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## Peripheral Formalin Injection Induces Long-Lasting Increases in Cyclooxygenase 1 Expression by Microglia in the Spinal Cord

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**Abstract:** Activated glia are a source of substances known to enhance pain, including centrally synthesized prostaglandins. We have previously shown that microglia are activated in the spinal cord following peripheral formalin injection. In the present study, we investigated cyclooxygenase (COX-1 and COX-2) expression in the spinal cord using immunohistochemistry and Western blots in the formalin pain model, to further understand how spinal glia modulate pain processing. We show that both COX-1 and COX-2 are constitutively expressed in the spinal cord. Hind paw formalin injection increased COX-1 expression, beginning at 1 day after injection and lasting at least 2 weeks, the duration of experiments. The COX-2 expression changed considerably less, with a significant increase of COX-2 protein level only observed at 2 h after injection. Double labeling studies showed that COX-1 was expressed in microglia and COX-2 was expressed in neurons. These data indicate that both COX-1 and COX-2 are increased in the spinal cord following formalin injection, but the time course and cellular sources are different, suggesting that both COX-1 (longer time points) and COX-2 (very short time points) may be involved in spinal modulation in the formalin pain model. Our study also suggests that spinal microglial activation may play a role in long-term hyperalgesia through the increased expression of COX-1.

**Perspective:** This article reports that COX-1 expression by microglia is increased in the spinal cord after peripheral formalin injection into the rat hind paw. This result could potentially help clinicians understand how COX-1 may be involved in pain processing and the role microglial activation plays in pain mechanisms.

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**Key words:** Formalin test, pain, prostaglandins, glia, COX-1, COX-2.

Prostaglandins (PG) are involved in peripheral mechanisms of hyperalgesia. Recently it has been increasingly accepted that prostaglandins also play a role in spinal mechanisms of hyperalgesia. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is synthesized in the spinal cord after acute nociceptive stimulation, peripheral inflammation, intrathecal injection of substance P, and mechanical injury to the spinal cord.<sup>6,11-13,15,16,24,25,30,31,38,41</sup> Spinally synthesized

and released PGE<sub>2</sub> has been shown to increase release of excitatory neurotransmitters in the spinal cord<sup>28,34</sup> as well as directly depolarize dorsal horn neurons,<sup>1</sup> therefore leading to central sensitization in the pain pathway, resulting in augmented pain responses to a normally painful stimuli (hyperalgesia) and pain responses to normally nonpainful stimuli (allodynia).

Prostaglandin E<sub>2</sub> is produced upon metabolism of arachidonic acid by cyclooxygenase (COX), an enzyme that consists of 2 isoforms, COX-1 and COX-2. Recently, COX-3, a COX-1 splice variant, has been shown to exist in the spinal cord and may therefore also play a role in central pain processing.<sup>5,27</sup> In general, COX-1 is constitutively expressed in almost all tissues and considered to be primarily responsible for prostaglandin synthesis required for cell homeostasis, and COX-2 is highly inducible in response to cytokines, growth factors, and other in-

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flammatory stimuli.<sup>32,33</sup> In inflammatory pain animal models, hind paw injection of complete Freund's adjuvant (CFA) or carrageenan produces mechanical allodynia and thermal hyperalgesia that is linked to increases of COX-2 mRNA and protein levels in the lumbar spinal cord.<sup>2,12,16,17,25,26</sup> The inflammation-induced pain behaviors could be attenuated or reduced by intrathecal administration of COX-2 inhibitors.<sup>16,25,26,38,40</sup> In contrast, COX-2 expression in the dorsal horn of the spinal cord does not markedly change after spinal nerve injury.<sup>4,42</sup> Also, the selective COX-2 inhibitor rofecoxib failed to attenuate pain behaviors in this model.<sup>4</sup> Interestingly COX-1 expression as demonstrated by immunohistochemistry was increased in the spinal dorsal horn after hind paw incision injury and spinal nerve ligation injury.<sup>43,44</sup> Intrathecally administering ketorolac, a relatively specific COX-1 inhibitor, and the specific COX-1 inhibitor SC569 dose dependently increased the mechanical withdrawal threshold in the incision pain model.<sup>43</sup> It is very likely that both COX-1 and COX-2 are inducible after peripheral nociceptive stimulation and that both are involved in spinal pain mechanisms although the cellular sources and the role in pain transmission may be different.

