

## Effect of Cyclooxygenase-1 Inhibition in Postoperative Pain Is Developmentally Regulated

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THE paw incision in the rat has been established as a reproducible model to study acute postoperative pain in adult animals and during development.<sup>1-3</sup> Age-dependent differences in behavioral responses using the paw incision model have been established by showing that 2-week-old animals recover more rapidly than older animals after paw incision as measured by withdrawal threshold to mechanical stimulation.<sup>2</sup> The etiology of this difference has not been studied.

One possible explanation is that similar tissue injury results in differences in the inflammatory response as a function of development. The production of cytokines is one marker of inflammation and developmental differences in production of tumor necrosis factor  $\alpha$  and interleukin  $1\beta$  have been found to be developmentally regulated after surgical tissue trauma.<sup>4</sup> However, the role of other inflammatory mediators in developmental responses to pain is unknown.

Cyclooxygenase (COX) enzymes and their products represent targets of intense interest in pain sensation and treatment. COX enzymes and prostaglandins have been studied extensively during the past 30 yr to determine their roles in the inflammatory response. During the past 10 yr, COX-2 has been given particular attention and has led to the development of selective compounds to maximize pain relief while reducing the negative effects caused by nonselective inhibition of COX isozymes.<sup>5</sup> COX-1 and COX-2 are constitutively expressed in the rat spinal cord, and intrathecal administration of COX-2 inhibitors reduces mechanical allodynia caused by peripheral inflammation from complete injection of Freund's adjuvant.<sup>6,7</sup> COX-2 has been shown to be expressed in the spinal cord after paw incision, but this expression only occurs for a very short time after the injury.<sup>8</sup> However, Zhu and Eisenach<sup>9</sup> recently showed that intrathecal injection of the selective COX-2 inhibitor NS-398 had minimal analgesic effects in an incisional model of postoperative pain. In contrast, spinal COX-1 inhibition, by intrathecal injection of the selective com-

pound SC560, produced a dose-dependent decrease in mechanical allodynia in adult animals after incisional surgery.<sup>10</sup> The authors also showed that COX-1 expression in the spinal cord increases in response to paw incision. COX-1 is also up-regulated in the spinal cord of rats in other models of pain, such as partial sciatic nerve ligation and L5-L6 spinal nerve ligation.<sup>9</sup> These findings suggest that COX-1 plays a potentially important role in the inflammatory response to peripheral tissue and nerve injury in mature rats.<sup>9-11</sup> The role of COX-1 in young animals and potential age-dependent behavioral responses to acute postoperative pain, however, have not been characterized. In this study, we hypothesize that development plays a role in age-specific behavioral responses to COX-1 inhibition as measured using response to mechanical and thermal stimuli.

### Materials and Methods

After approval from the Animal Care and Use Committee (Wake Forest University School of Medicine, Winston-Salem, North Carolina), male Sprague-Dawley rats at 2 and 4 weeks of age were studied (weight,  $33 \pm 3$  and  $93 \pm 4$  g, respectively). Two-week animals are preweanling, and 4-week animals are postweanling. After baseline testing, all animals were anesthetized with 2% halothane in oxygen under spontaneous ventilation through a nose cone. As previously described,<sup>1,2</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 foot for the differently sized and aged animals.<sup>2</sup> A small forceps was 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 thresholds 2 h after surgery were measured. This was followed by subcutaneous injection in the shoulder of 0.1, 0.3, or 1 mg/kg SC560 (a COX-1 selective inhibitor) in 87% dimethyl sulfoxide-13% deionized water (vehicle) or vehicle control in a volume 1  $\mu$ l/g body weight. All animals were included in the data analysis, and no animal in the study had a wound dehiscence or infection during the study.

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### Mechanical Stimulation Testing

All 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 by placing the filament on the footpad just anterior and lateral to the incision until the filaments bent. 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 done three times, with a positive response determined by brisk withdrawal of the foot from the filament. The force to withdrawal with a 50% probability was determined using the up-down method as previously described.<sup>12</sup> Mechanical withdrawal thresholds were determined before surgery, 2 h after surgery before injection of drug, and then at 1, 2, and 4 h after injection of drug. A person blinded to the treatment of the animals performed all sensory testing.

### Thermal Stimulation Testing

Withdrawal latencies were measured by placing the animals in a clear plastic box on a glass surface with a constant temperature of 30°C. A calibrated light heat source was focused on the hind paw, and time to paw withdrawal was measured. A 30-s exposure was not exceeded, to avoid tissue injury. The withdrawal latency was measured three times in each paw just anterior and lateral to the incision in the middle of the footpad. The three latencies were averaged for each animal at each time point. Withdrawal latencies were determined before surgery, 2 h after surgery, and at 2 and 4 h after injection of SC560 or vehicle. A person blinded to the treatment of the animals performed all thermal testing.

