g/ml for at least 2 h.¹⁸ A third group of animals received only saline (0.9% sodium chloride injection). There were eight animals in each of the three intrathecal injection groups. At 20 min after intrathecal drug injection, animals received bilateral foot incisions, and 3 h later, the spinal cords were rapidly removed and processed for Western blot analysis.

Statistical Analysis

After bilateral incision, the time course of the postincision COX-2 protein band optical density was evaluated with one-way ANOVA, followed by a two-sided Dunnett *post hoc* test using values obtained without foot incision as the comparative control (Statistical software; SPSS Inc., Chicago, IL). The effect of nerve block was compared with a two-sample *t* test. The effect of intrathecal agents was evaluated with one-way ANOVA, followed by a two-sided Dunnett *post hoc* test with the saline injection as the control. After unilateral incision, the 3-h postincision lumbar COX-2 protein optical density was compared with the preoperative control level using a two-sample *t* test. Graphs display optical density normalized to the mean optical density of control animals. All data are presented as mean \pm SE.

Results

Western blot analysis of spinal cord samples revealed two bands corresponding to the COX-2 standard (fig. 1).

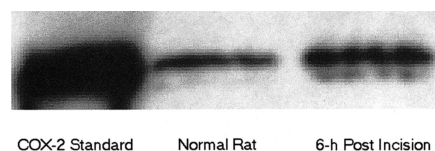


Fig. 1. Increase in lumbar spinal cyclooxygenase (COX)-2 protein after bilateral foot incision surgery in the rat. Representative Western blot of COX-2 protein in L4–L6 cord at 6 h after incision as compared with that of a normal, unoperated rat.

There was a darker band around 72 kD and a lighter band at 70 kD. Both bands were included for optical density analysis in these experiments, as previously done by other investigators.⁶

The time course of COX-2 protein change in the lumbar spinal cord after bilateral foot incision is shown in figure 2. COX-2 protein levels were increased at 3 h after surgery (1.32-fold) and remain elevated at 6 h (1.26-fold). At 12 h after incision and thereafter, however, COX-2 levels were not different from those of control. In comparison, COX-2 levels in the cervical cord did not increase at any of the study times after incision (fig. 3). After unilateral incision, the lumbar COX-2 protein level at 3 h was only 1.15-fold higher than that of control, which is not a significant increase.

Blocking the sensory impulses from the sciatic nerve on each side using the local anesthetic ropivacaine just before bilateral foot incision and for the next 3 h did not reduce COX-2 protein levels in the lumbar spinal cord at 3 h after incision compared with those in saline controls (fig. 4).

Intrathecal dexamethasone administered before bilateral foot incision reduced lumbar COX-2 protein levels at 3 h after surgery compared with those in control animals receiving an intrathecal saline injection (fig. 5). Intrathecal administration of the COX-2-specific inhibitor L-745,337 did not reduce COX-2 protein levels at 3 h after incision.

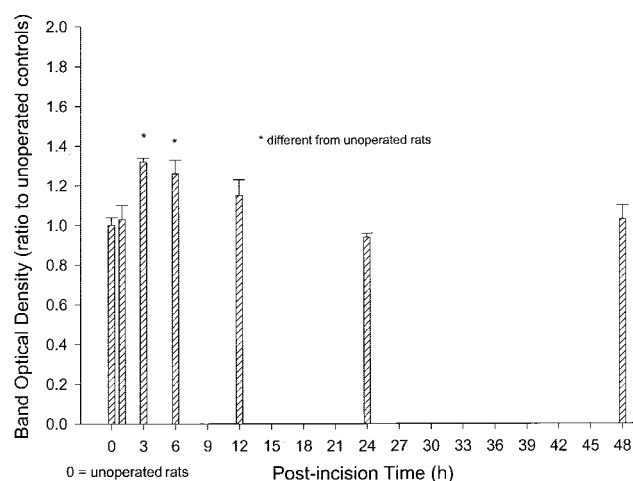


Fig. 2. Time course of lumbar spinal cord cyclooxygenase-2 protein optical density over a 48-h postsurgery period. *n* = 5 at each time point, with 8 unoperated control rats.

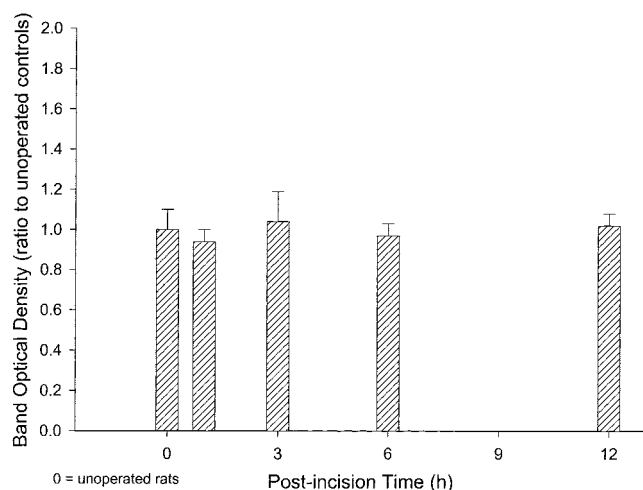


Fig. 3. Time course of cervical spinal cord (C3–C5) cyclooxygenase-2 protein optical density over a 12-h postsurgery period. $n = 5$ at each time point, with 8 unoperated control rats.

