Prostaglandin Synthase 1 Gene Disruption in Mice Reduces Arachidonic Acid–Induced Inflammation and Indomethacin-Induced Gastric Ulceration

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Summary

Cyclooxygenases 1 and 2 (COX-1 and COX-2) are key enzymes in prostaglandin biosynthesis and the target enzymes for the widely used nonsteroidal anti-inflammatory drugs. To study the physiological roles of the individual isoforms, we have disrupted the mouse *Ptgs1* gene encoding COX-1. Homozygous *Ptgs1* mutant mice survive well, have no gastric pathology, and show less indomethacin-induced gastric ulceration than wild-type mice, even though their gastric prostaglandin E_2 levels are about 1% of wild type. The homozygous mutant mice have reduced platelet aggregation and a decreased inflammatory response to arachidonic acid, but not to tetradecanoyl phorbol acetate. *Ptgs1* homozygous mutant females mated to homozygous mutant males produce few live offspring. COX-1-deficient mice provide a useful model to distinguish the physiological roles of COX-1 and COX-2.

Introduction

The cyclooxygenase isoforms, COX-1 and COX-2, catalyze the key step in the synthesis of prostaglandins. While COX-1 and COX-2 catalyze the same reaction, the conversion of arachidonic acid (AA) to prostaglandin H_2 (PGH_2), the isoforms appear to have different biological roles. COX-1 and COX-2 are also the target enzymes for the most widely used drugs in human medicine, nonsteroidal anti-inflammatory drugs (NSAIDs). Thus, there is considerable interest in understanding the physiological roles of COX-1 and COX-2 and in developing drugs that differentially inhibit them.

The COX isoforms are encoded by genes located on different chromosomes (Wen et al., 1993). The gene encoding COX-1 (*Ptgs1* in the mouse) spans about 22 kb (Yokoyama and Tanabe, 1989; Kraemer et al., 1992), from

which a message of 2.8 kb is derived (DeWitt and Smith, 1988; Merlie et al., 1988; Yokoyama et al., 1988). The gene encoding COX-2 (*Ptgs2* in the mouse) is about 8 kb (Xie et al., 1991; Kujubu et al., 1991; O'Banion et al., 1991) and produces a message of about 4.1 kb (Xie et al., 1991; Kujubu et al., 1991; O'Banion et al., 1991; Kujubu and Herschman, 1992; DuBois et al., 1994b). The COX-1 and COX-2 proteins from the same species are about 60% identical with the catalytic regions being conserved.

The genes encoding COX-1 and COX-2 differ in their regulation at the transcriptional level, and recent data reinforce the likelihood that the isoforms mediate different biological functions (Morita et al., 1995; Murakami et al., 1994). COX-1 appears to be constitutively synthesized in many tissues (Simmons et al., 1991; O'Neill and Ford-Hutchinson, 1993; Seibert et al., 1994), although its level of expression can vary with the state of differentiation or following cytokine or tumor promoter stimulation (Smith et al., 1993, 1994; Samet et al., 1995; Murakami et al., 1995). COX-1 is thought to carry out primarily "housekeeping" functions such as cytoprotection of the gastric mucosa, regulation of renal blood flow, and platelet aggregation (DeWitt and Smith, 1988, 1990; Merlie et al., 1988; Funk et al., 1991). In contrast, COX-2 message and protein are normally undetectable in most tissues; however, COX-2 expression in certain cell types can be rapidly induced by proinflammatory or mitogenic agents, including cytokines, endotoxins, tumor promoters, and mitogens (Xie et al., 1991; O'Banion et al., 1992; Hla and Neilson, 1992; Fletcher et al., 1992; DuBois et al., 1994a; Smith et al., 1994). Because of this rapid induction, the gene encoding COX-2 has been termed an immediate-early response or primary response gene (Simmons et al., 1989; Maier et al., 1990; Fletcher et al., 1992; Ryseck et al., 1992). COX-2 message and protein have been shown to be up-regulated in human colon cancers (Eberhart et al., 1994; Kargman et al., 1995) and in mouse skin papillomas and carcinomas (Muller-Decker et al., 1995).

The COX isoforms are the primary target enzymes for NSAIDs, which act by inhibiting the activity of the enzymes (Vane, 1971; Smith and Willis, 1971; Smith et al., 1990; Xie et al., 1992; Seibert et al., 1994; Masferrer et al., 1994; Mitchell et al., 1994; Seibert and Masferrer, 1994). Aspirin, the most common and best-studied NSAID, was originally shown to inhibit prostaglandin synthesis by Vane (1971). NSAIDs in common use today include aspirin, ibuprofen, and indomethacin, and all inhibit the COX enzymes. In addition to the use of NSAIDs as analgesics and for alleviation of acute and chronic inflammatory diseases such as arthritis (Levi and Shaw Smith, 1994; Simon, 1994), NSAIDs (in particular, aspirin) have proven effective for decreasing the frequency of heart attacks and strokes (Vane and Botting, 1992) and in reducing the incidence of colon cancer (Thun et al., 1991, 1993; Marnett, 1992). Some NSAIDs also inhibit chemically induced colon cancer in rodents (Rao et al., 1995, and references therein).

