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Prostaglandin Synthase 2 Gene Disruption Causes Severe Renal Pathology in the Mouse

Scott G. Morham,* Robert Langenbach,† Charles D. Loftin,† Howard F. Tiano,† Nectarios Vouloumanos,* J. Charles Jennette,* Joel F. Mahler,‡ Kimberly D. Kluckman,* Aric Ledford,* Christopher A. Lee,† and Oliver Smithies*

*Department of Pathology
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599-7525
†National Institute of Environmental Health Sciences
Laboratory of Experimental Carcinogenesis
and Mutagenesis
Research Triangle Park, North Carolina 27709
‡National Institute of Environmental Health Sciences
Laboratory of Experimental Pathology
Research Triangle Park, North Carolina 27709

Summary

The prostaglandin endoperoxide H synthase isoform 2, cyclooxygenase 2 (COX-2), is induced at high levels in migratory and other responding cells by proinflammatory stimuli. COX-2 is generally considered to be a mediator of inflammation. Its isoform, COX-1, is constitutively expressed in most tissues and is thought to mediate "housekeeping" functions. These two enzymes are therapeutic targets of the widely used nonsteroidal anti-inflammatory drugs (NSAIDs). To investigate further the different physiologic roles of these isoforms, we have used homologous recombination to disrupt the mouse gene encoding COX-2 (*Ptgs2*). Mice lacking COX-2 have normal inflammatory responses to treatments with tetradecanoyl phorbol acetate or with arachidonic acid. However, they develop severe nephropathy and are susceptible to peritonitis.

Introduction

Aspirin, one of the oldest and most extensively used synthetic drugs in the pharmacopoeia, belongs to the class of drugs known as nonsteroidal anti-inflammatory drugs (NSAIDs). Examples of other more recently introduced NSAIDs in common usage include ibuprofen, naproxen, indomethacin, and sulindac. In addition to their acute anti-inflammatory and analgesic actions, NSAIDs have been demonstrated to be effective against chronic diseases. For example, small daily doses of aspirin can decrease platelet aggregation, thereby reducing the incidence of thrombotic heart attacks and strokes by up to 50% (Meade, 1992). In addition, long-term use of aspirin in both women and men is associated with a 40% decrease in risk for fatal colon carcinoma (Thun et al., 1991, 1993). Nevertheless, the deleterious side effects of NSAIDs can be significant. These include a high incidence of gastrointestinal toxicity (Brooks and Day, 1991; Carson et al., 1987) and, under certain conditions, damage to renal function (Clive and Stoff, 1984). Indeed, renal problems for patients with a variety of diseases can become especially severe if prostaglandin synthesis is inhibited by NSAIDs (Whelton et al., 1990). The effects of NSAIDs, both therapeutic and toxic, can be attributed to a decrease in the production of prostaglandins (Vane, 1971; Flower, 1974).

The prostaglandins are a diverse group of autocrine and paracrine hormones that mediate many cellular and physiologic processes. Prostaglandin H_2 (PGH_2) is an obligate intermediate in formation of the prostaglandins. Prostaglandin synthase, an enzyme with two known isoforms, catalyzes formation of PGH_2 from arachidonic acid (AA). These prostaglandin synthases are the primary enzymatic targets for NSAIDs (Flower and Vane, 1972; Flower et al., 1972; DeWitt et al., 1993). We refer to the two prostaglandin synthase isoforms as cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2); their respective genes in the mouse are prostaglandin synthase 1 (*Ptgs1*) and 2 (*Ptgs2*). COX-1 and COX-2 are similar in both amino acid sequence and enzymatic function (O'Banion et al., 1992; Ryseck et al., 1992; Fletcher et al., 1992; Hsi et al., 1994), although their physiologic functions are thought to be quite different (Smith et al., 1994).

COX-1 is constitutively expressed in most tissues (Simmons et al., 1992; O'Neill and Ford-Hutchinson, 1993), but at different levels in various cell types. Enzyme expression usually remains at fairly constant levels, although dramatic changes can occur in certain cells after stimulation with growth factors or other agents (DeWitt, 1991; Smith et al., 1993). Immunofluorescence data indicate that COX-1 is the isoform expressed in kidney, stomach, vascular smooth muscle (Smith et al., 1994), and platelets (Funk et al., 1991).

