# Nociception in cyclooxygenase isozyme-deficient mice

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**Prostaglandins formed by cyclooxygenase-1 (COX-1) or COX-2 produce hyperalgesia in sensory nerve endings. To assess the relative roles of the two enzymes in pain processing, we compared responses of COX-1- or COX-2-deficient homozygous and heterozygous mice with wild-type controls in the hot plate and stretching tests for analgesia. Preliminary observational studies determined that there were no differences in gross parameters of behavior between the different groups. Surprisingly, on the hot plate (55°C), the COX-1-deficient heterozygous groups showed less nociception, because mean reaction time was longer than that for controls. All other groups showed similar reaction times. In the stretching test, there was less nociception in COX-1-null and COX-1-deficient heterozygotes and also, unexpectedly, in female COX-2-deficient heterozygotes, as shown by a decreased number of writhes. Measurements of mRNA levels by reverse transcription–PCR demonstrated a compensatory increase of COX-1 mRNA in spinal cords of COX-2-null mice but no increase in COX-2 mRNA in spinal cords of COX-1-null animals. Thus, compensation for the absence of COX-1 may not involve increased expression of COX-2, whereas up-regulation of COX-1 in the spinal cord may compensate for the absence of COX-2. The longer reaction times on the hot plate of COX-1-deficient heterozygotes are difficult to explain, because nonsteroid anti-inflammatory drugs have no analgesic action in this test. Reduction in the number of writhes of the COX-1-null and COX-1-deficient heterozygotes may be due to low levels of COX-1 at the site of stimulation with acetic acid. Thus, prostaglandins made by COX-1 mainly are involved in pain transmission in the stretching test in both male and female mice, whereas those made by COX-2 also may play a role in the stretching response in female mice.**

Prostaglandins (PGs), mostly $PGE_2$ and prostacyclin, are important mediators of inflammation, pain, and fever. They are synthesized in tissues by the constitutive enzyme cyclooxygenase-1 (COX-1) and by its isoform, COX-2, which can be induced with cytokines, growth factors, or other inflammatory stimuli (1). PGs are hyperalgesic and enhance nociception produced by other mediators such as bradykinin (2). The nonsteroid anti-inflammatory drugs are analgesic by preventing the synthesis of PGs (3).

Noxious stimuli cause tissue damage and release pain-producing substances that activate nociceptors on the terminals of sensory nerve fibers. PGs increase neuronal activity in nociceptive nerve fibers by raising cAMP levels and lowering the activation threshold for opening of tetrodotoxin-resistant sodium channels in the neuronal membrane. In the skin, nociceptors on myelinated, medium-velocity Aδ fibers respond rapidly with a sensation of "sharp" pain, whereas those on the slow-conducting C fibers mostly respond with delayed, "dull" pain (4–6). Visceral pain is generally diffuse (poorly localized) and often referred to other intact tissues (7). There is evidence for the presence of polymodal C fibers and Aδ fibers in the gut (8–10). Painful sensations reach higher brain centers by a number of spinal nerve pathways, some of which are monosynaptic and others, polysynaptic (11). All sensory pathways synapse in the dorsal horn of the spinal cord, and most have synapses in the thalamus and periaqueductal gray matter of the midbrain. COX-1 and COX-2 both have been identified in the brain and spinal cord of humans and rats; COX-2, as well as COX-1, is constitutively expressed (12–14). It is likely, therefore, that PGs made by both COX enzymes are involved in hyperalgesia. The nonselective COX inhibitors, such as ibuprofen and aspirin, have long been in use as analgesics and act peripherally (15), because they are highly polar and cross the blood–brain barrier with difficulty. The recently developed selective COX-2 inhibitor, rofecoxib, is less polar and crosses the blood–brain barrier easily. It has been registered for clinical use for the alleviation of pain.