From the combined evidence of NSAID effects on rodent and human colon cancers, it has been suggested that randomized prevention trials with humans be initiated (Heath et al., 1994). However, while NSAIDs have many beneficial effects, they can also cause adverse side effects, the most common of which are gastrointestinal ulceration and nephrotoxicity (Clive and Stoff, 1984; Black, 1986; Brooks and Day, 1991; Price and Fletcher, 1990; Simon, 1994).

Since the discovery of COX-2, the identification of drugs that selectively inhibit this isoform has become the focus of NSAID development (Xie et al., 1992; Meade et al., 1993; Seibert and Masferrer, 1994; Mitchell et al., 1994; Masferrer et al., 1994). The rationale for this is that COX-1 is not elevated during inflammation and that, as a necessary housekeeping gene, its inhibition by NSAIDs may be the cause of their adverse side effects. In contrast, COX-2 is normally nondetectable in most tissues, but is rapidly elevated during inflammation (Masferrer et al., 1994; Crofford et al., 1994; Vane et al., 1994; Mitchell et al., 1994; Seibert et al., 1994; Vane, 1994; Simmons et al., 1991), and its inhibition by NSAIDs is thought responsible for their therapeutic effects.

The relative biological contributions of the COX-1 and COX-2 isoforms in the maintenance of normal physiological functions and in diseased states is not, however, entirely clear. Most of the current knowledge has come from studies of NSAID inhibition, COX-2 induction, or both. As an alternative approach to understand the roles of these enzymes better, we have used gene targeting to generate a mouse strain that is unable to synthesize COX-1. The development of COX-2-deficient mice is reported by Morham et al. (1995 [this issue of Cell]). With these mice, we hope to learn more about the roles of the two isoforms in normal physiology and in various inflammatory disorders and to understand better the etiology of the therapeutic effects and deleterious side effects of NSAIDs. In the present study, we report the generation of mice lacking COX-1 and some of their phenotypic characteristics.

Results

Cloning of the 3' Region of the Ptgs1 Gene

The 3' region of the mouse Ptgs1 gene was cloned using a 357 bp probe synthesized from strain 129 mouse embryonic stem (ES) cell DNA. The probe was made by polymerase chain reaction (PCR) with primers for the 5' end of exon 11, the sequences of which were based on the human COX-1 gene structure (Yokoyama and Tanabe, 1989) and the mouse cDNA sequence (DeWitt et al., 1990). This probe detected only a single band on Southern blots of mouse ES cell DNA digested with Xbal, HindIII, BglII, BamHI, or SacI, indicating that it was specific for a single gene. The 15 kb fragment cloned was subsequently shown to be from Ptgs1, and not the isoform Ptgs2, by the following. First, three different primer pairs specific for sequences in exon 11 of Ptgs1 were used for PCR, and the predicted product sizes were obtained. Second, about 100 bp of DNA 3' of the ClaI site in exon 11 (Figure 1A) was
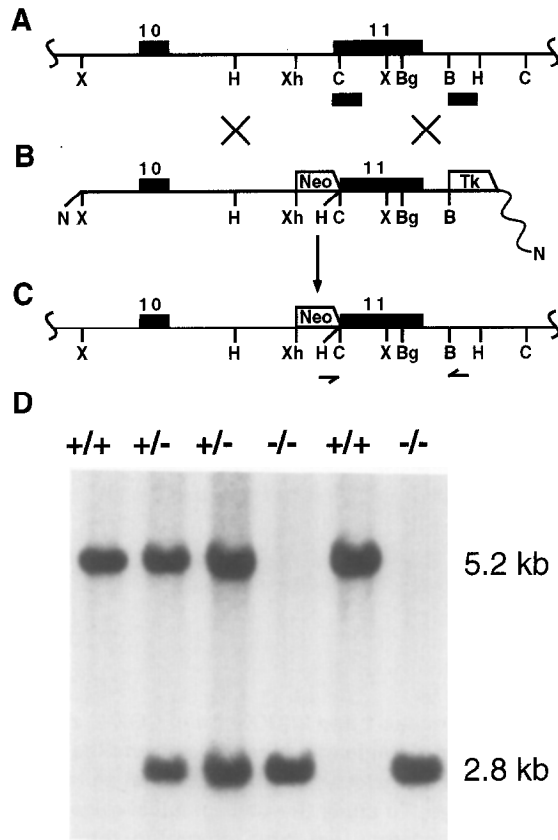

Figure 1. Targeted Disruption of the Mouse Ptgs1 Gene

(A) The 3' region of the Ptgs1 gene. Closed boxes represent exons, and bars below represent the probes. Restriction sites: Bg, BglII; B, BamHI; C, ClaI; H, HindIII; X, Xbal; Xh, Xhol.

(B) Targeting construct. Phosphoglycerolkinase-promoted neomycin and herpes simplex thymidine kinase genes are represented by Neo and Tk, respectively. Wavy lines represent plasmid (not to scale). N shows the linearizing NotI restriction site.

(C) Targeted Ptgs1 locus. Diagnostic PCR primers are indicated by arrows.

(D) Southern blot of HindIII-digested targeted allele (2.8 kb) and wild-type allele (5.2 kb) mouse tail DNA. Wild type (+/+), heterozygotes (+/−), and homozygote mutants (−/−) are shown.

sequenced from the fragment and shown to match the published sequence of Ptgs1 (DeWitt and Smith, 1990). Third, PCR with primers specific for the 5' and 3' ends of Ptgs1 exon 10 produced the correct sized product from the cloned fragment. Fourth, the 2.4 kb PCR fragment used to diagnose targeted clones (Figure 1C) was digested by BglII into fragments of the predicted sizes.

