

# Developmental Differences in Spinal Cyclooxygenase 1 Expression after Surgical Incision

Douglas G. Ririe, M.D.,\* Heather D. Prout, B.S.,† David Barclay, M.D.,‡ Chuanyo Tong, M.D.,\* Marina Lin, M.S.,§ James C. Eisenach, M.D.∥

**Background:** Systemic administration of a cyclooxygenase 1 (COX-1) inhibitor reduces hypersensitivity to mechanical stimuli after incisional paw surgery in 4-week-old, but not 2-week-old, animals. The purpose of the current study was to test whether this developmental difference was reflected by differences in COX-1 expression in the spinal cord after surgery.

**Methods:** Rats 2 and 4 weeks of age, paralleling infant and child human neurologic developmental stages, were used. A paw incision was made under general anesthesia and the withdrawal thresholds were measured before and after systemic and intrathecal administration of a COX-1 selective inhibitor (SC560). Immunohistochemistry was used to assess COX-1 protein in the spinal cord, and real-time polymerase chain reaction was used to quantify gene expression of COX-1 mRNA.

**Results:** Systemic and intrathecal administration of SC560 produced an increase in withdrawal threshold in the 4-week-old, but not in the 2-week-old, animals. Intrathecal SC560 increased withdrawal thresholds in the 4-week-old animals at a dose 100-fold less than with systemic administration. Cyclooxygenase 1 protein in the spinal cord was increased ipsilateral to surgery in the 4-week-old, but not in the 2-week-old, animals. Cyclooxygenase 1 mRNA was increased in the 4-week-old animals in the spinal cord ipsilateral to surgery relative to the contralateral side of the spinal cord, but not in the 2-week-old animals.

**Conclusions:** These results suggest that developmental differences in COX-1 expression in the spinal cord likely explain the lack of efficacy of COX-1 inhibitors in the 2-week-old rats. Whether this reflects a deficit in factors that stimulate COX-1 expression or a difference in response to these factors is not addressed, but should similar deficits occur in humans, COX-1 inhibitors may exhibit reduced efficacy in infants.

TISSUE trauma from surgery results in a localized inflammatory response, as well as direct neural injury to afferents innervating the site of injury. As such, peripheral and central nervous system signaling, which underlies pain after surgery, may differ from other types of inflammation, such as chemically induced inflammation.<sup>1</sup> Recent pain research has focused on neural-immune interactions in both the periphery and the spinal cord that induce hypersensitivity to thermal and mechanical stimuli.<sup>2</sup> As part of this, resident immune cells in the spinal

cord, microglia, become activated in many persistent pain states and elaborate several factors that result in central sensitization, including prostaglandins.<sup>3</sup>

Age-dependent differences in behavioral responses using the paw incision model have been established.<sup>4-6</sup> Age-dependent differences in baseline thermal and mechanical sensitivity exist.<sup>4</sup> In addition, developmental differences in resolution of hypersensitivity after paw incision have been shown whereby the resolution of the hypersensitivity in the very young rats occurs earlier than in the adult rats.<sup>4,5</sup> The hypersensitivity to paw incision in the very young also has been shown to be more sensitive to preemptive effects of local anesthetic nerve blockade.<sup>5</sup> Cyclooxygenase 1 (COX-1) has been shown to be upregulated in the spinal cord after the paw incision, and intrathecal inhibition of COX-1 reduces mechanical allodynia in the mature animal.<sup>7</sup> Spinal COX inhibition also reduces allodynia from nerve injury.<sup>8</sup> In contrast to these observations in adults, the effect of COX-1 selective inhibition in reducing mechanical allodynia after paw incision does not occur in very young animals.<sup>6</sup> This could be a result of differences in expression of the COX-1 or it could be that the increase in COX-1 expression in the younger animals occurs to a similar extent, but does not produce hypersensitivity after incision. Therefore, we hypothesized that developmental differences in response to inhibition of COX-1 are a result of developmental differences in COX-1 expression in the spinal cord in response to the pain signals from the periphery.