### Statistics

Data are presented as mean  $\pm$  SE. Withdrawal thresholds and thermal latencies were analyzed using repeated measures analysis of variance between groups of similar ages for time and treatment effects using analysis of variance. Differences were assessed using Fisher protected least significant differences. Multiple comparisons were adjusted for using the Bonferroni correction where appropriate. Significance was  $P < 0.05$ .

## Results

### Mechanical Stimulation

The withdrawal thresholds to mechanical stimulation before surgery were  $5.9 \pm 0.4$  for the 2-week-old animals ( $n = 10$ ) and  $19 \pm 0.8$  g for the 4-week-old animals ( $n = 8$ ), which are significantly different ( $P < 0.05$ ). After surgery, withdrawal thresholds decreased significantly in both age groups ( $P < 0.05$ ), and the relative decreases at each age were similar (67% for the 2-week-old animals

### Threshold Response vs Time (SC560 1 mg/kg)

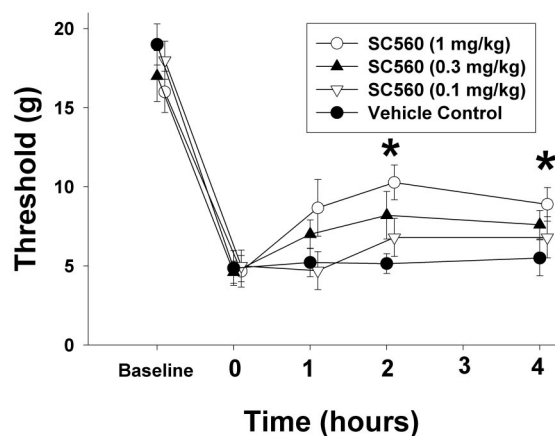


Fig. 1. Mechanical withdrawal threshold in grams versus time for the cyclooxygenase-1 selective inhibitor SC560 (open circles, 1 mg/kg [ $n = 8$ ]; closed triangles, 0.3 mg/kg [ $n = 8$ ]; open triangles, 0.1 mg/kg [ $n = 8$ ] or vehicle control (open circles [ $n = 8$ ]) in 4-week-old animals. Baseline is withdrawal threshold before surgery. Time 0 is 2 h after recovery from surgery and just before administration of study drug or vehicle. A dose-dependent increase drug effect was seen. Actual thresholds were only significantly different from baseline for the 1-mg/kg dose at 2 and 4 h as indicated by the asterisk, which is  $P < 0.05$  compared with vehicle control.

and 79% for the 4-week-old animals). SC560 in 4-week-old animals produced a dose-dependent increase in withdrawal threshold ( $P < 0.05$ ;  $n = 8$  each dose) (fig. 1). This was significantly different from the control vehicle animals, in which no response was found over the course of the study ( $P < 0.05$ ;  $n = 8$ ). The maximum analgesic effects of SC560 occurred at 1 mg/kg, and the peak effect was 2 h after administration in the 4-week-old animals. In contrast, SC560 was ineffective over this dose range in the 2-week-old animals, and there was no difference when compared to vehicle control ( $n = 10$  for each). The response to the highest dose of SC560 (1 mg/kg) versus control over time is presented for the 2-week-old animals (fig. 2). The 2- and 4-week-old animals were compared for changes from baseline threshold at the 2-h time point for SC560. The 2- and 4-week-old vehicle control groups had no significant change in threshold over time. COX-1 inhibition produced an increase in threshold in the 4-week-old animals compared with the control group ( $P < 0.05$ ), whereas it produced no effect in the 2-week-old animals.

### Thermal Stimulation

The baseline withdrawal latencies before surgery were different for the 2-week-old animals ( $18.8 \pm 1.1$  s;  $n = 10$ ) and the 4-week-old animals ( $12.2 \pm 0.5$  s;  $n = 8$ ) ( $P < 0.05$ ). Withdrawal latencies to thermal testing decreased significantly after surgery in both age groups, with the relative decreases in latencies being similar (78% in the 2-week-old animals and 70% in the 4-week-