Vector Construction and Targeting

The targeting strategy for disruption of the Ptgs1 gene is shown in Figure 1. Since aspirin inactivates COX-1 by acetylating Ser-530 (Humes et al., 1981; Roth et al., 1983; DeWitt et al., 1990), we disrupted the gene prior to the codon for this amino acid in exon 11. The targeting vector was designed to replace about 1 kb of intron 10, together with the splice junction and first 44 bp of exon 11, with the neomycin resistance (Neo) gene (Figure 1B). If a pro-

tein were made from the resulting disrupted gene, it would lack the carboxy-terminal 120 amino acids, including Ser-530. Alternate splicing to eliminate the *Neo* gene would result in the elimination of 14 amino acids and loss of proper reading frame.

The plasmid was linearized with NotI and electroporated into strain 129–derived E14TG2a ES cells. Following electroporation, G418 and ganciclovir selection was started (Mansour et al., 1988), and 6 of 96 doubly resistant colonies isolated were positive for the expected 2.4 kb PCR product indicated in Figure 1C. Using the exon 11 probe indicated in Figure 1A, we confirmed targeting in these PCR-positive clones by detection on Southern blots of the expected 2.8 kb HindIII fragment. The same 2.8 kb band was also detected with a 510 bp BamHI–HindIII fragment that hybridizes to a genomic region 3′ to the targeting construct (Figure 1A).

Production of Animals

Cells from two of the targeted clones were injected into C57BL/6J (B6) blastocysts, resulting in the birth of four male chimeras. One chimera produced heterozygous F1 129/B6 offspring after mating to B6 females. From the first seven F2 litters obtained by mating F1 heterozygous siblings, 14 wild-type, 31 heterozygous, and 16 homozygous mutant weanling pups were obtained, in agreement with Mendelian expectations (p > 0.9). Southern blots of HindIII-digested tail DNA from wild-type F2, heterozygous F2, and homozygous mutant F2 mice are shown in Figure 1D.

General Health of the COX-1-Deficient Mice

The COX-1-deficient mice develop normally and appear healthy. Necropsy and microscopic examination of selected tissues (liver, spleen, kidney, gastrointestinal tract, reproductive tract, heart, and lungs) from three homozygous mutant males and three homozygous mutant females, aged 2–5 months, revealed no significant pathology. However, in three of six kidneys examined from homozygous mutant mice, a minimal change was present, characterized by one or two small foci per section of basophilic, immature tubules. The size and frequency of these lesions did not change with age. All six wild-type age-matched controls did not show these foci. There were no consistent or remarkable findings in other tissues examined, including the glandular and nonglandular stomach.

Northern Blot Analysis of the COX-1 Message

The effect of *Ptgs1* gene disruption on *COX-1* mRNA was analyzed by determining the level of message in the colons and kidneys of wild-type, heterozygous, and homozygous mutant F2 mice using the 357 bp probe described above. Figure 2A shows that the level of full-length (2.8 kb) message is reduced to approximately half normal in the heterozygous mice. No 2.8 kb message is detected in the homozygous mutant mice. Similar results (data not shown) were obtained when the 1.7 kb fragment of mouse cDNA (Oxford Biomedical) was used as the probe. When the blots were rehybridized to a probe specific for the *Neo* gene,
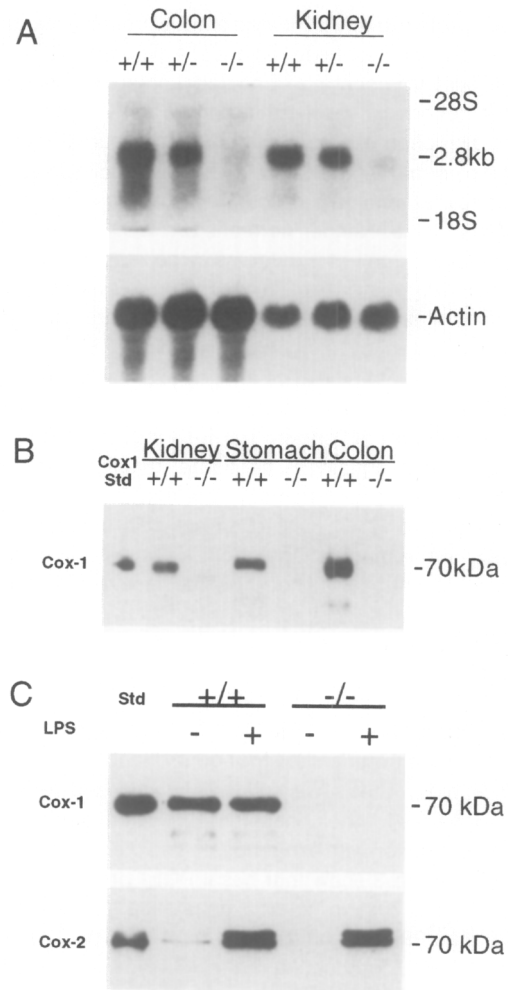