COX-2 has a markedly different expression pattern. Normally, the enzyme is undetectable in most tissues, but it can be expressed at high levels in macrophages and certain other cell types after induction with a variety of substances, including inflammatory mediators and mitogens (Lee et al., 1992; Fu et al., 1990; Evett et al., 1993; Kujubu et al., 1991; Masferrer et al., 1992; Sano et al., 1992; O'Sullivan et al., 1992a, 1992b). The inhibition of COX-2 expression by anti-inflammatory glucocorticoids lends further credence to a presumed role for COX-2 in inflammation (O'Banion et al., 1992; Kujubu et al., 1991; Kujubu and Herschman, 1992; Masferrer et al., 1992). COX-2 is also found to be up-regulated in human colorectal adenomas and adenocarcinomas (Eberhart et al., 1994). Other traits that distinguish *Ptgs2* from *Ptgs1* are inhibition of *Ptgs2* transcription by interleukin-10 (Mertz et al., 1994) and transforming growth factor β1 (Reddy et al., 1994).

In addition to the cellular traits that distinguish the two isoforms from one another, individual NSAIDs inhibit COX-1 and COX-2 in characteristically different ways. For example, the acetylation of Ser-530 in COX-1 by aspirin

irreversibly inactivates the enzyme (Humes et al., 1981; Rome and Lands, 1975; Rome et al., 1976; Roth et al., 1983), while acetylation of the corresponding residue in COX-2 causes the enzyme to catalyze formation of 15-hydroxy-eicosatetraenoic acid instead of PGH_2 (Meade et al., 1993; O'Neill et al., 1994; Cromlish et al., 1994). Ibuprofen acts as a reversible inhibitor approximately equipotent on either isoform, while 6-methoxy-naphthyl-acetic acid (the active metabolite of nambutone) preferentially inhibits COX-2 (DeWitt et al., 1993; Smith et al., 1994). COX-2 inhibitors have anti-inflammatory properties with little gastrointestinal ulceration (Masferrer et al., 1994; Futaki et al., 1994), while COX-1 inhibitors have anti-inflammatory properties but tend to cause gastrointestinal distress as a side effect (Brooks and Day, 1991; Clive and Stoff, 1984).

The above findings have led to the view that the constitutively expressed enzyme, COX-1, is involved in producing prostaglandins for cellular "housekeeping" functions, including gastric cytoprotection, vascular homeostasis, and normal renal maintenance. The inducible enzyme, COX-2, is thought to be involved in inflammation, cellular differentiation, and mitogenesis (DeWitt et al., 1993). However, despite intense investigation of prostaglandin synthesis and function, clear models for the individual roles of COX-1 and COX-2 remain elusive. It is therefore of great interest to investigate further the roles of the individual COX isoforms in vivo. To do this, we have generated mice in which *Ptgs2* has been disrupted by gene targeting and describe herein the consequences of the resulting lack of COX-2.

Results

Vector Construction and Gene Targeting

The mouse *Ptgs2* gene was isolated as a λ bacteriophage clone containing a 16 kb fragment of BglII-digested genomic DNA. This DNA fragment was obtained from the mouse strain 129-derived embryonic stem (ES) cell line E14TG2a. The probe used to identify the λ clone was a 408 bp fragment specific to exon 10 of *Ptgs2* and was derived by polymerase chain reaction (PCR) amplification of mouse genomic DNA using primers made from the published sequence of *Ptgs2*. The single PCR fragment obtained was demonstrated by digestion with ClaI and NcoI to be from *Ptgs2* (data not shown); sites for these enzymes are not present in the corresponding *Ptgs1* region. A *Ptgs2* cDNA clone (O'Banion et al., 1992) was used as a probe to confirm the identity of the λ clone. Successful amplifications using primer sets specific to exons 1, 4, 7, 8, and 10 of *Ptgs2* were used to establish the identity of the genomic clone further and to distinguish it from *Ptgs1* (data not shown).

Figure 1B depicts the targeting construct used to inactivate *Ptgs2* in the ES cell line E14TG2a. As illustrated, a neomycin resistance gene (*Neo*) has been inserted between a 6.0 kb EcoRI fragment and a 1.8 kb BstXI–EcoRI fragment, both obtained from the 16 kb genomic clone of *Ptgs2*. This insertion interrupts the coding sequence in exon 8 (Figure 1B), thereby disrupting *Ptgs2* (Figures 1A and 1C). In addition to the insertion, we introduced a 104