### Threshold Response vs Time (SC560 1 mg/kg)

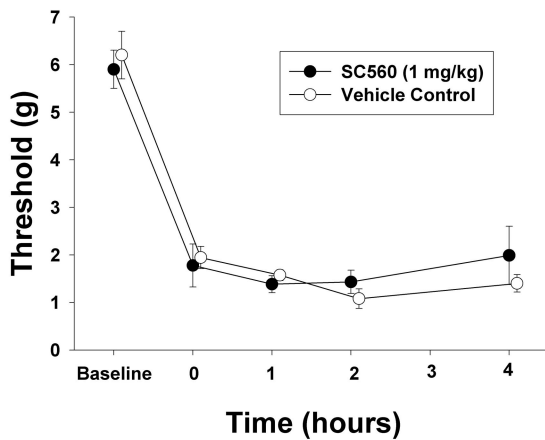


Fig. 2. Mechanical withdrawal threshold *versus* time for SC560 (1 mg/kg) and vehicle control in 2-week-old animals. There was no effect of SC560 ( $n = 10$ ) or vehicle ( $n = 10$ ) on withdrawal threshold in 2-week-old animals at any time.

old animals). The dose of SC560 that had a maximum effect on mechanical testing (1 mg/kg) produced an increase in withdrawal latency to thermal testing in the 2-week-old animals over time ( $n = 10$ ;  $P < 0.05$ ) (fig. 3). There was no effect on thermal withdrawal latency in vehicle control animals ( $n = 10$ ). In contrast, no effect of 1 mg/kg SC560 on thermal withdrawal latency was found in the 4-week-old animals in the treated ( $n = 8$ ) and control animals ( $n = 8$ ).

### Thermal Latency vs Time (SC560 1 mg/kg)

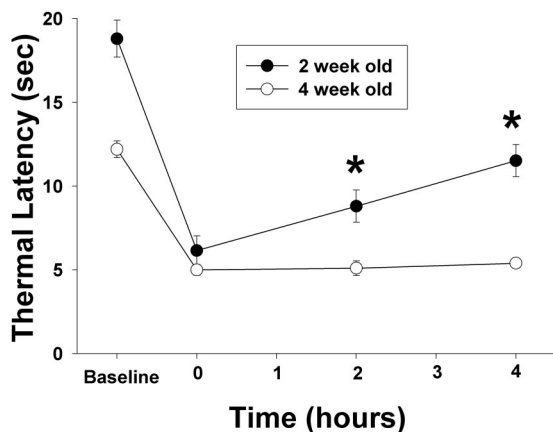


Fig. 3. Thermal withdrawal latency *versus* time for the cyclooxygenase-1 inhibitor (SC560) in 2- and 4-week-old animals. The dose of SC 560 was 1 mg/kg. There was a significant increase in thermal latencies in the 2-week-old animals ( $n = 10$ ) over time ( $P < 0.05$  compared with predrug value). There was no effect in the 4-week-old animals ( $n = 8$ ). There was a significant difference between age groups ( $P < 0.05$ ). \*  $P < 0.05$  compared with vehicle control for the 2-week-old animals.

### Discussion

In the 4-week-old rats, COX-1 inhibition demonstrated a significant reduction in postoperative hypersensitivity to local mechanical stimulation near the wound, whereas the 2-week-old animals had no such response. This suggests that developmental changes between 2 and 4 weeks of age in the rat result in a role for COX-1 in the generation of postoperative mechanical allodynia. However, the effect of COX-1 inhibition on hypersensitivity to thermal stimulation near the surgical wound was quite different. COX-1 inhibition produced a significant reduction in postoperative thermal hypersensitivity only in the 2-week-old animals, with no such effect in the 4-week-old animals. This discrepancy may relate to different fiber types stimulated by these modalities, to neural development of the sensory system during this period of rapid change, or to differences in the inflammatory response and chemical mediators from the surgical incision at different times in development.

Responses to mechanical and thermal stimuli reflect activation of different nerve fiber types. The punctate mechanical stimulation in the current study primarily activates A fibers, whereas the signal from slow skin heating is carried primarily by C fibers.<sup>13</sup> Interestingly, differences in A fibers and C fibers exist during development. A- $\beta$  fibers exhibit greater extension into lamina II in the spinal cord in early life, which resolves as development occurs, so that adult A- $\beta$  fibers terminate primarily in lamina III-V.<sup>14</sup> In contrast, C fibers seem to have their terminal connection in the spinal cord established very early in development, before the age of testing in our study. However, functional maturation of C fibers is delayed, and many chemicals in C fibers do not reach adult level until some weeks after birth.<sup>15-17</sup> Related to these functional changes, the receptive field does not seem to expand in response to injury until probably after 2 weeks of age.<sup>18</sup> These differences may contribute to the observed differences in responses of the differently aged animals.

In addition to the neuroanatomic differences that may contribute to the observed differences, effects of COX-1 inhibition on mechanical withdrawal thresholds may occur *via* other mechanisms in the spinal cord. Certainly, differential expression of COX-1 in the spinal cord may occur between 2 and 4 weeks of age. This is a time during development when many systems are developing and changing rapidly. Differences in immune function that may alter the inflammatory response to tissue trauma from surgery; developmental differences in opioid, *N*-methyl-D-aspartate receptors, and other receptors systems; and neuroanatomical changes are all occurring.<sup>14,15,18-21</sup> Therefore, it is likely that changes are also occurring in expression of COX-1 in response to surgical injury. This could be either from specific developmental regulation of COX-1 expression or from differences in



the immune response to the tissue trauma resulting in changes in COX-1 expression.