In the central nervous system, COX-1 is constitutively present in ependymal cells, vascular endothelia, microglia, astrocytes, and some neurons under normal conditions.<sup>18</sup> Cyclooxygenase 1 was up-regulated after hind paw incision injury and spinal nerve ligation injury, and the sources of COX-1 were from glial cells as judged by cell morphology.<sup>43,44</sup>

There is mounting evidence that glia within the spinal cord create and maintain pathologic pain, and it has been hypothesized that activated glia can release a variety of substances known to enhance pain although the details by which they enhance pain are not yet clear.<sup>22,23,35</sup> In our previous studies, hind paw injection of formalin produced long-lasting hyperalgesia and associated microglial activation in the lumbar spinal cord.<sup>10,11</sup> From that evidence, we hypothesized that microglial activation may play a role in spinal pain mechanisms through the release of COX-1 enzyme. In the present study, we investigated COX-1 as well as COX-2 expression in the lumbar spinal cord by immunohistochemistry and Western blot analysis, using a formalin pain animal model, to further understand how spinal glia modulates pain processing.

## Materials and Methods

### *Animals and Treatments*

Experiments were performed on adult (200-225 g) male Sprague-Dawley rats. All rats were housed at a constant temperature of 22°C on a 12/12-h light/dark cycle with food and water available ad libitum. Experimental rats were given subcutaneous injections of 100 µL 5% formalin (diluted in .9% saline) into the plantar surface of the right hind paw, and control rats were injected with 100 µL .9% saline instead of formalin. Injections were made without anesthesia. The treat-

ment and care of all animals used in the study were approved by, and followed the guidelines of, the Peking University Medical Sciences Center Animal Welfare Committee.

### *Immunohistochemistry*

Twenty-four rats (4 rats at each time point) were given subcutaneous injections of 100 µL 5% formalin into the right hind paw. Tissue was collected from these rats at hours 2 and 6 and days 1, 3, 7, and 14 after formalin injection. Rats were killed with an overdose of pentobarbital sodium and then subjected to transcardiac perfusion (250 mL body-temperature .1 mol/L phosphate-buffered solution (PBS) pH 7.4 followed by 300 mL ice-cold 4% paraformaldehyde/4% sucrose in .1 mol/L PBS pH 7.4). Four control rats were injected with 100 µL .9% saline and killed and perfused at 1 day after formalin injection. After perfusion, the lumbar spinal cord (L4-5) was removed, postfixed in 4% paraformaldehyde fixative for 4 h, and then placed in a 30% sucrose solution (in .1 mol/L PBS) overnight at 4°C. Forty-micrometer-thick tissue sections were cut transversely on a cryostat and processed for COX-1 and COX-2 immunohistochemical staining using the avidin-biotin technique (Elite Kit; Vector, Burlingame, CA).

For COX-1 immunohistochemistry, sections were preincubated with .1 mol/L PBS pH 7.4 containing 5% normal horse serum (NHS) for 60 min at room temperature after being pretreated with .3% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min. Then tissues were incubated for 3 days at 4°C in a mouse monoclonal anti-ovine COX-1 antibody (1:300, catalog no 160110; Cayman, Ann Arbor, MI) diluted in PBS containing .3% Triton-X 100 and 5% NHS. The antibody is known to cross-react with ovine, human, bovine, rat, murine, and monkey COX-1. Subsequently, the sections were incubated with the secondary biotinylated horse antimouse IgG (rat adsorbed; Vector Lab) overnight at 4°C. Sections were washed 3 times with PBS containing .3% Triton-X 100 between incubation steps. 3,3'-diaminobenzidine (DAB) was used as the chromagen. For COX-2 immunohistochemistry, the staining procedure was the same as for COX-1 except for the following procedures: 1) Blocking buffer was 10% normal goat serum (NGS) in PBS containing .3% Triton-X 100; 2) primary antibody polyclonal rabbit anti-murine COX-2 (1:1000, catalog no 160126; Cayman) diluted in PBS containing .3% Triton-X 100 and 10% NGS was used and incubated at 4°C for 2 days; and 3) the secondary biotinylated goat anti-rabbit IgG was incubated at 4°C overnight. The antibody to COX-2 is an affinity-purified polyclonal antibody raised against a peptide corresponding to amino acids 584-598 of murine COX-2 and is known to cross-react with homologous regions of human, ovine, rat, and murine COX-2 but not COX-1. Control sections without primary antibodies, omission of primary antibody or replacement of primary antibody by normal horse/goat serum were always negative. All of the tissues for each time point (6 experimental rats and 1 saline-injected control rat)