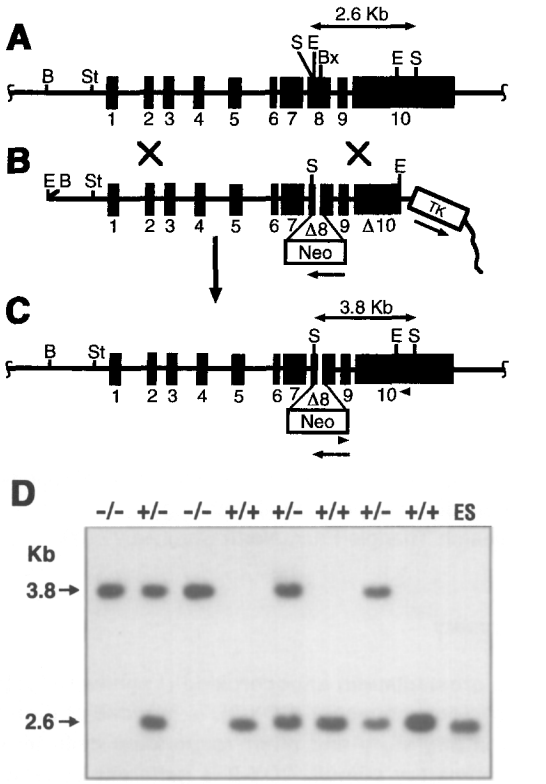


Figure 1. Disruption of the Mouse *Ptgs2*

(A) The endogenous *Ptgs2* locus. The numbered closed boxes denote exons. The double arrow indicates the endogenous SacI fragment characteristic of the wild-type gene. B, Bx, E, S, and St indicate the BglII, BstXI, EcoRI, SacI, and StyI sites, respectively.

(B) Targeting construct. *Neo* and *TK* indicate neomycin resistance and thymidine kinase genes, oriented as indicated by the arrows. The wavy line indicates plasmid sequence (not drawn to scale). Δ8 represents exon 8 after deletion of the region between the EcoRI and BstXI with subsequent insertion of *Neo*. Δ10 indicates the truncated exon 10. The most 5′ EcoRI site in the construct comes from the λ phage vector.

(C) The targeted *Ptgs2* locus with *Neo* disrupting exon 8. PCR primers used to identify targeted cell lines are shown as right and left arrowheads. The double arrow indicates the SacI fragment characteristic of the targeted *Ptgs2* gene.

(D) Autoradiogram from a Southern blot of mouse F2 genomic DNA digested with SacI, showing the expected fragments for the wild-type (2.6 kb) and targeted alleles (3.8 kb) as indicated by the arrows. The blot was probed with a radiolabeled SacI fragment from a cDNA of *Ptgs2*; this fragment includes exons 8 and 9 and a 5′ portion of exon 10. ES denotes DNA from the parental ES cell line; (+/+), (+/−), and (−/−) denote tail DNA from wild-type, heterozygous, and homozygous mutant F2 mice, respectively.

bp deletion in exon 8 that eliminated the nucleotides encoding Tyr-371 and His-374, both crucial for COX activity (Shimokawa et al., 1990; Shimokawa and Smith, 1990). Nucleotide sequence data spanning the junction of the *Neo* gene with exon 8 were obtained and demonstrated that the sequence at the junction corresponds to *Ptgs2* (data not shown).

Of 192 G418–ganciclovir-resistant clones isolated, 11 were determined to have undergone homologous recombination. This determination was made by PCR amplification of a fragment characteristic of the desired gene tar-

geting event. Cell lines having undergone the planned targeting event display a 2.0 kb PCR product when PCR is performed using the two primers illustrated in Figure 1C. Genomic DNAs from the PCR-positive cell lines were digested with SacI for Southern blot analysis. Targeted clones showed the expected presence of a 2.6 kb DNA fragment corresponding to the wild-type *Ptgs2* allele and a 3.8 kb DNA fragment corresponding to the targeted allele.

Two targeted cell lines were injected into C57BL/6J (B6) blastocysts. Both gave rise to male chimeric animals that, when mated to B6 females, subsequently passed the targeted allele to their F1 129/B6 offspring. F1 heterozygotes were interbred, and Southern blot analysis of genomic DNA from their F2 progeny showed the 2.6 kb and/or 3.8 kb DNA fragments expected for the wild-type (+/+), heterozygous (+/−), and homozygous mutant (−/−) genotypes (Figure 1D).

Genotypic Ratios from F1 × F1 Matings

Matings between heterozygous F1 animals produced F2 progeny in the following numbers (wild type:heterozygous: homozygous): females, 15:31:11; males, 28:20:12. The genotypes of the female progeny did not differ from Mendelian expectations (χ^2, $p > 0.5$). However, males showed significant deviation from normal Mendelian ratios (χ^2, $p < 0.001$).