## Materials and Methods

### Behavior

After approval from the Animal Care and Use Committee (Wake Forest University, Forsyth County, Winston-Salem, NC), male Sprague-Dawley rats at 2 and 4 weeks of age were studied (weights,  $33 \pm 3$  and  $93 \pm 4$  g, respectively). Two-week-old animals are not yet weaned, and 4-week-old animals are weaned. After baseline testing, all animals were anesthetized with 2% halothane in oxygen under spontaneous ventilation through a nose cone. As previously described,<sup>4,9</sup> the plantar aspect of the left hind paw was prepared in a sterile manner with a 10% povidone-iodine solution. A midline incision from the heel to the base of the toes was performed using a no. 11 blade with sterile technique. Rather than a fixed-length incision, this created an incision that was relatively comparable as a function of the size of the paw for the different size and age animals.<sup>4</sup> A small forceps was

\* Associate Professor, † Medical Student, ‡ Anesthesia Resident, § Research Associate, ∥ Professor, Department of Anesthesiology and Center for the Study of Pharmacological Plasticity in the Presence of Pain, Wake Forest University School of Medicine, Winston-Salem, North Carolina.

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Address correspondence to Dr. Ririe: Department of Anesthesiology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina 27157-1009. dririe@wfuwmc.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

used to elevate the flexor tendon from the heel to the toes. The incision was closed with 5.0 nylon on an FS-2 needle using two inverted mattress sutures.

All the animals had a 2-h recovery period before testing. Preweanling animals recovered in the cage with their mothers. Baseline withdrawal thresholds 2 h after surgery were measured. The animals then underwent a second brief general anesthetic with halothane and received systemic or intrathecal SC560. Systemic administration was *via* a subcutaneous injection in the shoulder of vehicle or 10  $\mu\text{g}$  of SC560 (a COX-1 selective inhibitor) in 87% dimethyl sulfoxide/13% deionized water (vehicle) or vehicle control in a volume 1  $\mu\text{l/g}$  body weight. The intrathecal administration consisted of prepping the back with alcohol and placing a 30-gauge needle in the subarachnoid space as evidenced by a tail flick as previously described.<sup>10</sup> Our experience with this method using administration of local anesthetic has led to spinal blockade in 10 of 10 animals of 2 weeks of age and less. All animals were included in the data analysis, and no animal in the study had a wound dehiscence or infection during the study.

#### *Mechanical Stimulation Testing*

Animals were placed in a plastic cage with a mesh floor. They were acclimated to the environment for 20 min before testing. Mechanical allodynia was assessed using calibrated von Frey filaments to determine withdrawal thresholds to application of the filament on the footpad just anterior and lateral to the incision until the filaments bent. This was carried out by a person blinded to the treatment. The von Frey filaments used were 3.84, 4.08, 4.31, 4.56, 4.74, 4.93, 5.18, 5.46, and 5.88, corresponding to 0.5, 0.9, 1.7, 3.7, 5.5, 8.0, 12.4, 21.5, and 53.0 g, respectively. This was carried out three times, with a positive response determined by brisk withdrawal of the foot from the filament. The force resulting in withdrawal with a 50% probability was determined using the up-down method as previously described.<sup>11</sup> Mechanical withdrawal thresholds were determined before surgery, 2 h after surgery before injection of drug, and then at 1 h, 2 h, and 4 h after injection of drug.

#### *Immunohistochemistry*

All animals underwent a left paw incision as described above. Control animals without surgery and treated animals at 5 and 24 h after surgery were killed and were perfused with 4% paraformaldehyde. Spinal cords were dissected and cryopreserved in 30% sucrose. Spinal cord sections were cryosectioned and stored at  $-80^{\circ}\text{C}$ . Free-floating sections were processed in batches together to avoid processing effects and were stained as described previously.<sup>7,8</sup> Briefly, after treatment with  $\text{H}_2\text{O}_2$  and blocking with goat serum, COX-1 mouse monoclonal primary antibody (1:1000) and a biotinylated secondary goat antimouse antibody (1:200) were used to visualize

the staining with ABC reagent and were developed by 3,3'-diaminobenzidine with nickel enhancement. Sections then were dehydrated and covered with Permount (Biomedica Corporation, Foster City, CA) and coverslips. Digital images were captured using a microscope. Slides from the lumbar segment L5 were used for quantification. Cells in the spinal cord dorsal to the central canal staining positive for COX-1 were counted by a blinded observer. Ten to 15 spinal cord slices were counted from each animal and were averaged to give the number of positive cells per slice for each animal.