were processed for immunohistochemical staining together to ensure consistency in staining.

For double immunofluorescence, 30- $\mu$ m-thick spinal sections from formalin-injected animals at 2 h, day 3, and day 14 were incubated with polyclonal antiCOX-1 antibody (1:400, catalog no 160109; Cayman), or COX-2 antibody (1:400, catalog no 160126; Cayman) for 2 nights at 4°C and monoclonal neuronal-specific nuclear protein (NeuN) (neuronal marker, 1:800; Chemicon), GFAP (astrocyte marker, 1:400; Dako), and OX-42 (microglia marker, 1:200; Serotec) for 1 night at 4°C, followed by a mixture of FITC- and TRITC-conjugated secondary antibodies (Jackson) for 40 min at 37°C. The stained sections were examined with Bio-Rad confocal microscopy, and images were captured with a CCD spot camera.

### Western Blotting

The animals were killed by decapitation at 2 and 6 h and 1, 3, 7, and 14 days after injection (6 rats at each time point). Another 6 control rats were injected with saline in the paw, instead of formalin and were killed 1 day after injection. Lumbar enlargement segments of the spinal cord were removed, quickly frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for later use. The L4-5 spinal cord segment was first isolated. Then the spinal segment was cut into a left and right half from the ventral midline. Finally, the right half was further split into the dorsal and ventral horn at the level of the central canal. The dorsal horns were homogenized in 50 mmol/L Tris/HCl, 2 mmol/L EDTA, pH 7.4, 1 mmol/L phenylmethyl-sulfonyl fluoride, and 10  $\mu$ g/mL leupeptine and sonicated  $4 \times 15$  s at 30% power. After removal of cellular debris, protein from combined nuclear and microsomal fractions was obtained by centrifugation at 100,000g for 1h. A 60  $\mu$ g sample of protein that was quantified by BCA protein assay kit (PIERCE) was submitted to SDS-polyacrylamide gel electrophoresis (5% stacking, 12% separation gel) and transferred to PVDF filter (Millipore) by semidry blotting. The membrane was blocked with 5% bovine serum albumin (BSA)/Tris-buffered saline (TBST) pH 7.6 for 1 h at room temperature and incubated overnight at 4°C with the rabbit polyclonal antiCOX-1 antibody (Cayman) diluted 1:500 in 5% BSA/TBST or with the rabbit polyclonal antiCOX-2 antibody (Santa Cruz) diluted 1:500 in 5% BSA/TBST. Immunoreactive proteins were detected using horseradish peroxidase-conjugated antirabbit secondary antibody and visualized using chemiluminescence reagents provided with the luminol reagent (Santa Cruz) according to the manufacturer's protocol and exposed onto films for 1-10 min.

### Statistical Analysis

For the quantification of Western blotting results, the COX-1 and COX-2 bands were scanned into a computer and the density of the blot calculated by Total LAB 1.00 software. Values for COX-1 and COX-2 were

corrected for  $\beta$ -actin values and expressed as fold increase compared with the respective saline-injected controls with. All data were reported as mean + SEM. Six rats were included in each time point and differences between time points were compared using one-way analysis of variance (ANOVA) with post hoc Bonferroni test. The criterion for statistical significance was  $P < .05$ .