Figure 2. Northern and Western Blot Analyses

(A) Northern blot of total RNA from colon and kidney of wild-type (+/+), heterozygous (+/−), and homozygous mutant (−/−) mice. The blot was probed with a 357 bp PCR fragment from the *COX-1* cDNA.
(B) Western blot of microsomal protein from kidney, stomach, and colon of wild-type (+/+) and homozygous mutant (−/−) mice. The COX-1 standard is in the left lane. The protein was detected with a polyclonal antibody to COX-1.
(C) Western blot of total protein from control and LPS-stimulated peritoneal macrophages from wild-type (+/+) and homozygous mutant (−/−) mice. (Top) COX-1 levels in control and LPS-stimulated peritoneal macrophages from wild-type and homozygous mutant mice; a COX-1 standard is on the left; the protein was detected with an antibody to COX-1. (Bottom) COX-2 levels in control and LPS-stimulated peritoneal macrophages from wild-type and homozygous mutant mice; a COX-2 standard is on the left; the protein was detected with a polyclonal antibody to COX-2.

a band at 1.1 kb was detected (data not shown) in the heterozygous and homozygous mutant RNA samples; as expected, the band was more intense in the homozygous mutant sample. The *Neo* probe did not hybridize with any band detected by the *COX-1* probes.

Western Blot Analysis of the COX-1 Protein

A polyclonal antibody against residues 274–289 of COX-1 (Morita et al., 1995) was used to determine the level of

COX-1 protein or any truncated product from the disrupted gene that includes these residues. Amino acids 274–289 would still be present in a truncated protein if it were made, as the codons for these amino acids are 5' to the site of gene disruption. Western blot analysis (Figure 2B) shows that the normal 70 kDa COX-1 protein is readily detectable in kidney, stomach, and colon microsomes of wild-type F2 *Ptgs1* mice. Neither normal-sized COX-1 protein nor any smaller fragment is detected in the same tissues from the homozygous mutant mice. Figure 2C (upper panel) demonstrates that COX-1 protein levels are not significantly affected by lipopolysaccharide (LPS) in macrophages from wild-type mice and, as expected, are not detectable in the homozygous mutant mice. The bottom panel of Figure 2C shows that LPS induces the COX-2 protein about equally in peritoneal macrophages from COX-1 wild-type and homozygous mutant mice. COX-2 message and PGE_2 production are also induced about equally in the macrophages from wild-type and homozygous mutant mice (data not shown). These results indicate that disruption of *Ptgs1* prevents constitutive synthesis of COX-1 but does not alter *Ptgs2* inducibility in macrophages.

PGE_2 Production in Peritoneal Macrophages

To determine the effect of *Ptgs1* gene disruption on prostaglandin biosynthesis, we isolated and analyzed peritoneal macrophages for their basal (not LPS-stimulated) level of PGE_2 production with exogenous AA as the substrate. The data in Figure 3 show that basal PGE_2 production is reduced about 70% in heterozygous mice and is reduced more than 99% in homozygous mutant mice. The basal levels of PGE_2 production in the wild-type and heterozygous mice were not altered by a 6 hr incubation in medium containing dexamethasone, indicating that PGE_2 production is due to COX-1 and not to COX-2, the production of which is inhibited by dexamethasone. These data show that basal PGE_2 production is virtually absent in macrophages from the *Ptgs1* disrupted mice and that COX-2 contributes little to basal levels of PGE_2 from exogenous AA in unstimulated macrophages.

Gastric Ulceration

The COX-1 homozygous mutant mice from F2 and subsequent generations did not have gross or microscopic gastric lesions; therefore, the absence of COX-1 alone is not sufficient to cause lesions. Because COX-1 is a target for NSAIDs (Xie et al., 1992; Seibert et al., 1994; Masferrer et al., 1994; Mitchell et al., 1994; Seibert and Masferrer, 1994) and because NSAID inhibition of COX-1 has been thought causal in the induction of gastrointestinal lesions, we investigated the possibility that COX-1-deficient mice have altered sensitivity to indomethacin, an NSAID known to induce stomach ulceration in mice (Yokoyama et al., 1985; Rainsford, 1987; Ettarh and Carr, 1993). First, a dose response was determined by administering indomethacin to heterozygous F2 mice by gavage at doses ranging from 10 to 80 mg/kg (data not shown). Doses of 10 and 20 mg/kg were found to be in the lower, but still
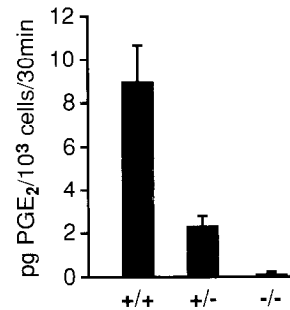

Figure 3. Production of PGE_2 by Peritoneal Macrophages

PGE_2 levels were determined by radioimmunoassay, and the data are expressed as picograms of PGE_2 per 10^3 cells. Data are for macrophages from five mice for each genotype (wild type, heterozygous, and homozygous mutant). Data are presented as the mean ± SEM.