To investigate the role of the two isoforms of COX in pain transmission, we have used the hot-plate test as a model of "sharp," rapidly transmitted pain and the stretching test, also called abdominal constriction or writhing (16), as a model of slowly developing, diffuse pain in COX-1- or COX-2-deficient homozygous and heterozygous mice and wild-type controls. A preliminary observational study was performed to assess any gross differences in the behavior of the knockout compared with control animals. The levels of the mRNA for the two COX enzymes also were measured in the pawpads and spinal cords of the enzyme-deficient and control animals.

## Materials and Methods

**Animals.** Strain C57/DBA1 of COX-1-deficient, COX-2-deficient, and wild-type (WT) mice used in these studies was developed recently at the Memphis Veterans Affairs Medical Center. The strain was created by back-crossing C57BL/J6 COX-deficient mice with WT DBA/1 mice for six generations followed by extensive intercrossing. Adult mice of this strain did not exhibit reduced longevity or the severe renal pathology described for the original COX-2-deficient C57BL/J6 strain (17). However, both the COX-1-null and COX-2-null female mice were infertile.

Animals were housed in Plexiglas cages at 25°C ± 1°C and kept on a 12-h light/12-h dark cycle. Food and water were available *ad libitum*. All experimental mice were offspring of a number of simultaneous matings and, at 6–8 weeks of age, weighed 18–23 g. The experiments were performed by using animals of genotypes COX-1- and COX-2-deficient homozygotes and heterozygotes and their wild-type control littermates. The genotype of each animal was determined by PCR.

**Genotyping.** DNA from tails was extracted by using the Dneasy Tissues Kit from Qiagen (Chatsworth, CA). Briefly, samples from tail were lysed by using proteinase K, and the lysate was loaded on to a minicolumn. After the column was washed, DNA was eluted in water or a buffer ready for PCR. Three primers were used in the same PCRs for identification of the COX-1 or

Abbreviations: PG, prostaglandin; COX, cyclooxygenase; WT, wild type; RT-PCR, reverse transcription–PCR.

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COX-2 allele. The WT allele 5′ primer (COX1–5′) AGGAGATGGCTGCTGAGTTGG, the mutant allele 5′ primer (COX1-Neo) GCAGCCTCTGTTCCACATACAC, and the 3′ primer (COX1–3′) AATCTGACTTTCTGAGTTGCC were used to yield a fragment of 600 or 700 bp for the COX-1 WT or mutant allele, respectively. The WT allele 5′ primer (COX2-Δ9) ACACACTCTATCACTGGCACC, the mutant allele 5′ primer (NeoPro) ACGCGTCACCTTAATATGCG, and the 3′ primer (TGC2–3) GTACGGCTTCAGGGAGAA yield a fragment of 600 or 800 bp for the COX-2 WT or mutant allele, respectively.

**Behavior.** Mice were placed singly in the center of an open field measuring 25 × 25 cm and observed for 5 min. The amounts of rearing, grooming, locomotion, defecation, urination, and jumping were measured either by the number of times the behavior occurred or with a visual score (+, ++, or +++).

**Analgesia.** *Hot-plate test.* The reaction time of each animal was measured in an apparatus made by Columbus Instruments (Columbus, OH) consisting of a flat surface measuring 25 × 25 cm, maintained at 55°C, and surrounded by a transparent, plastic box. Animals were placed on the heated surface and removed immediately after they licked the footpad of any paw. The time spent on the hot plate (reaction time) was recorded.

*Stretching test.* Animals were injected i.p. with 0.1 ml/10 g 1.2% acetic acid and then placed singly in a plastic animal cage measuring 25 × 50 cm. Responses were measured by following the method first described by Collier *et al.* (16). Stretching responses, defined as constriction of the abdomen with stretching of the hind limbs, were counted for 15 min after the i.p. injection. Animals were killed immediately after each 15-min experiment.