## Results

### *Plantar Hind Paw Formalin Injection Induced Long-Lasting Increased COX-1 Expression by Microglia*

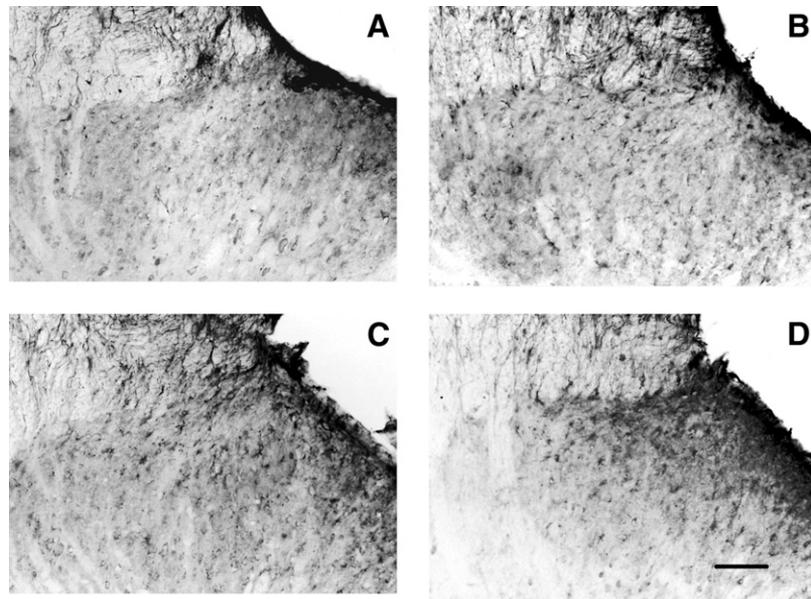
Cyclooxygenase 1 was constitutively expressed and faint labeling was observed in the spinal cord in saline-injected control rats (Fig 1A). The COX-1 immunoreactivity was located mainly in cells with glial morphology in both the dorsal and the ventral horn, and in the white matter as well. The COX-1-immunoreactive (COX-1-IR) glial-like cells were distributed throughout the gray matter but were much more obvious in the superficial dorsal horn (Fig 1).

Hind paw formalin injection dramatically increased COX-1 expression as observed with immunohistochemistry (Fig 1) and Western blot analysis (Fig 2). Increases were observed on day 1 after injection and lasted at least 2 weeks, the length of the experiments. The COX-1 immunoreactivity was increased more in the medial portion of the ipsilateral spinal dorsal horn. The increased COX-1-IR cells appeared to be in an activated state (retracted processes, swollen cell bodies, and clearly hypertrophic) and intensely stained (Figs 1C and 1D).

To identify the cell types that expressed COX-1 after peripheral formalin injection, we performed immunofluorescence staining for COX-1 (Fig 3A) and double immunostaining of COX-1 on days 3 and 14 after injection with several cell-specific markers: NeuN (neurons), GFAP (astrocytes), and OX-42 (microglia). Cyclooxygenase 1 did not colocalize with either NeuN or GFAP (Figs 3B and 3C) but colocalized with OX-42 (Figs 3D-3F). At 2 weeks, COX-1 expression still colocalized only with OX-42 (Fig 3G), indicating no change in the types of cells expressing COX-1. These results indicated that spinal COX-1 was expressed by microglia.

### *Plantar Hind Paw Formalin Injection Induced a Rapid Increase in COX-2 Expression by Neurons*

COX-2 was also constitutively expressed in the spinal cord in control rats (Fig 4A). Following hind paw formalin injection, COX-2 expression was slightly increased in the ipsilateral side of the spinal dorsal horn as observed with immunohistochemistry (Fig 4B) but changed considerably less at several later time points. By double labeling COX-2 and NeuN (neuronal marker), COX-2 was completely colocalized with NeuN (Figs 4C-4E), not with either GFAP or OX-42. By Western blotting, a significant



**Figure 1.** COX-1 immunoreactivity (IR) changes over time in the ipsilateral side of the spinal dorsal horn following formalin injection. (A) Saline-injected control animal: small cells in the dorsal horn slightly stained. (B) COX-1 IR mildly increased at day 1 after formalin injection. (C) Glia-like cells deeply stained with COX-1, and morphology clearly hypertrophic, in the superficial and medial portion of the spinal dorsal horn at day 3. (D) Strong increases in COX-1 IR seen in the superficial dorsal horn at 2 weeks. Scale bar 80  $\mu\text{m}$ .

increase of COX-2 protein level in the dorsal spinal cord was detected only at 2 h after injection (Fig 5).