detectable, response range. The data in Figure 4 show that F2 and F3 wild-type and homozygous mutant mice treated with 20 mg/kg have about an equal number of ulcers, although the percent of surface area ulcerated is somewhat reduced in the homozygous mutant mice (p <
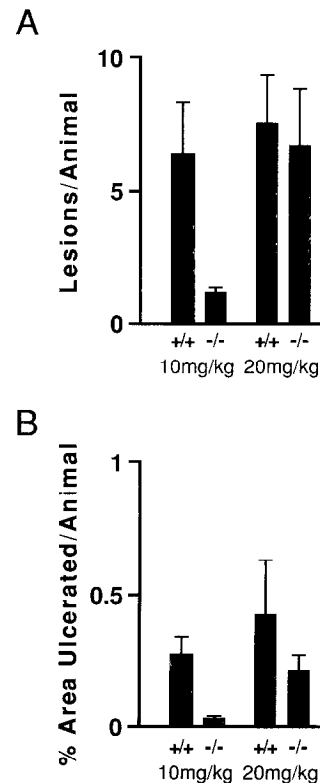

Figure 4. Induction of Stomach Ulceration by Indomethacin in Wild-Type and Homozygous Mutant Mice

(A) Data are expressed as the number of lesions of glandular stomach ulcers per animal at 10 and 20 mg/kg indomethacin. (B) Data are expressed as the percent ulcerated area of the total glandular stomach surface area at 10 and 20 mg/kg indomethacin. For (A) and (B), 10 mg/kg wild type (n = 9), homozygous mutant (n = 6); 20 mg/kg wild-type (n = 7), homozygous mutant (n = 8). Data are presented as the mean ± SEM.
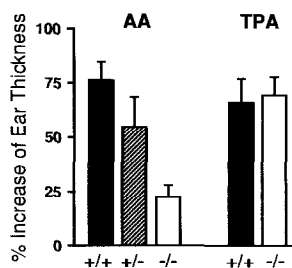
Figure 5. Induction of Ear Inflammation by AA and TPA

Data are expressed as the percent increase over pretreatment ear thickness. For AA and TPA treatments, five and four mice, respectively, of the F2 genotype indicated (wild type, heterozygous, homozygous mutant) were used. The mean control ear thickness was 0.22 ± 0.02 mm. Data are presented as the mean ± SEM.
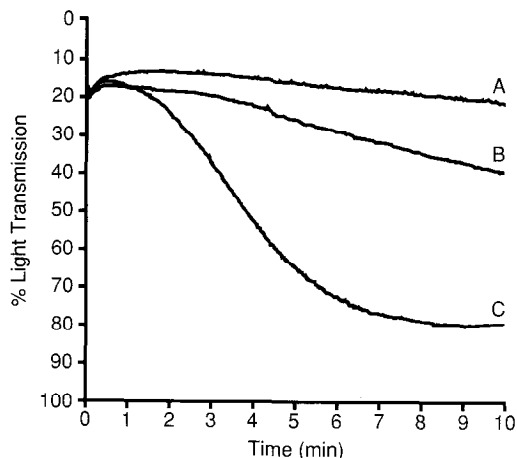

Figure 6. AA-Induced Aggregation of Platelets from Wild-Type F2 and Homozygous F2 Mutant Mice

(A) Solvent control with platelets from wild-type mice. (B) Platelets plus AA from homozygous mutant mice. (C) Platelets plus AA from wild-type mice.

0.36). At the 10 mg/kg dose, the homozygous mutant mice had statistically fewer lesions (p < 0.04), and a lower percent of stomach surface area was ulcerated (p < 0.06) than in the wild-type mice.

A possible means whereby gastric cytoprotection might be achieved in the COX-1 homozygous mutant mice is via a compensatory production of prostaglandins by the COX-2 isoform. To investigate this possibility, we determined PGE_2 levels in the glandular stomachs of COX-1 homozygous mutant mice and compared these levels with those in wild-type mice, untreated or after gavage with 40 mg/kg of indomethacin. Untreated wild-type levels were 113,430 ± 5,430 pg per milligram of tissue (n = 2); treated wild-type were 353 ± 126 pg per milligram of tissue (n = 2); homozygous mutant levels were 713 ± 265 pg per milligram of tissue (n = 2). Thus, it appears that COX-2 is contributing little to PGE_2 production in the stomach of COX-1 homozygous mutant mice.

Ear Inflammation

A standard ear swelling assay (Gad et al., 1986; Opas et al., 1985) was used to determine whether mice with the Ptgs1 gene disruption had an altered inflammatory response to chemical challenge. The mice used in these studies were littermates from the F2 generation. Figure 5 shows that when AA was administered topically, the homozygous mutant mice had a significantly reduced (p < 0.002) inflammatory response (about 30% normal). The heterozygous mice also had a decreased response, although it did not reach statistical significance (p < 0.26). In contrast, inflammation in response to the potent tumor promoter tetradecanoyl phorbol acetate (TPA) did not differ in the wild-type and homozygous mutant mice. By 6 hr after treatment with AA, ear thickness had returned to normal, while TPA-treated ears remained inflamed for at least 18 hr after treatment.

In gene targeting experiments in which breeding is not confined to a single inbred strain of mice, the cosegregation of strain differences in genes linked to the target locus must be considered (Smithies and Maeda, 1995). In the present instance, this complication is effectively eliminated by the observations of Morham et al. (1995); their

wild-type F2 Ptgs2 animals, in which the wild-type Ptgs1 locus and linked genes from the B6 and 129 strains can occur in all combinations, did not differ significantly in response to AA from our F2 wild-type animals carrying only the Ptgs1 B6/B6 combination.