**Reverse Transcription–PCR (RT-PCR).** The construction and *in vitro* transcription of the mutant mouse COX-1 and COX-2 as internal standards for mRNA semiquantitation as well as RNA extraction, RT-PCR, and gel electrophoresis were similar to the methods described previously (18). The sets of primers used in this study were based on the cloned mouse COX-1 and COX-2 cDNA sequences (19, 20) as follows: for generating internal standard, for COX-1, 5′-AGGAGATGGCTGCTGAGTTGGCCAGCACGTTCGGTGGTGAC-3′ (sense) and 5′-ATCTGACTTTCTGAGTTGCC-3′ (antisense); COX-2, 5′-ACACACTCTATCACTGGCACC-3′ (sense) and 5′-TTCAGGGAGAAGCGTTTGCACAAACTGAGTGAGTCCATGTT-3′ (antisense); for reverse transcription, COX-1, CAACCAGAAATCTGACTTTCTGA-3′ (antisense); for generating internal standard for COX-2, 5′-GTACGGCTTCAGGGAGAA-3′ (antisense); for PCR, COX-1, 5′-AGGAGATGGCTGCTGAGTTGG-3′ (sense) and 5′-AATCTGACTTTCTGAGTTGCC-3′ (antisense) (602 or 437 bp for COX-1 WT or internal standard, respectively); for PCR, COX-2, 5′-ACACACTCTATCACTGGCACC-3′ (sense) and 5′-TTCAGGGAGAAGCGTTTGC-3′ (antisense) (274 or 143 bp for COX-2 WT or internal standard, respectively). All PCRs were performed by using a DeltaCycler II System (Ericomp, San Diego). The plasmids were purified by using the Qiagen Plasmid Kit. *Eco*RI, *SSt*I, or *Ban*I was used for restriction mapping of purified plasmids of COX-1 or COX-2, respectively. Total RNA was extracted by following the modified method of Chomczynski and Sacchi (21), using Tri-Reagent according to the manufacturer's protocol (Sigma). cDNA samples were amplified by PCR, using Ready-To-Go PCR Beads (Amersham Pharmacia) in a total volume of 25 ml. To remain in the linear range during amplification, reactions were carried out for 32 cycles (94°C for 15 s; 60°C for 15 s; and 72°C for 1 min; 7 min for the last extension) and 35 cycles (94°C for 15 s; 55°C for 15 s; and 72°C for 1 min; 7 min for the last extension) for COX-1 and COX-2 mRNA measurements, respectively. Results from three repeated PCRs from each cDNA sample were used for statistical analyses. All data were expressed as means ± SEM and subjected to one-way ANOVA. When appropriate, posthoc analysis was done by using the Student–Newman–Keuls test. A significance level of $P < 0.05$ was applied to all data.

**Materials.** Analytical-grade glacial acetic acid (Sigma) was diluted with pyrogen-free saline to provide a 1.2% solution for i.p. injection. All other compounds used were obtained from Sigma unless otherwise designated. The mice were obtained from a colony maintained at the Memphis Veterans Affairs Medical Center. All experiments were performed at the Memphis Veterans Affairs Medical Center in accordance with procedures approved by the Animal Safety Committee.

## Results

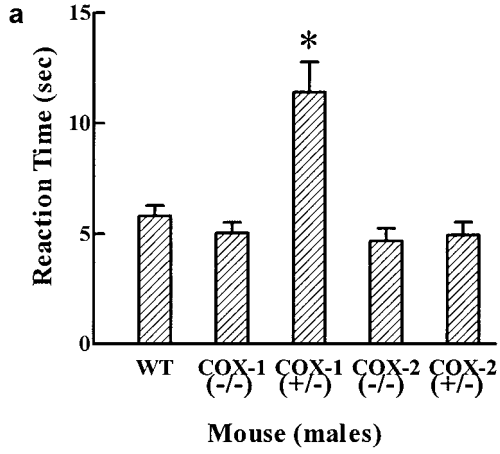
**Behavior.** By simple observation, the only difference between the WT and gene-deficient groups of mice in the behavior parameters being studied (results not shown) was that the COX-2-deficient animals appeared to be less excitable and easier to handle than the other groups. This docile behavior was most obvious in female COX-2-null mice.