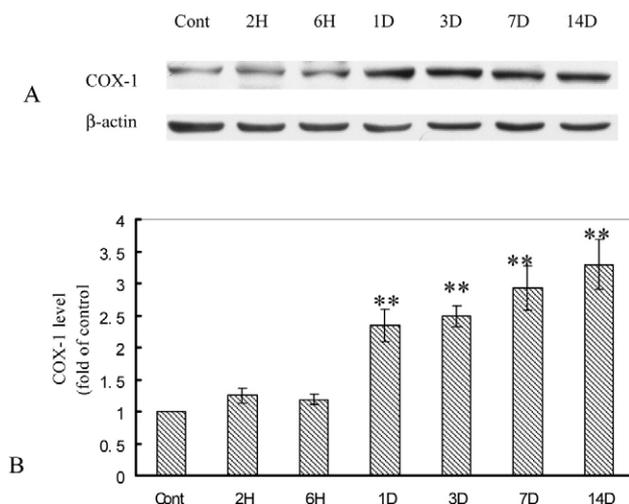
## Discussion

In the present study, hind paw formalin injection caused a rapid short-duration increase (within 2 h) of COX-2 protein expression by neurons in the spinal cord. It also caused a later dramatic increase in COX-1 expression in microglia on the ipsilateral side of the dorsal horn, predominantly in the medial portion of the superficial

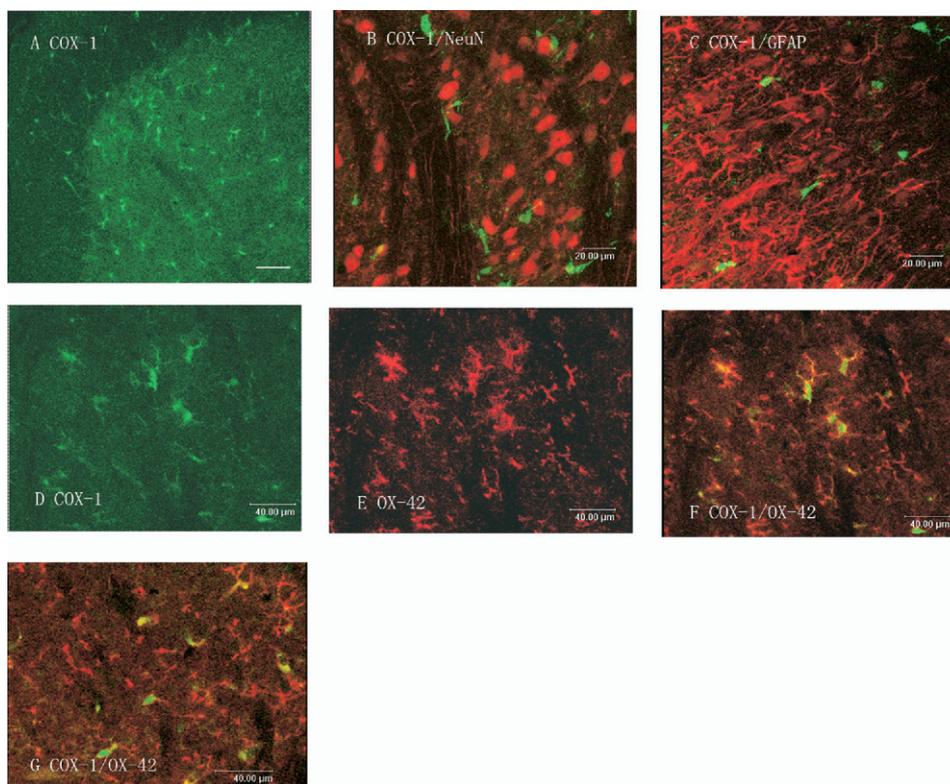
lumbar dorsal horn, the region representing the paw of the hindfoot. The increase of COX-1 protein in the spinal dorsal horn began at 1 day after injection and lasted at least 2 weeks. The time course of spinal COX-1 expression caused by formalin injection closely matched the long-term pain behavior and microglia activation observed with OX-42 labeling which we and others have reported previously.<sup>9,11,29,37</sup>