Discussion

The increase in COX-2 protein in the lumbar spinal cord after bilateral foot incision is qualitatively similar to the upregulation of COX-2 protein observed after peripheral inflammatory injection.^{4,5} However, there are some quantitative differences. First, in the bilateral foot incision model, the peak value of spinal COX-2 protein is reached at 3–6 h after surgery; by 12 h, COX-2 levels are no longer elevated. In contrast, injection of the inflammatory combination of kaolin and carrageenan into a knee joint produced an increase in COX-2 protein levels by 3 h, which reached a peak at 12 h.⁴ In another study with CFA injection into the rat hind paw, lumbar spinal COX-2 protein levels reached peak value at 12 h.⁵ The relatively rapid return of spinal COX-2 protein levels to

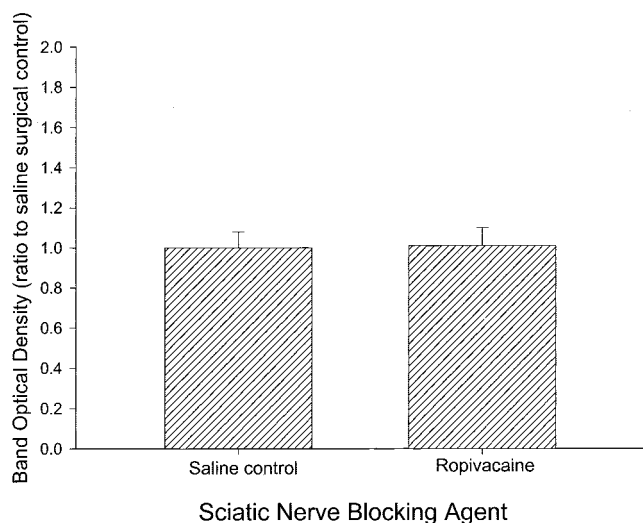


Fig. 4. Lumbar cyclooxygenase-2 protein optical density at 3 h after incision with ropivacaine blockade of the sciatic nerve compared with incision rats with saline injected into the sciatic notch. $n = 9$ per group.

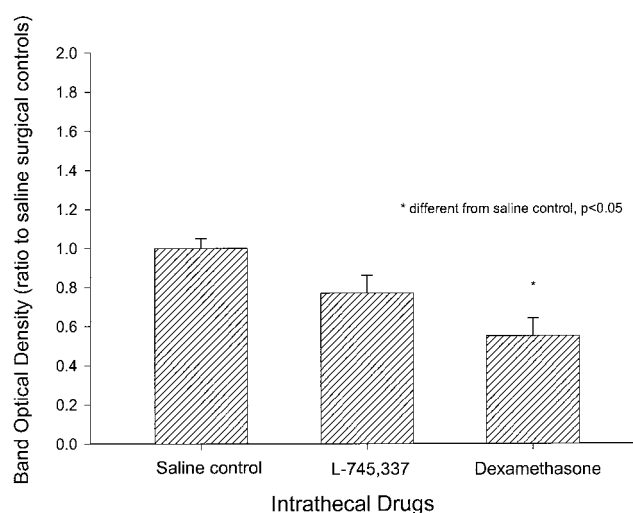


Fig. 5. Lumbar cyclooxygenase-2 protein optical density at 3 h after incision with intrathecal injection of drug or saline control 20 min before incision. Intrathecal drugs were L-745,337 (40 μ g) or dexamethasone sodium phosphate (8 μ g). $n = 8$ per group.

baseline in our foot incision experiment may explain why no overall increase was seen in the lumbar COX-2 protein level measured at 24 h after abdominal incision in sheep.¹⁵ However, the abdominal incision study did show a localized increase of COX-2 protein immunoreactivity in lamina 5 but not in lamina 1 or 2 at 24 h, so there may be localized COX-2 increases in the rat spinal cord that persist beyond 6 h.

Second, the maximum ratio of lumbar COX-2 protein to control (observed at 3 h) is only 1.32 in response to bilateral foot incision. In the kaolin and carrageenan knee injection model, lumbar COX-2 protein increased sixfold.⁴ In the CFA hind paw inflammatory model, the lumbar COX-2 protein level increased twofold.⁵

The relatively small increase in spinal COX-2 protein seen with bilateral plantar foot incision suggests that these skin/muscle incisions alone are a much weaker stimulus to the cytokine-cyclooxygenase-prostaglandin pathway than direct injection of agents (e.g., CFA, carrageenan) chosen purposely to elicit a vigorous immunogenic response. A more invasive type of surgery or a procedure stimulating a greater inflammatory response may produce larger increases in spinal COX-2 protein. Notably, clinical studies have shown that plasma cytokine levels after cardiac bypass surgery¹⁹ are much higher than after hip replacement surgery.²⁰ This is supported by our unilateral incision experiment, in which lumbar COX-2 protein levels at 3 h were not significantly elevated, in contrast to the upregulation of COX-2 protein at 3 h after bilateral incision.