**Analgesia.** *Hot-plate test.* Reaction time on the hot plate was delayed in both male and female COX-1-deficient heterozygous animals compared with WT controls, showing decreased nociception. Thus, mean reaction time ± SEM was 5.8 ± 0.48 s in male WT mice, and this increased significantly to 11.38 ± 1.37 s in male COX-1-deficient heterozygotes. Female WT mice had a mean reaction time of 7.08 ± 0.61 s, and this was increased significantly to 10.73 ± 1.60 s in female COX-1-deficient heterozygotes. The mean reaction times of COX-1-null, COX-2-null, and COX-2-deficient heterozygote males and females did not differ from the WT controls (Fig. 1).

*Stretching test.* Decreased nociception, as shown by a reduction in the mean number of writhes in 15 min, was evident in COX-1-null and COX-1-deficient heterozygote males and females compared with WT controls. Thus, the mean number of writhes ± SEM was 17.5 ± 1.57 for WT male mice, and this fell significantly to 2.8 ± 2.8 for COX-1-null and to 4.6 ± 1.21 for COX-1-deficient heterozygous males. Similarly, the mean number of writhes ± SEM was 12.8 ± 1.94 for WT female mice, and this was reduced significantly to 2.8 ± 0.99 for COX-1-null and to 3.86 ± 1.39 for COX-1-deficient heterozygous females. The number of writhes in COX-2-deficient heterozygous females also was reduced significantly compared with WT controls to a mean ± SEM of 5.6 ± 1.7, indicating less nociception in these animals (Fig. 2). There was no difference from WT controls in the number of writhes in COX-2-null and COX-2-deficient heterozygote males or in COX-2-null females.

**Expression of COX-1 and COX-2 in Pawpads and Spinal Cords.** *COX-1 mRNA analyses.* Reduced mRNA levels for COX-1 were observed in pawpads of COX-1-deficient heterozygous animals compared with WT controls, whereas the levels in COX-2-null and COX-2-deficient heterozygotes were similar to controls. Spinal cord tissue of COX-2-null and COX-2-deficient heterozygous animals expressed levels of COX-1 mRNA higher than the control values measured in WT mice (Fig. 3*a*). These high COX-1 mRNA levels also were evident from the gel electrophoresis data in Fig. 4*a*. In the case of COX-2-null mice, the raised COX-1 mRNA levels may illustrate a regulatory mechanism to compensate for the absence of COX-2.

*COX-2 mRNA analyses.* The spinal cords of COX-1-null, COX-1-deficient heterozygote, and COX-2-deficient heterozygote mice showed no difference in COX-2 mRNA levels from WT controls (Figs. 3*b* and 4*c*). However, COX-2-deficient heterozy-



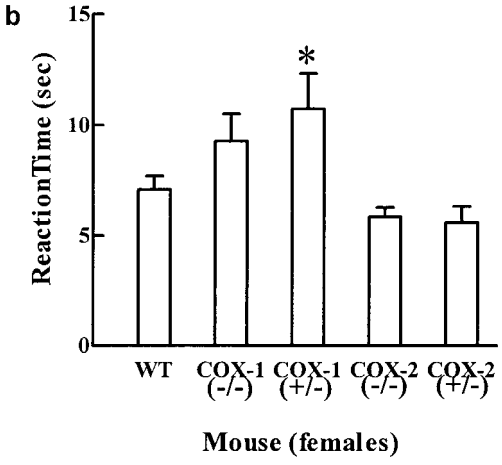
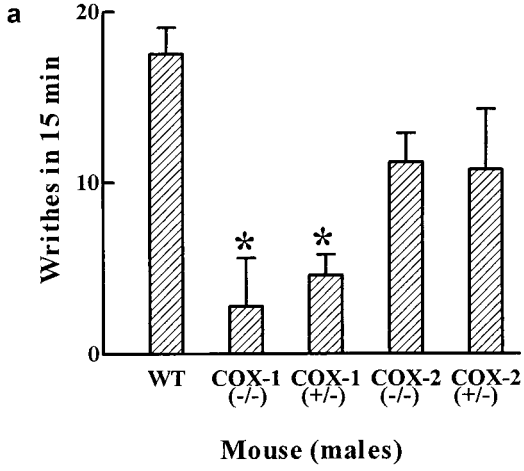