#### *Gene Expression and Real-time Polymerase Chain Reaction*

For the mRNA expression using reverse transcription and real-time polymerase chain reaction (PCR), the animals underwent anesthesia and surgery as above, and at baseline, 6 h and 1, 3, and 7 days the spinal cords were isolated, separated into right and left lumbar regions, and flash-frozen and stored at  $-80^{\circ}\text{C}$ . Briefly, tissue was thawed to  $4^{\circ}\text{C}$  and total RNA was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH). After the RNA was isolated, the experiments were processed in batches together to avoid processing effects. Reverse transcription was carried out using 1  $\mu\text{g}$  total RNA and a mixture of random hexamers and oligo dT primers, reverse transcription buffer, 2  $\mu\text{l}$  of dNTPs (5 mM each), and Omniscript reverse transcriptase (Applied Biosystems, Foster City, CA). The reaction was allowed to incubate at  $37^{\circ}\text{C}$  for 1 h, after which the Omniscript reverse transcriptase is inactivated by incubating at  $95^{\circ}\text{C}$  for 5 min.

TaqMan real-time PCR assays for COX-1 were performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Each RT-PCR consisted of 25  $\mu\text{l}$  of TaqMan universal PCR master mix (Applied Biosystems), 5  $\mu\text{l}$  forward primer, 5  $\mu\text{l}$  reverse primer (2  $\mu\text{M}$  each), 5  $\mu\text{l}$  TaqMan probe (2.5  $\mu\text{M}$ ), 5  $\mu\text{l}$  reverse transcription product, and 5  $\mu\text{l}$   $\text{H}_2\text{O}$ . The reaction is started at  $50^{\circ}\text{C}$  for 2 min,  $95^{\circ}\text{C}$  for 10 min, followed by 30 to 40 cycles of denaturation at  $95^{\circ}\text{C}$  for 15 s, and annealing/extending at  $60^{\circ}\text{C}$  for 1 min per cycle.

Gene-specific forward and reverse primers and the 5' end-labeled probes were designed using Primer Express Software 2.0 (Applied Biosystems), according to the software guidelines (forward primer, TGGCGTTGCTCATC-CATCT; reverse primer, GAAACTGGAGCGAGAGACTC-CCT; target, CTCCCAGAGTCATGAGTC). All primers and probes were purchased from Applied Biosystems. Internal standard 18S ribosomal RNA also was determined for each sample. Relative mRNA levels were determined by the  $\Delta\Delta$  Ct method because efficiencies for both 18S and COX-1 were equal. The COX-1 expression in the left spinal (surgery) cord was divided by the COX-1 in the right spinal (nonsurgery) cord to allow

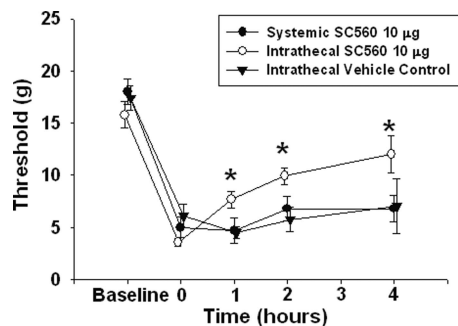


Fig. 1. Effects of selective cyclooxygenase 1 (COX-1) inhibition using SC560 after surgery in 4-week-old animals. The mechanical withdrawal threshold is presented over time. Baseline is before surgery and time 0 is 2 h after surgery and before injection of SC560. Intrathecal administration of 10 µg SC560 produced a time-dependent increase in withdrawal threshold ( $P < 0.05$ ) from baseline. At 1, 2, and 4 h, the intrathecal injection of 10 µg SC560 produced a higher withdrawal threshold than vehicle or systemic administration subcutaneously of the same 10-µg dose (\* significantly different from vehicle control).

comparison of the data from the 2-week-old and 4-week-old animals directly.

#### Statistical Analysis

Data are presented as mean  $\pm$  SE. Withdrawal thresholds were analyzed using repeated measures analysis of variance between groups of similar ages for time and treatment effects, followed by Fisher's protected least significant differences. Data for the immunohistochemistry are presented as means with SEM and were analyzed using a paired two-sample *t* test. The real-time PCR data were analyzed using analysis of variance with Fisher's protected least significant difference to test for effects. Multiple comparisons were adjusted for using the Bonferroni correction where appropriate. All results were considered significant if the *P* value was less than 0.05.