### *The Formalin Test as Both a Short-Term Inflammatory Pain and a Long-Term Injury Pain Model*

Injection of formalin into the rat's hind paw produces 3 phases of nociceptive behavior. Most reports have used only the first 2 phases of the formalin test to study both peripheral and central hyperalgesia. These first 2 phases are used extensively as an inflammatory pain model.<sup>7</sup> The first phase lasts 5-10 min and consists of paw guarding, flinching, and biting. Following a quiet period, the second phase begins, lasts 1-1.5 h, and consists of overt paw licking and biting. Peripheral inflammatory processes are involved in the second phase and the central sensitization observed during this period plays an important role in the formalin-induced hyperalgesia.<sup>36</sup> After the second phase, the animal emits no further overt pain behavior. The injected surface of the foot becomes anesthetic at this time, suggesting nerve as well as tissue damage. However, testing of the opposite surface of the foot (inject dorsal, measure plantar) demonstrates that the paw remains hyperalgesic and allodynic for 8-20 h. At 24 h, the rat exhibits normal pain behavior, but at 48 h after injection, hyperalgesia and allodynia are again detected, and these pain behaviors remain for up to 4



**Figure 2.** Western blot bands (A) and quantification analysis (B) reveal that COX-1 protein levels increase from 1 day and remain elevated for 2 weeks after injection. The  $\beta$ -actin data shown are used as a comparison standard. One-way ANOVA:  $F = 15.42$ ;  $df = 41$ . \*\* $P < .01$ , compared with control.



**Figure 3.** Formalin injection induces microglia expressing COX-1 in the ipsilateral side of the spinal dorsal horn. Immunofluorescence staining for COX-1 in the medial portion of the spinal dorsal horn (A). Scale bar 100  $\mu$ m. (B-C) Double immunofluorescence labeling of COX-1 with NeuN (red, B), a marker of neurons, and GFAP (red, C), a marker of astrocytes. (D-F) COX-1 immunofluorescence in activated microglia labeling intensively with OX42: COX-1 (green, D), OX42 (red, E), and merged (yellow, F). (G) Double labeling of COX-1 and OX-42 on day 14 after injection.

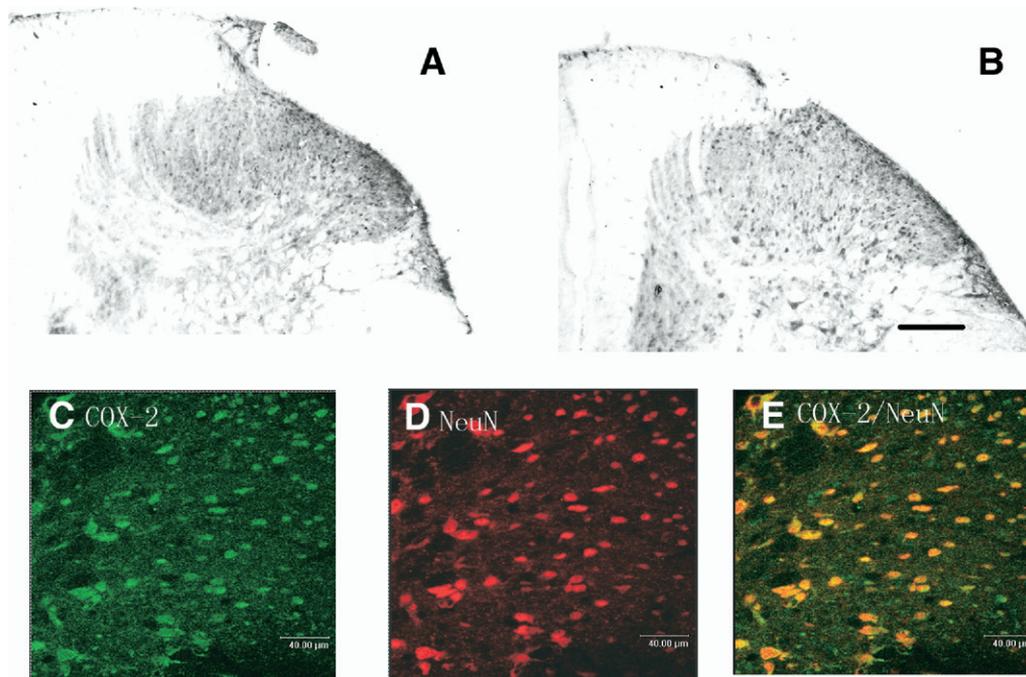
weeks.<sup>10,11</sup> We interpret this long-term pain as having some similarities and some differences to that produced by incision injury and nerve injury models. In those models, microglial activation and COX-1 over-expression in the spinal dorsal horn is also found.<sup>43,44</sup> In our formalin model, we have previously reported microglial activation beginning at day 1 after injection.<sup>10,11</sup> The present study demonstrates increasing microglia expression of COX-1 in the spinal dorsal horn at 1 day after injection. Because pain behavior does not appear until a day after the initial COX expression increases (pain behavior does not increase until day 2), it appears that sufficient COX-1 expression may be required to synthesize enough PG to enhance central sensitization in the pain pathway, ultimately resulting in augmented pain responses. As with some other injury models, the formalin model represents a mixed inflammatory, tissue injury, and nerve injury model.