Platelet Aggregation

COX-1 is considered the key enzyme for generating prostaglandins involved in platelet aggregation (Funk et al., 1991). We therefore investigated the ability of platelets from wild-type and homozygous mutant mice to aggregate in vitro. The curves in Figure 6 show that platelets from homozygous mutant mice aggregate more slowly and to a lesser extent in response to AA than do platelets from wild-type mice.

Reproductive Capability of Homozygous Mutant Mice

Table 1 lists the frequency of live births and the litter sizes resulting from different heterozygous and homozygous matings. When homozygous mutant females are mated with homozygous mutant males, almost all of the pups are found dead, even though the litter sizes are normal. The cause of pup death has not been determined. In con-

Table 1. Effect of Parental Genotype on Litter Size and Pup Survival

Pairing		Postnatal Survivors per Total Births[a]	Average Litter Size[a]
Male	Female		
Homozygous × homozygous[b]		4 of 39	7.8 ± 1.2
Heterozygous × homozygous		20 of 26	6.5 ± 1.0
Homozygous × heterozygous		27 of 31	7.8 ± 0.5

[a] Data are based on four litters for the heterozygous × homozygous and five litters for the homozygous × homozygous pairs mated.
[b] Four different homozygous females, one of which produced two litters.

trast, when homozygous mutant females are mated with heterozygous males, the number of surviving pups and the litter sizes are close to normal, with the number of pups having the heterozygous and homozygous mutant genotypes being essentially equal. The breeding of homozygous mutant males to heterozygous females likewise results in normal pup survival and litter size. These data indicate that both homozygous mutant males and homozygous mutant females are fertile, but that pup survival is decreased when homozygous mutant mice are mated to each other; in this situation, neither the female nor the pups have functional COX-1.

Discussion

In the present study, we have used homologous recombination to disrupt the mouse Ptgs1 gene that encodes COX-1. The major findings are that COX-1-deficient mice are generally healthy, do not have spontaneous stomach ulcers, and show less gastric ulceration than wild-type mice after gavage with indomethacin. The homozygous mutant mice also have a reduced inflammatory response to AA, and homozygous mutant × homozygous mutant matings result in reduced pup survival.

Because the stomach and kidney are two tissues where COX activity had been thought essential for proper function (Robert, 1975, 1979; Clive and Stoff, 1984; Black, 1986; Brooks and Day, 1991; Price and Fletcher, 1990; Simon, 1994), the lack of pathology in these tissues of COX-1-deficient mice was surprising. Furthermore, based on the hypotheses that COX-1 is a housekeeping enzyme and that NSAID inhibition of COX-1 is responsible for stomach ulceration, we had expected that COX-1 deficiency might lead to spontaneous stomach ulceration or bleeding in the homozygous mutant mouse. But the gastrointestinal tissues of the homozygous mutants were not distinguishable from wild type. Thus, absence of COX-1 is not sufficient to cause stomach ulceration in mice. Furthermore, measurement of PGE_2 in the glandular stomach of COX-1 homozygous mutant mice indicates that PGE_2 levels per milligram of tissue are less than 1% of the levels observed in wild-type mice. This reduction is consistent with that observed in peritoneal macrophages from homozygous mutant mice (Figure 3) and is approximately equal to the levels observed in the stomachs of wild-type mice treated with 40 mg/kg indomethacin. The low level of PGE_2 in the glandular stomach of the homozygous mutant mice coupled with the finding that COX-2 is undetectable by Western blot analysis (data not shown) suggests that compensation by COX-2 is not a significant factor in this tissue in COX-1 homozygous mutant mice.

Because of the absence of spontaneous gastric ulceration in the COX-1-deficient mice, we determined whether they have an altered sensitivity to NSAID-induced gastric ulceration. For this we chose indomethacin, which is widely used in NSAID studies of gastric ulceration and is known to induce ulceration in the mouse stomach (Yokoyama et al., 1985; Rainsford, 1987; Ettarh and Carr, 1993). Our experiments confirm that wild-type mice are sensitive to

stomach ulceration by indomethacin gavaged at either 10 or 20 mg/kg, doses commonly used to cause ulceration in rats (Futaki et al., 1993; Rainsford, 1993; Beck et al., 1990). Surprisingly, we found that the homozygous Ptgs1 homozygous mutants are less sensitive to indomethacin-induced stomach ulceration than are the wild-type mice: the absence of COX-1 decreases rather than increases the incidence of ulceration after indomethacin treatment. Thus, these observations suggest that indomethacin-induced gastric ulceration may be due to mechanisms other than (or in addition to) COX-1 inhibition. Alternatively, in the development of gastric ulcers, lack of COX-1 activity due to gene disruption may not be equivalent to the inhibition of COX-1 activity by indomethacin. As described by Morham et al. (1995), mice deficient in COX-2 also show no spontaneous stomach ulcers or overt intestinal lesions. These data emphasize that the relationship between inhibition of COX activity and ulceration is complex, and they illustrate that the COX-1- and COX-2-deficient mice provide novel ways of studying isoform-specific NSAIDs and for identifying mechanisms in addition to COX inhibition that may be involved in the ulcerative process.