## Results

#### Behavior

Baseline withdrawal thresholds were  $7.2 \pm 0.6$  g in the 2-week-old animals and  $18.6 \pm 1.2$  g in the 4-week-old animals before surgery. After paw incision, the withdrawal thresholds decreased to  $2 \pm 0.3$  g in the 2-week-old animals and to  $4.6 \pm 1.3$  g in the 4-week-old animals. Administration of SC560 systemically in a dose of 10 µg (100 µg/kg) subcutaneously produced no significant increase in withdrawal threshold, whereas intrathecal administration of the same dose produced an increase in withdrawal threshold to a maximum effect of  $12.2 \pm 1.4$  g at 4 h (fig. 1). The difference between the subcutaneously administered systemic SC560 and the intrathecally administered SC560 was significant. However, the same dose of 10 µg (300 µg/kg) in the 2-week-old animals produced no effect on mechanical thresholds when administered either systemically or intrathecally (fig. 2).

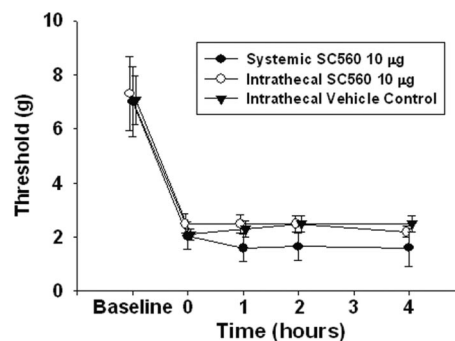


Fig. 2. Effects of selective cyclooxygenase 1 (COX-1) inhibition using SC560 after surgery in 2-week-old animals. The mechanical withdrawal threshold is presented over time. Baseline is before surgery and time 0 is 2 h after surgery and before injection of SC560. Systemic and intrathecal administration of the same dose as that in the 4-week-old animals (10 µg) produced no change in withdrawal threshold from baseline or from vehicle control. This is different than the response in the 4-week-old animals ( $P < 0.05$ ).

#### Immunohistochemistry

Cyclooxygenase 1 immunoreactivity in the dorsal horn of the spinal cord in 2-week-old and 4-week-old animals ipsilateral and contralateral to the paw incision are shown in figure 3. These are representative photomicrographs of COX-1 immunoreactivity at 24 h after surgery for the 2- and 4-week-old animals. The COX-1 immunoreactivity cells are smaller in the 2-week-old than the 4-week-old. There is a clear difference in COX-1 immunoreactivity between the ipsilateral and contralateral (fig. 3, A and B) spinal cord in the 4-week-old animals, whereas in the 2-week-old animals, there was no difference in COX-1 immunoreactivity of the cells between the ipsilateral and contralateral spinal cord (fig. 3, C and D).

Quantification of the COX-1 immunoreactivity was car-

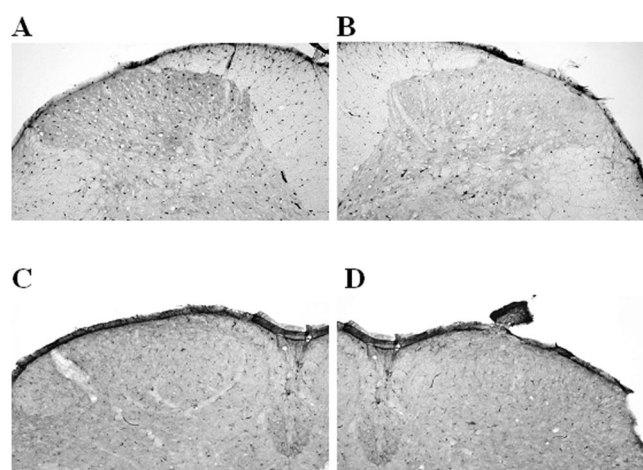
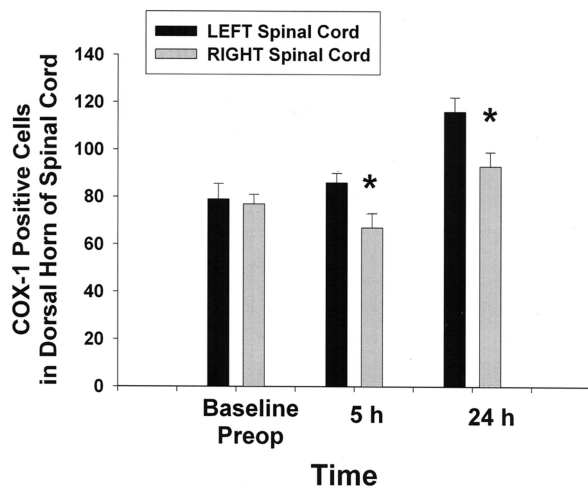