**Fig. 1.** Reaction times of COX-deficient male (*a*) and female (*b*) mice in the hot-plate test. The time taken (s) for COX-1-deficient heterozygous mice to lick their paws on a hot plate at 55°C was greater than the time taken by WT animals. The increase in reaction time in COX-1-deficient heterozygotes indicated less nociception in these animals. Histograms represent means for 5 animals (males) and 7–10 animals (females) ± SEM; *, $P < 0.05$ when compared with WT controls.



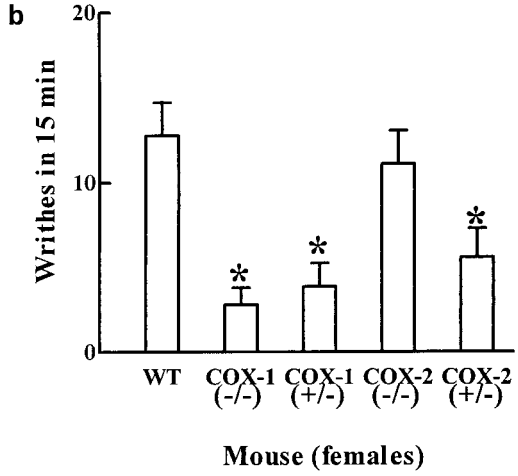




**Fig. 2.** Writhing responses in COX-deficient male (*a*) and female (*b*) mice. The number of writhing responses in 15 min in the stretching test for COX-1-deficient homozygous or heterozygous mice injected i.p. with 1.2% acetic acid was less than the number of responses by WT animals. This indicated a decrease in nociception in these mice. COX-2-deficient heterozygous females also showed fewer writhing responses and decreased nociception. Histograms represent means for 4–6 animals (males) and 7–10 animals (females) ± SEM; *, $P < 0.05$ when compared with WT controls.

gous mice and COX-1-deficient heterozygotes expressed lower levels of COX-2 mRNA in pawpads than WT controls (Figs. 3*b* and 4*d*). COX-2 mRNA levels in pawpads of COX-1-null mice were similar to those of WT controls.

## Discussion

The concept of a constitutive COX-1 and an inducible COX-2 now has been firmly established. However, COX-2 is also constitutively expressed in human brain in amounts equal to COX-1 (12), and it is the predominant isoform in the spinal cord of the rat (13). Rat stomach tissue microsomes constitutively express about 5% of COX as COX-2 protein (22), and the macula densa of the rat kidney expresses constitutive COX-2 that is up-regulated by salt deprivation (23). We also have found COX-2 mRNA in the spinal cord and in the pawpad skin of control WT mice. In animals in which the COX-2 gene had been deleted, COX-1 mRNA was up-regulated in the spinal cord. This was *in vivo* confirmation of the *in vitro* finding that cultured lung fibroblasts obtained from COX-2-deficient mice expressed greater amounts of COX-1 than control WT cells that express both enzymes (24). Thus, the increase in COX-1 enzyme compensated for the loss of COX-2. However, there was no reciprocal increase in COX-2 to compensate for the loss of COX-1 enzyme from the spinal cords of COX-1-deficient mice as measured by COX-2 mRNA.