The kidneys of the COX-1-deficient mice showed only minimal abnormalities even at 5 months of age. Therefore, absence of COX-1 in the kidney is not deleterious under normal physiological conditions.

Prostaglandins have key functions in various stages of the reproductive process, ranging from ovulation and spermatogenesis to parturition (Thorburn, 1991, 1992; Zahradnik et al., 1992). Which COX isoform is involved in each of these stages is not known, except for ovulation, when it appears that COX-2 is the important form (Sirois et al., 1992). Our studies show that neither male nor female fertility appears to be affected by lack of COX-1 (Table 1), but they clearly show that complete lack of COX-1, such as occurs in homozygous mutant × homozygous mutant matings, severely impedes survival of pups perinatally. Because prostaglandins are known to be involved in the initiation of labor (Kelly, 1994), it may be the onset of labor that is impaired. However, the normal litter size and pup survival seen when heterozygous males are mated with homozygous mutant females show that absence of COX-1 synthesis in the mother can be overcome by the presence of COX-1 in as few as 50% of the pups or in their placental material. From these matings, heterozygous and homozygous mutant pups are born in about equal numbers. The mating of homozygous mutant males with heterozygous females likewise results in normal litter sizes and pup survival. The most likely explanation for these observations is that prostaglandins from the COX-1 pathway in either the maternal or fetal tissues are essential for normal parturition. Further studies are needed to elucidate the roles of the COX isoforms in pregnancy and parturition, and the COX-deficient mice should provide useful models for such studies.

The decrease in AA-induced platelet aggregation seen in our COX-1-deficient mice accords well with existing data that platelets contain the constitutively produced isoform

COX-1 (Funk et al., 1991). Since platelets lack nuclei, this deficiency cannot be overcome by any compensatory induction of *Ptgs2* transcription followed by COX-2 synthesis.

The COX-1-deficient mice clearly have a reduction in their responses to AA, although their responses to TPA do not differ from wild type. However, the COX-2-deficient animals are as sensitive to inflammation caused by both AA and TPA as are wild-type mice. A possible reason for the differences between AA and TPA effects in the COX-1-deficient mice is that AA in wild-type mice (and COX-2-deficient mice) can be immediately metabolized by the constitutive COX-1 enzyme to PGH_2 and subsequently to PGE_2, which contributes to edema and inflammation. Since this cannot occur in the COX-1 homozygous mutant mice, less inflammation occurs. TPA, on the other hand, does not interact directly with COX-1 but is known to induce the synthesis of other enzymes, including COX-2 in vitro (Kujubu et al., 1991; DuBois et al., 1994a) and in vivo (Muller-Decker et al., 1995). The finding that TPA-induced inflammation is equal in COX-2 homozygous mutant mice and wild-type mice indicates that COX-2 is not essential for this type of inflammation to occur in the skin of these mice. Thus, it appears that COX-1 can contribute to inflammation. More detailed studies of the responses of the COX-1- or COX-2-deficient mice will be needed to indicate the relative roles of the two isoforms following different types of inflammatory stimuli.

The major conclusions from the present study are that lack of COX-1 does not cause spontaneous gastric ulceration and decreases indomethacin-induced ulceration, that it decreases inflammatory responses to AA, that it decreases platelet aggregation, but that it has no other overt systemic effects except those associated with parturition. Lack of COX-2, as reported by Morham et al. (1995), also does not cause spontaneous stomach ulceration, but, in contrast with lack of COX-1, it has no effect on inflammatory responses to AA, causes severe kidney disease, and leads to spontaneous peritonitis in some animals.

Experimental Procedures

Targeting Vector Construction
A Charon 35 genomic library containing DNA from E14TG2a mouse ES cells (Hooper et al., 1987) was screened with a 357 bp probe (see below) for the 5' end of exon 11 of the mouse *Ptgs1* gene. A clone containing approximately 15 kb of the 3' end of the *Ptgs1* gene was isolated, and overlapping 6 kb XbaI and ClaI fragments (Figure 1A) were subcloned into Bluescript. A 4.3 kb NotI–XhoI fragment of the XbaI subclone was inserted into the pPNT vector (Tybulewicz et al., 1991) 5' to the *Neo* gene, and a 2.3 kb BamHI fragment from the ClaI subclone was inserted 3' to the *Neo* gene to produce the targeting construct (Figure 1B).

Cell Culture and Targeting
E14TG2a ES cells were cultured by conventional methods on feeder cells. The targeting vector was linearized with NotI and electroporated at 5 nM into about 10^8 trypsinized ES cells using a 1 s pulse at 300 V and 200 µF. Positive/negative selection was performed with G418 and ganciclovir (Mansour et al., 1988) with ganciclovir providing about a 10-fold enrichment over G418 alone. Ganciclovir- and G418-resistant colonies were isolated 8–9 days after electroporation and transferred individually to 24-well plates previously seeded with feeder cells. The cells were trypsinized 2 days later and reseeded into 6-well plates. After 2 days the cells were trypsinized, and about half were frozen at $-80°C$. DNA isolated from the remaining half was used for PCR and genomic Southern blot analysis.