Fig. 3. Representative photomicrographs of cyclooxygenase 1 (COX-1) immunoreactivity at 24 h after surgery for the 2- and 4-week-old animals. The COX-1 immunoreactivity cells are less prominent in the 2-week-old spinal cord slices compared with the 4-week-olds. There is a clear difference in COX-1 immunoreactivity between the ipsilateral (A) and contralateral (B) spinal cord in the 4-week-old animals, whereas in the 2-week-old animals, there is no difference in immunoreactivity of the cells between the ipsilateral (C) and contralateral spinal cord (D).



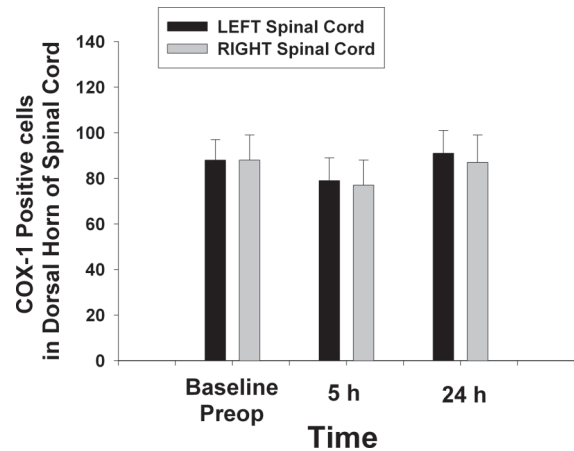


**Fig. 4.** Spinal cord cyclooxygenase 1 (COX-1) immunoreactivity after surgery in 4-week-old animals. COX-1 immunoreactivity in the spinal cord of 4-week-old animals after surgery. There was no difference between left and right COX-1 positive cells in the spinal cord before left paw surgery. However, there was a significant increase in COX-1 positive cells on the left (surgery side) compared with the right (nonsurgery side) at both 5 and 24 h after surgery (\* $P < 0.05$ ;  $n = 6$ ). Ten to 15 spinal cord slices were counted from each animal and were averaged to give the number of positive cells per slice for each animal.

ried out. COX-1 immunoreactivity was present in spinal cord in both the 2- and 4-week-old animals in the absence of surgery. The number of immunostained cells in the dorsal horn of the spinal cords at baseline was  $84 \pm 9$  in the 2-week-old animals and  $79 \pm 6$  in the 4-week-old animals. After paw incision, an increase in COX-1 immunoreactivity was seen in the dorsal horn of the spinal cord of the 4-week-old animals ( $n = 6$  at each time point) ipsilateral to surgery. This increase in COX-1 immunoreactivity in the 4-week-old animals was significant at 6 and 24 h after incision when compared with the contralateral side ( $P < 0.01$ ; fig. 4). The maximum increase in immunoreactivity was seen at 24 h. However, no difference in COX-1 immunoreactivity was seen over time in the dorsal horn of the spinal cord in the 2-week-old animals ipsilateral to surgery when compared with the contralateral side or baseline ( $n = 6$  at each time point) after surgery (fig. 5). The location of the staining in the dorsal horn was uniform across the groups for the 2- and 4-week-old animals, with COX-1 immunoreactivity cells being equally distributed in superficial (I-II) and deep (III-V) laminae in the dorsal horn over time.