Injection of carrageenan into the rat footpad produced hyperalgesia and an increase in PG levels in the spinal cord. The selective COX-2 inhibitor, celecoxib, but not the selective COX-1 inhibitor, SC560, reduced hyperalgesia in the footpad and prevented the rise of PG levels in the spinal cord (25). This observation suggests that COX-2 is involved in mediating both a peripheral and a central neurological component of inflammatory pain. To evaluate further the role of COX-1 and COX-2 isoenzymes in nociception, we have used two models of analgesia in COX-1-deficient and COX-2-deficient mice. The reaction time on a heated metal plate normally is prolonged by centrally acting, morphine-like analgesics but not by nonsteroid anti-

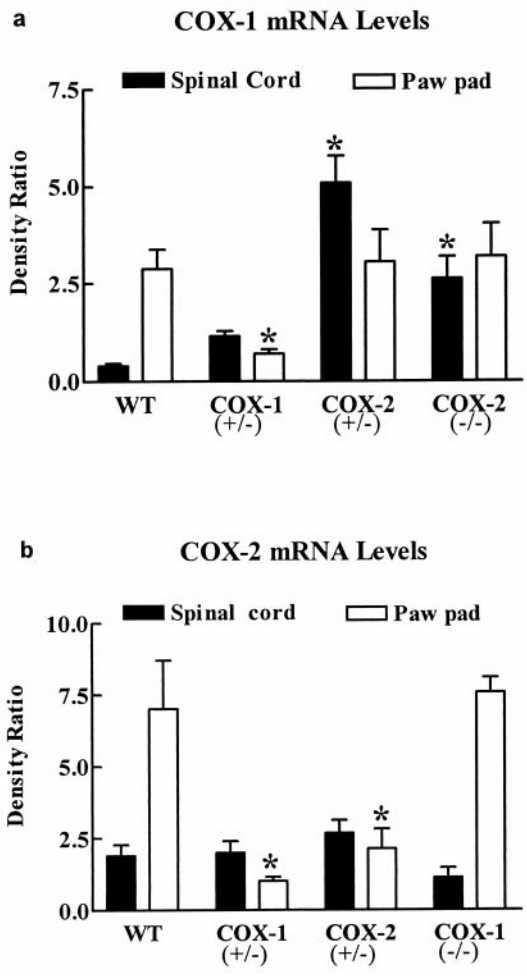



**Fig. 3.** COX-1 (*a*) and COX-2 (*b*) mRNA levels in spinal cords and skin of pawpads of WT and COX-deficient mice. The levels of COX mRNA present in tissue are expressed as ratios of densitometric measurements of samples compared with their internal standard. mRNA for COX-1 was not found in COX-1-null mice, and mRNA for COX-2 was not found in COX-2-null mice. Data are presented as mean ± SEM from 6–12 determinations on samples from three to four animals. *, $P < 0.05$ when compared with WT controls.

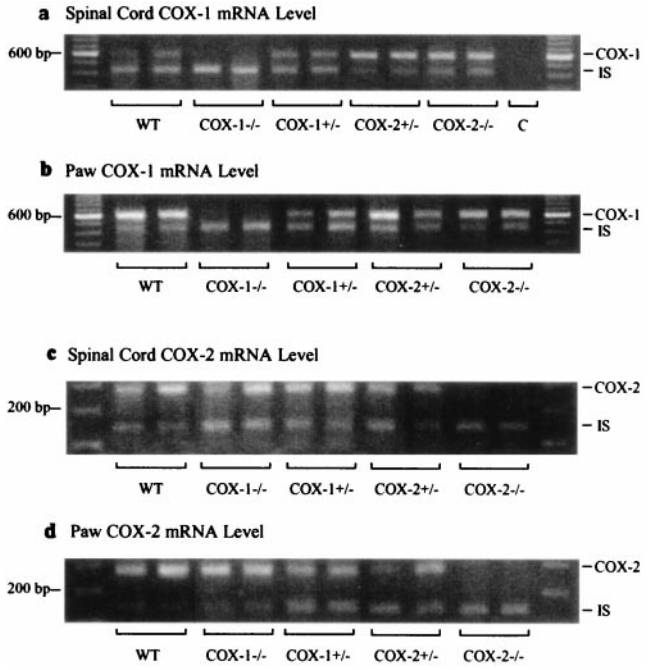



**Fig. 4.** Gel electrophoresis of RT-PCR-amplified COX-1 or COX-2 mRNA and their corresponding internal standard cRNAs (IS) in spinal cord (*a* and *c*) or pawpads (*b* and *d*) of WT and COX-1- or COX-2-deficient mice. Control (C) samples without RNA were used to verify that RT-PCR was free from contamination.