PCR and Southern Blot Analysis
PCR was the initial screen for identifying targeted ES cells and mice carrying the disrupted *COX-1* gene (Kim and Smithies, 1988). A primer specific for the *Neo* gene and a second primer specific for a genomic sequence 3' to sequences in the targeting construct (Figure 1C) produced a single 2.4 kb band diagnostic of targeting. Genomic Southern blots were produced by standard techniques and probed with a random primer ^{32}P-labeled probe (Stratagene Prime-It II) made from the 357 bp PCR fragment specific for the 5' region of exon 11. DNA was isolated from mouse tails to determine genotype.

Northern Blot Analysis
Total RNA was isolated from tissues by homogenizing them in TRIzol (Life Technologies) or from cells by scraping directly into TRIzol as recommended by the supplier. Each sample (15 µg) was electrophoresed in a 2.2 M formaldehyde–0.9% agarose gel. After capillary transfer, the blot was hybridized with a random primer ^{32}P-labeled probe made from the exon 11–specific 357 bp PCR fragment or a 1.7 kb *COX-1* cDNA fragment (Oxford Biomedical Research Corporation). Blots were stripped and probed for actin to ensure equal loading of RNA.

Western Blot Analysis
To prepare microsomes, we homogenized tissues in buffer (0.1 M Tris–HCl [pH 7.4], 2 mM EDTA, 10 mg/ml leupeptin, 20 mg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride) and then sonicated them at 30% power (Fisher Scientific, Model F50) three times for 15 s. The homogenates were centrifuged at 10,000 × g for 15 min at 4°C. The resulting supernatants were centrifuged at 100,000 × g for 1 hr at 4°C, and the microsomal pellets were sheared in buffer (100 mM Tris [pH 6.8], 8% SDS, 20% glycerol) with a 25-gauge needle. An aliquot was removed for protein determination (Bio-Rad DC) before boiling with bromophenol blue (0.05% [w/v]) and 2-mercaptoethanol (6% [v/v]).

For immunoblot analysis, 40 µg of microsomal protein (kidney and stomach), 20 µg of microsomal protein (colon), or 10 µg of protein from cell lysate (macrophages) were separated by SDS–PAGE using the Mini-PROTEAN II electrophoretic apparatus (Bio-Rad). Proteins were transferred onto Hybond–ECL nitrocellulose (Amersham) using the Mini Trans-Blot electrophoretic transfer cell system (Bio-Rad). Membranes were blocked in 5% nonfat milk–Tris-buffered saline with 0.1% Tween 20 (TBST) before incubating with a rabbit antibody to murine COX-1 provided by Dr. D. DeWitt (Morita et al., 1995) or to COX-2 (Cayman Chemical). Blots were incubated with anti-rabbit IgG horseradish peroxidase–linked secondary antibody (Boehringer Mannheim) in TBST and 1% nonfat milk. Chemiluminescent detection was performed using reagents from Amersham, and bands were visualized after exposure to Hyperfilm–ECL (Amersham).

Indomethacin-Induced Stomach Ulceration
Indomethacin was suspended in 1% methyl cellulose at the concentration of 1 or 2 mg/ml and given at the stated doses by gavage. All animals were fasted 16–18 hr prior to gavage. Animals were euthanized using CO_2 6 hr after treatment, and their stomaches were removed and opened along the lesser curvature. Stomach lesions were scored as described by Ghanayem et al. (1987). The number of lesions were counted, an enlarged image of the formalin-fixed glandular stomach and of each individual lesion was traced, and the area of each lesion was determined using a computer-assisted image analysis system. The total area of the stomach was traced and measured. The area of all lesions in each stomach was calculated and divided by the area of the glandular stomach to derive the percent of area with lesions. All samples were scored blind. No ulceration was shown by eight wild-type mice that received vehicle alone.

Mouse Ear Inflammation Assay
AA (2 mg per 10 µl) or TPA (1 µg per 10 µl) in acetone was applied

to the inside of the left ear and 10 μl of acetone was applied to the right ear as described by Opas et al. (1985). Ear swelling was determined after 2 hr for AA or 6 hr for TPA by the method of Gad et al. (1986).

Macrophage Isolation and PGE₂ Analysis

Peritoneal macrophages were isolated and LPS stimulated by modification of the procedure of Watanabe et al. (1994). Thioglycolate-elicited macrophages were isolated by peritoneal lavage with 5 ml of cold RPMI 1640 medium. Macrophages were seeded at 1×10^7 to 2.5×10^7 cells per 60 mm dish, depending on yield, and allowed to attach for 2 hr in a humidified incubator with 5% CO_2 in air. The plates were then washed with Hank's balanced salt solution to remove nonadhering cells, and medium containing 1% serum with or without LPS (10 μg/ml) was then added. Attached cell numbers were determined by counting with an eyepiece micrometer. After a 6 hr incubation, the medium was removed and replaced for 30 min with medium containing 10 μM AA. Subsequent analysis for PGE₂ in the medium was by a competitive radioimmunoassay (Amersham).

Platelet Aggregation

Platelet aggregation was carried out as described by Paigen et al. (1987) using AA to induce aggregation. For each assay, blood was pooled from two mice (about 1 ml in total volume) and centrifuged to prepare platelet-rich and then platelet-poor plasma. The assay was conducted with 300 μl of plasma at 3×10^8 platelets per milliliter. Aggregation was induced by adding 15 μl of Na–AA (22 mg/ml in Na_2CO_3 buffer). Turbidity was measured with a Lumi-Aggregometer.

Acknowledgments

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