### ***COX-2 Is Increased in Neurons at Early Time Points During Inflammation Caused by the Formalin Model and Other Inflammatory Pain Models***

As reported by others,<sup>3,20,25</sup> COX-2-immunostained cells in the spinal cord were neurons. As in other reports, we found little or no COX-2 in glial cells at any time before or after our formalin injury model.

COX enzymes have been previously studied extensively with inflammatory models. These models used Freund's adjuvant or carrageenan injections to produce a relatively selective inflammation of peripheral tissues and/or joints. In the central nervous system, constitutive COX-2 is considered the predominant isoform of COX and is highly regulated by different mediators.<sup>14,19,25</sup> After experimental induction of peripheral inflammation, a significant induction of COX-2 gene expression and protein expression and synthesis of PGs in the spinal cord has been observed in several laboratories.<sup>2,8,12,13,16,17,25,26,38</sup> The induction of COX-2 mRNA is rapid, being maximal at 2-4 or 6 h after CFA or carrageenan injection.<sup>2,12,17,25</sup> The maximum elevation of COX-2 protein levels was observed 12 h after the induction of arthritis or CFA-induced hind paw inflammation,<sup>8,25</sup> with a slow return to baseline by about 72 h.<sup>2</sup>

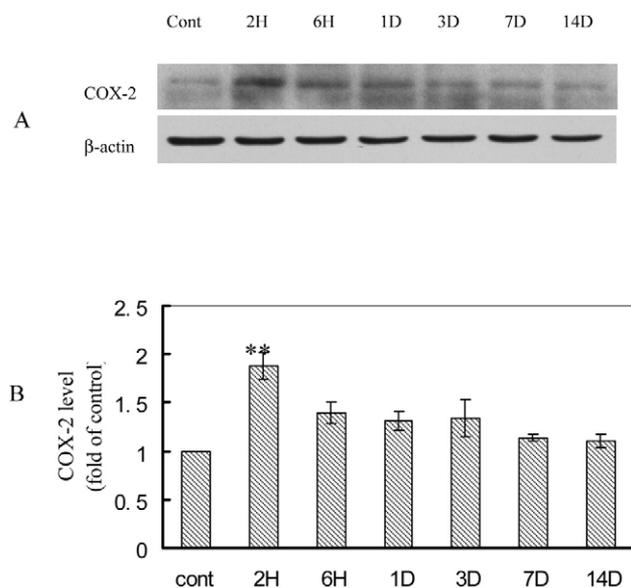
Using the mixed model of formalin paw injection, intraperitoneal administration of ibuprofen reduced paw flinching and blocked the elevated levels of PGE<sub>2</sub>-LI from the spinal cord.<sup>21</sup> Both the nonselective COX inhibitor indomethacin and selective COX-2 inhibitor NS-398 administered intrathecally or intraperitoneally 10 min before formalin injection, inhibited the formalin induced phase 2 flinching behavior in a dose-dependent manner.<sup>39</sup> These reports suggest that



**Figure 4.** COX-2 is expressed in the ipsilateral side of the spinal dorsal horn in a saline-injected animal (A) and at 2 h after formalin injection (B), showing that COX-2-IR cells increased in both the superficial and the deep dorsal horn. Scale bar 160  $\mu$ m. (C-E) Double immunofluorescence labeling of COX-2 (green, C) at 2 h after formalin injection with NeuN (red, D), a marker of neurons. COX-2 was completely merged with NeuN (yellow, E).