#### Real-time PCR

The baseline relative expression of COX-1 in the 2-week-old animals was  $0.25 \pm 0.12$  and  $0.35 \pm 0.18$  in the left and right spinal cord, respectively, whereas in the 4-week-old animals, the baseline relative expression of COX-1 was  $0.59 \pm 0.61$  and  $0.51 \pm 0.53$  in the left and right spinal cord, respectively. Expression of left *versus* right in the same animal yielded an expression ratio of  $0.98 \pm 0.25$  for the 2-week-old animals and  $1 \pm 0.15$  for

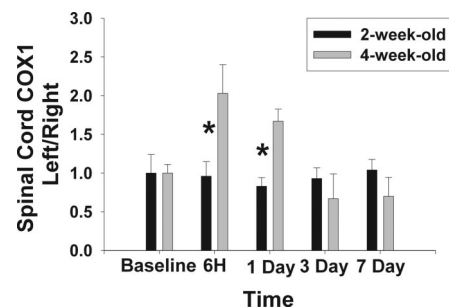


**Fig. 5.** Cyclooxygenase 1 (COX-1) immunoreactivity in the spinal cord of 2-week-old animals after surgery. There was no difference in COX-1 positive cells between left (surgery) and right spinal cords at any time ( $n = 6$ ). Ten to 15 spinal cord slices were counted from each animal and were averaged to give the number of positive cells per slice for each animal.

the 4-week-old animals. There was an increase in expression of COX-1 mRNA in the spinal cord of the 4-week-old animals ipsilateral to the paw incision as measured by relative real-time PCR (fig. 6). The expression of COX-1 is presented as an increase in COX-1 ipsilateral to paw incision relative to the contralateral side. The maximum increase in COX-1 expression of the 4-week-old animals was seen at 6 h after surgery and was twofold that of the contralateral spinal cord. This returned to baseline by 3 days. However, whereas COX-1 expression was present in the 2-week-old spinal cord, there was no increase in expression of the COX-1 in the 2-week-old spinal cord ipsilateral to the surgery at any time after paw incision.

#### Discussion

In 4-week-old rats, systemic administration of a COX-1 inhibitor produced a dose-dependent reduction in post-



**Fig. 6.** Cyclooxygenase 1 (COX-1) mRNA expression by reverse transcription real-time polymerase chain reaction after surgery. Data are presented as relative expression of COX-1 in the left side of the spinal cord (surgery side) divided by the relative expression of COX-1 in the right side of the spinal cord (nonsurgery side) for each animal at each time point ( $n = 8$ ). There is a nearly twofold increase in expression of COX-1 in the 4-week-old animals at 6 and 24 h after surgery (\* $P < 0.05$ ). There is no change in the 2-week-old spinal cord at any time after surgery.

operative hypersensitivity as measured by withdrawal thresholds.<sup>6</sup> However, systemic administration of a COX-1 inhibitor in the 2-week-old group produced no such reduction in hypersensitivity. This suggests that there was less COX-1 activity in the 2-week-old animals compared with the 4-week-old animals after surgery, or that COX-1 activity fails to induce hypersensitivity at the younger age. The current results in the 4-week-old animals agree with previous studies in adult rats,<sup>7</sup> indicating that the major site for the antiallodynic effect of COX-1 inhibitors after incisional surgery is in the central nervous system, probably the spinal cord. This is consistent with the high lipid solubility of the SC560, allowing distribution into the central nervous system when given systemically and potentially having its effect centrally.<sup>12</sup>

The lack of efficacy of the intrathecal administration of the COX-1 inhibitor in the 2-week-old animals suggests that either the same activation of COX-1 does not occur in the 2-week-old animals or that the increase in activity fails to induce hypersensitivity in the 2-week-old animals. The COX-1 protein is present in the 2-week-old animals at baseline by real-time PCR and by immunohistochemistry. However, the increase in gene expression as measured by increase in messenger RNA for COX-1 in conjunction with the increase in COX-1 staining in cells in the dorsal horn of the spinal cord ipsilateral to the surgery occurred only in the 4-week-olds and not in the 2-week-old animals. This strongly suggests that the cause of the differential effects of COX-1 inhibition after paw incision is the result of the developmental difference in induction of COX-1 expression after peripheral trauma between 2 and 4 weeks of age. This suggests that the event that induces an increase in COX-1 in the spinal cord is under developmental regulation.

The notion that COX-1 activity and expression changes as a function of age is not novel. COX-1 activity and expression as well as prostaglandin E<sub>2</sub> production have been shown to increase with increasing age.<sup>13</sup> Although these same observations have not been extended to the very young, increases in COX-1 during development have been seen in other tissues consistent with developmental regulation of COX-1 expression.<sup>14</sup> The lack of increased expression of COX-1 after peripheral trauma and inflammation in the young in our study further suggests that the younger animals either lack a response element or do not receive the same signals in the spinal cord to induce COX-1 as the older animals.