Increased expression of COX-1 in the spinal cord of the mature animals after paw incision suggests that the effects of COX-1 inhibition on mechanical thresholds are spinally mediated.<sup>9,10</sup> This increase in COX-1 expression in microglia in the spinal cord may be responsible for the effects of SC560 in the 4-week-old animals in our study. However, COX-1 inhibition in the 2-week-old group produced no significant reduction in mechanical allodynia. Therefore, it is possible that COX-1 is not expressed in the spinal cord microglia in response to the paw incision in the 2-week-old animals as it is in older animals. Because 4-week-old animals are anatomically similar to adults in regard to A- $\beta$  fiber connections in the dorsal horn, it is possible that these neuroanatomical connections are important for COX-1 up-regulation. In contrast, lack of A- $\beta$  fiber connections to the deeper laminae of the dorsal horn, either from normal development or from the injury, may explain why 2-week-old animals did not respond to treatment with the SC560 (COX-1-specific inhibitor).

The paradox of the ability of COX-1 inhibition using SC560 to produce a totally opposite effect on thermal latency when compared to mechanical thresholds with respect to development is unexpected. The developmental differences in A fiber-mediated responses can be explained by recognizing that COX-1 may be up-regulated in the spinal cord of the adult rat but not in the younger rat. However, this does not explain the differences in C fiber-mediated thermal responses. Ma and Eisenach<sup>22</sup> observed COX-1 expression in the epidermis of the adult rat paw after surgical injury following the Brennan model. Therefore, one possible explanation is that COX-1 is expressed in the periphery in response to surgery in greater quantities in the 2-week-old animals compared with the 4-week-old animals, or there may be a difference in response to a product of COX-1 in the periphery.

Developmental expression of prostaglandin receptors for COX-1 products in the spinal cord could also explain age-specific thermal hypersensitivity responses to COX-1 inhibition. The prostaglandin receptor EP<sub>3</sub> has been shown to mediate thermal hyperalgesia pain sensitivity while EP<sub>1</sub> receptors seem to modulate mechanical allodynia in response to PGE<sub>2</sub> administered intrathecally.<sup>23</sup> Inhibition of COX-1 in the 2-week-old animals produced thermal latency increases, whereas there was no effect in the 4-week-old animals. Therefore, there may be a developmental shift in expression of EP receptor subtypes, which may explain the paradoxical behavioral responses to thermal and mechanical stimuli.

Age-dependent differences in the inflammatory response to injury and wound healing exist. Differences in cytokine production and other neurotrophic factors in response to injury occur.<sup>24</sup> Evidence suggests that ther-

mal sensitivity after incision may be mediated through one of these neurotrophic factors, in particular nerve growth factor.<sup>25</sup> Nerve growth factor and its receptor TrkA expression have also been shown to be altered during development and as a function of tissue injury.<sup>26,27</sup> Therefore, it is possible that the COX enzyme products are linked to either the expression or the effect of nerve growth factor on thermal sensitivity in the younger animals and not in the older animals. This provides an exciting avenue for further investigation into the differential age-dependent effects of inflammation in pain and possibly even wound healing.

Controversy still exists regarding the role for COX-1 and COX-2 in different models of pain.<sup>28,29</sup> The differences may be related in part to differences in models and types of pain as well as measurement of pain and dosing of the inhibitors. In addition, there is most likely a role for inhibition of both enzymes to provide the most efficacious pain relief, and there is clearly a developmental difference in response at least to COX-1.<sup>30</sup> Therefore, despite the fact that others have demonstrated a lack of effect of spinal COX-2 inhibition in the acute postoperative incision model of pain, it is still important to elucidate the role of COX-2 in developmental modulation of pain.<sup>31</sup>

The results of COX-1 inhibition in the modified postoperative pain model suggest there is a reciprocal relation regarding COX-1 expression as a function of development. Whether these reciprocal responses to COX-1 inhibition between the 2- and 4-week-old rats are a result of age-specific developmental expression in the peripheral or central nervous system is unclear. Further studies of COX-1 expression in the spinal cord and periphery as a function of development will be important in elucidating the etiology of the differences in behavioral responses to postoperative pain as a function of age. Knowledge of the anatomical and biologic mechanisms behind these responses will help in understanding postoperative pain during development and may lead to more developmentally appropriate treatment of acute postoperative pain.

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