COX-2 is important in mediating at least early pain behavior evoked by the formalin pain model.

Consistent with the results of these inflammation studies, in the present study COX-2 was induced rapidly and was significantly elevated at 2 h after formalin injection.



**Figure 5.** Western blot bands (A) and quantification analysis (B) reveal COX-2 protein levels increase only at 2 h after injection. The  $\beta$ -actin data shown are used as a comparison standard. One-way ANOVA:  $F = 6.806$ ;  $df = 34$ .  $^{**}P < .01$ , compared with control.

### ***COX-1 Is Increased at Later Time Points and Remains Increased for Several Days or Weeks in the Formalin Model and in Injury and Neuropathic Pain Models, Possibly Mediating This Enhanced Pain***

Unlike COX-2, we found that COX-1 was expressed by microglia and expression increased at a later time point and remained elevated for at least 2 weeks after formalin injection. When administered either before or after formalin injection into the hindpaw, intrathecal administration of the specific COX-1 inhibitor SC-560 (100  $\mu$ g) increased paw withdrawal threshold to control levels on day 3 after formalin injection. Normally, formalin injection causes greatly decreased paw withdrawal thresholds on day 3 after injections. However, the COX-2 inhibitor NS-398 (50  $\mu$ g) had no such effect (data not shown). These data suggest that COX-1, but not COX-2, may mediate long-term enhanced pain. Cyclooxygenase 2, however, may mediate early pain enhancement, as described in the preceding section. These data also suggest that COX-2 expression at an early time point is not necessary for later COX-1 expression or long-term pain enhancement.

Cyclooxygenase 1 has also been found to increase in the ipsilateral spinal dorsal horn days after paw incision injury and peripheral nerve injury. As in our experiments, COX-1 elevation was associated with mechanical hypersensitivity, and preoperative inhibition of COX-1 reduced the hyperalgesia.<sup>43-45</sup> Moreover, COX-2 expression in the

spinal dorsal horn did not markedly change following spared nerve injury (SNI) neuropathic pain model.<sup>4</sup>

Thus, it is possible that COX-2 expression is important in mediating enhanced pain caused by peripheral inflammation at least at early time points, whereas COX-1 expression is important in mediating pain caused by nerve injury.

However, it is possible that both COX-2 and COX-1 play important roles in long-term hyperalgesia, at least in the formalin model. Previous studies in our laboratory have shown that afferent input during the first 2 h after the formalin injection is necessary for the development of long-term hyperalgesia and allodynia.<sup>10</sup> Thus, it is possible that neural events during the first 2 h are necessary but not sufficient for long-term pain and that the expression of both COX-2 in neurons for short-term pain and COX-1 by microglia in the longer term are necessary and sufficient for the

establishment of the entire pain behavior profile in the formalin injection model. The COX enzymes do not function in isolation but catalyze the production of PGE<sub>2</sub>, and through this mechanism they may enhance the release of other pain modulators by spinal astrocytes and microglia, such as interleukin 1 $\beta$ , tumor necrosis factor  $\alpha$ , nitric oxide, and adenosine triphosphate.<sup>22,23,35</sup>

In summary, the present data confirmed that both COX-1 and COX-2 were constitutively expressed in the spinal cord.<sup>2,3,8,20,38</sup> Both COX-1 and COX-2 are increased in the spinal cord after formalin injection, but the time course and cellular sources are different, suggesting that both COX-1 and COX-2 may be involved in different modes of spinal pain modulation in the formalin pain model. Our study also suggests that spinal microglial activation may play a role in pain processing through the increased expression of COX-1.

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