The control of COX-1 expression is poorly understood, and its regulation clearly differs from COX-2. Nitric oxide increases activity of COX-1 without increasing expression level in adult animal models of inflammation.<sup>15</sup> However, nitric oxide synthetase is developmentally regulated and may be involved in the developmental differences in activity of COX-1. In the spinal cord in lamina II, nitric oxide synthetase is low in the newborn, but gradually increases to adult level by 3 weeks of age.<sup>16</sup> Be-

cause the differences in response to SC560 occur between 2 and 4 weeks postnatally, nitric oxide synthetase could play a role by limiting increases of activity of the enzyme. This does not explain the differences in expression between the different ages of development. We did not measure enzyme activity in the current study, although the inhibition of allodynia by SC560 in the older animals is consistent with the possibility that there is not only increased COX-1 protein, but enhanced COX-1 activity in the older animals that is not present in the younger animals.

Cyclooxygenase 1 expression is increased in the 4-week-old animals after surgery as measured by increased mRNA as well as immunohistochemistry. The knowledge of the regulation of expression and induction of COX-1 in the central nervous system, and in particular the spinal cord, is limited. Classically, in tissues throughout the body, it has been thought that COX-2 is the inducible isoform and COX-1 is the constitutively expressed isoform, but evidence suggests that both isoforms are constitutively expressed in the spinal cord.<sup>17-19</sup> In addition, the idea that COX-1 is an inducible isoform in many tissues, including the spinal cord, has gained acceptance.<sup>6,20,21</sup>

Cyclooxygenase 1 and COX-2 seem to be under different control such that one can be induced without the other being affected. COX-2 has been shown to be upregulated in the presence of inflammatory insults or nerve injury, whereas COX-1 is not.<sup>22,23</sup> COX-1 is increased in the spinal cord after surgical incision, and the pain from surgical incision is reduced by intrathecal administration of selective COX-1 inhibitors, but not by selective COX-2 inhibition.<sup>6,7,20</sup> Therefore, the generation of the pain from inflammation or nerve injury seems to be distinct from that generated from surgical incision. This is consistent with other data whereby the mechanism of pain from surgical incision seems to be distinct from the mechanism of the inflammatory pain.<sup>24-26</sup>

The cells that have an increase in COX-1 in the spinal cord are thought to be microglia.<sup>17,20</sup> Activation of microglia in the central nervous system from peripheral stimulation must result from some signal arising from the peripheral neurons. The exact signal activating microglia and how this is coupled to activate COX-1 upregulation is unclear. However, there are a number of factors that may activate microglia centrally. One possible mediator of microglia activation is fractalkine and the fractalkine receptor.<sup>27</sup> In neuropathic pain, the fractalkine receptor is upregulated in the microglia and may allow these cells to be activated by the fractalkine. Whether fractalkine is involved in incisional pain is not known, but will be of interest. Another such target for glial activation may be the muscarinic acetylcholine receptors that seem to be present in microglia in other areas of the central nervous system and may play a role in upregulation of COX-1, probably *via* nitric oxide synthetase.<sup>21</sup> Elucidation of the

incoming signal and the transduction pathway to the glia will be crucial to understanding the age-dependent differences in expression of COX-1 in younger animals.

This study has focused on the local spinal cord effects of the surgical incision and the role of COX-1. The role of COX-2 and even possibly the COX-3 isoform has not been elucidated centrally or in the periphery in animals of different ages. Whether this lack of efficacy of central COX-1 inhibition occurs in human infants for mechanical allodynia after surgery is unknown. Furthermore, whether the laboratory markers of thermal hypersensitivity and mechanical allodynia in the animal are similar in the human after surgery, particularly at very young ages, is unknown. COX inhibition is a cornerstone of adjunctive pain management in the postoperative period in patients of all ages. Studies to determine the regulation of COX-1 in the spinal cord will be important to the understanding of postoperative pain during development and in the adult. Understanding the differences in response to drugs during development and the role of the isoforms are important for improving understanding and treatment of pain and analgesia in response to surgical trauma in the young.

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