The rapid onset of COX-2 protein increase (3 h) in the foot incision model might be attributed to the instant nociceptive stimuli produced by injuring peripheral nerve endings. However, local anesthetic blockade of

the sciatic nerve before and during a 3-h postoperative period did not decrease lumbar COX-2 protein compared with that in incisional control animals. Samad *et al.*⁵ have reported that a 24-h block of the sciatic nerve after CFA injection into the hind paw had only a moderate effect on the large mRNA upregulation that usually occurs. They concluded that circulating proinflammatory cytokines are the more important mechanism in central upregulation of COX-2 that occurs in response to a peripheral inflammatory stimulus.

Intrathecal administration of dexamethasone decreased the lumbar COX-2 protein upregulation compared with saline-administered incision animals. This is consistent with the findings of Hay and de Belleruche,¹² who reported that subcutaneous dexamethasone given 30 min before intraplantar injection of CFA prevented the increase in lumbar COX-2 mRNA usually seen in that model. A COX-2-selective inhibitor (L-745,337) did not decrease spinal COX-2 protein levels in our study, consistent with the study of Hay *et al.*,³ in which systemic indomethacin or COX-2-selective inhibitors had no effect in reducing COX-2 mRNA at 4 h after CFA injection. It may seem unlikely that a COX-2 inhibitor, which binds to the COX-2 molecule, would also block the induction of COX-2; however, COX-2 produces prostaglandins that may have a positive feedback effect (e.g., with interleukin-1 β as intermediary) to stimulate more COX-2 production.^{5,7} Our results do not support this mechanism whereby a COX-2 inhibitor could cause feedback reduction in COX-2 production.

We did not observe any increase in cervical COX-2 protein after surgery. This is different from the findings of Samad *et al.*,⁵ who reported an increase in cervical COX-2 mRNA after inflammation, although the increase was less than in the lumbar cord. Because the lumbar COX-2 protein only increased 1.32-fold in our experiments, it is possible that an increase at the cervical level would be too small after limited incisions to be significantly different compared with that in controls. Another possibility is that cytokines or growth factors are taken up by the peripheral nerve at the incision site and stimulate COX-2 upregulation in the lumbar spinal cord. For example, when brain-derived neurotrophic factor is applied to sciatic nerve terminals, catalytic activity of trk receptors increases at distant points in the nerve within 60 min.²¹ This could explain increased 3-h postincision lumbar COX-2 protein with unchanged cervical concentrations, without dependence on nerve conduction. Finally, nerve conduction may still be responsible for the selective lumbar upregulation if all fibers (e.g., c-fibers) were not inhibited in the sciatic nerve block experiment, because we only monitored mechanical hypersensitivity during the 3 h between foot incision and removing the spinal cord.

At 3 h after bilateral foot incision, the animals demonstrated mechanical hypersensitivity (a low 20-mN force

threshold) in both legs as well as COX-2 upregulation. However, it is not well understood how this hypersensitivity is related to the upregulation of spinal COX-2 protein. Certainly, there are other factors not measured in the present study that could be closely linked to hyperalgesia. The levels of prostaglandins (e.g., prostaglandin E₂ [PGE₂]) in the spinal cord could be important in the induction and maintenance of incisional pain, because intrathecal injection of PGE₂ produces acute pain in rodents.²² In an inflammatory pain model, COX-2 protein levels increased fourfold starting at 3 h after kaolin and carrageenan injection and PGE₂ measured by intraspinal probes increased by 6 h.⁴ In a similar model, COX-2 protein levels in the spinal cord and PGE₂ levels in the cerebrospinal fluid increased at 6 h after CFA injection.⁵ Although COX-2 enzyme activity is an important determinant of PGE₂ production, one limitation of our Western blot analysis is that it does not indicate protein activity. COX-1 upregulation does not occur in inflammatory pain models,²⁻⁵ but it may have a role in surgical incision models, because after unilateral hind paw incision, COX-1 protein slowly increased in the lumbar dorsal horn, reaching a peak at 48 h and remaining elevated for 5 days.²³ It should also be noted that the precision of the Western blot analysis might be improved if the optical densities of COX-2 were referenced to another control protein.⁵

In summary, we have demonstrated that surgical incision causes upregulation of lumbar spinal cord COX-2 protein. These increases in spinal COX-2 are sustained for a few hours immediately after incision and can be attenuated with steroid pretreatment but not with peripheral nerve blockade by local anesthetic. Additional studies are necessary to determine whether more extensive surgical trauma induces correspondingly larger increases in the spinal COX-2 response and whether therapeutic approaches focused on these spinal changes have benefits for management of postsurgical pain.

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