inflammatory drugs, whereas the number of writhing responses to i.p. acetic acid injection are reduced not only by morphine but also by peripherally acting, anti-inflammatory analgesics such as indomethacin (16). In the hot-plate test, there were comparable responses in COX-1-deficient, COX-2-deficient homozygous mice and the WT controls. However, the COX-1-deficient heterozygotes clearly exhibited less nociception (longer reaction times) than control animals. One possible explanation is that when the COX-1 enzyme is absent, compensatory mechanisms are triggered during fetal development, and reactions of these homozygous mice are normal. When the amount of COX-1 enzyme is merely reduced, as in the heterozygous mice, the trigger for compensation does not occur and the lower PG production causes prolonged reaction times. RT-PCR studies have confirmed the low COX-1 mRNA levels in the pawpads of COX-1-deficient heterozygous mice, but this peripheral COX-1 may not be involved in the nociceptive response to thermal stimulation. The increase in COX-1 mRNA in the spinal cords of COX-2-deficient homozygotes may constitute compensation for the absence of COX-2. However, reduction of central COX-1 enzyme levels is compensated by mechanisms that do not involve COX-2. No gross differences in behavior were noted between the different groups of mice that could account for different responses to nociceptive stimuli.

In the writhing test, it was clear that decreased COX-1 levels resulted in decreased nociception in COX-1-deficient heterozygotes and an even greater decrease in COX-1-null homozygotes. Thus, compensatory mechanisms did not operate to produce normal responses to the i.p. injection of acetic acid. The writhing response to acetic acid is brought about by the release of prostacyclin synthesized by cyclooxygenase in the abdominal cavity of the mice (26). Up-regulation of COX-1 mRNA in the spinal cords of COX-2-deficient homozygotes and heterozygotes may explain the normal writhing responses to acetic acid in male mice. However, in female mice, writhing was reduced in COX-2-deficient heterozygotes, indicating incomplete compensation for the absence of COX-2 in the spinal cords. It also may confirm the studies of Smith *et al.* (25), who demonstrated a central component of inflammatory pain mediated by COX-2. COX-1 mRNA levels were determined only in male mice, so it is unknown whether they would be low in the female COX-2 heterozygous animals in contrast to the high values recorded in male mice. Gender differences may exist in pain-processing pathways. Recent studies have demonstrated that female patients experience pain relief with κ agonist opiate analgesics such as pentazocine, whereas male patients do not (27). κ opiate receptors together with COX-2 mRNA have been identified in the dorsal horn of the spinal cord, and these mechanisms may interact differently in male and female animals (28).

We have demonstrated that increased levels of COX-1 mRNA above control values in the spinal cord of mice may compensate for the absence of COX-2 in COX-2-null and COX-2-deficient heterozygous males to maintain normal responses to thermal and chemical stimulation. No increase occurred in COX-2 mRNA to compensate for the absence of COX-1. Thus, other mechanisms of compensation must be involved. A graded reduction in the number of writhing responses in COX-1-deficient

heterozygous animals and COX-1-null mice correlated with the reduction in COX-1 mRNA levels, demonstrating an absence of compensatory mechanisms in the sensory pathways of these mice. Writhing responses also were reduced in female COX-2-deficient heterozygous mice, possibly because of reduction in COX-2 levels in the spinal cord. Thus, peripheral COX-1 mediates nociception in slowly developing pain in mice, the writhing response, and central COX-1 may be involved in rapidly transmitted pain caused by thermal stimulation, the hot-plate test. The analgesic potency of a range of nonsteroid anti-inflammatory drugs in relieving tooth-extraction pain in humans correlates closely with increasing selectivity toward COX-1 rather than COX-2 (29, 30). COX-2, most likely in the spinal cord, may mediate nociception in the writhing response of female mice, supporting the concept of a gender difference in pain perception.

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