Lipopolysaccharide Induction of *Ptgs2* mRNA in Peritoneal Macrophages

Ptgs2 is an inducible gene with only slight transcriptional activity in the absence of stimulation. Lipopolysaccharide (LPS) induction of macrophages normally elicits a marked increase in *Ptgs2* mRNA levels resulting in increased PGE_2 production (Lee et al., 1992). We therefore compared the responses of peritoneal macrophages from F2 wild-type animals with F2 mutants homozygous for the gene disruption. The Northern blot in Figure 2A illustrates the levels of *Ptgs2* mRNA that were detected in peritoneal macrophages from wild-type and homozygous F2 animals before and after induction with LPS. In the wild-type macrophages, the level of detectable mRNA (4.7 kb in size) increases 13-fold, as determined by densitometry. In the homozygous mutant macrophages, no mRNA of the normal size is found, either before or after LPS treatment. However, a very low level of aberrant 6 kb transcript can be detected. This transcript also hybridizes to a *Neo* probe (data not shown) and represents an anomalous mRNA containing both *Neo* and *Ptgs2* sequences. Thus, mutant macrophages synthesize no normal *Ptgs2* mRNA before or after LPS induction.

LPS Induction of COX-2 Protein in Peritoneal Macrophages

To confirm further the inactivation of *Ptgs2* in homozygous mutants, we measured the levels of cellular COX-2 protein by Western blot analysis. The Western blot in Figure 2B illustrates the levels of enzyme in macrophages as detected by a polyclonal antibody specific to COX-2. COX-2 migrates in this electrophoretic system as a doublet (due to glycosylation) approximately 70–72 kDa in size (Pritchard et al., 1994). The wild-type macrophages show a greater than 10-fold increase in COX-2 levels after LPS treatment, as determined by densitometry. The homozygous mutant macrophages fail to show any immunoreactive COX-2 before or after LPS induction, as expected from the absence of *Ptgs2* mRNA. We conclude that our gene targeting has abolished any detectable production of COX-2 by the *Ptgs2* gene. In contrast, levels of COX-1 in homozygous macrophages were unchanged in treated and untreated cells and were equivalent for both wild-type or homozygous mice (data not shown).

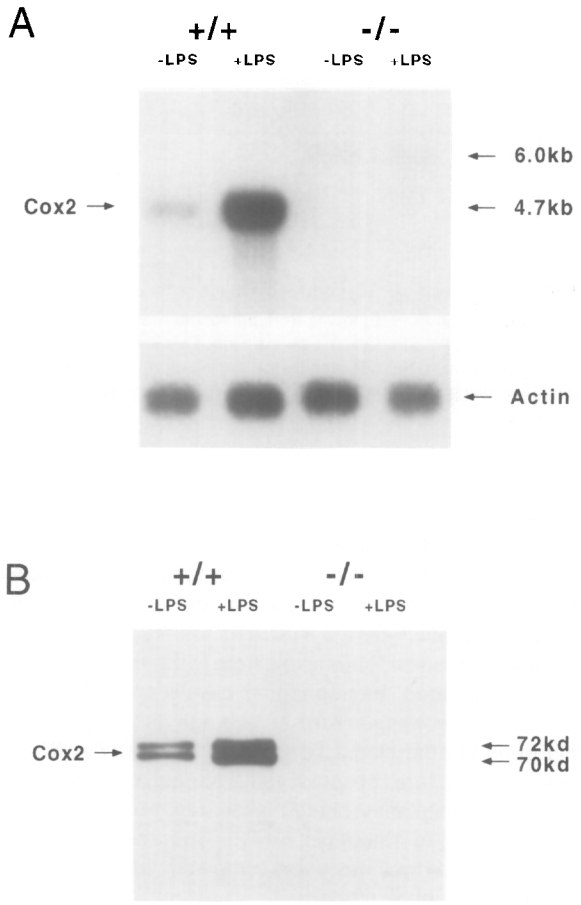


Figure 2. Expression of *Ptgs2* mRNA and Protein from Macrophages before and after Induction with LPS

(A) Autoradiogram from a Northern blot of total RNA isolated from macrophages induced (plus) or uninduced (minus) with LPS. The probe was made from a SacI fragment of a *Ptgs2* cDNA (Oxford Biomedical Research). (+/+) or (−/−) denotes RNA from a wild-type F2 animal or a homozygous *Ptgs2* disrupted animal, respectively. The Cox2 at the left indicates the position of *COX-2* mRNA; mRNA sizes are indicated on the right.

(B) A Western blot of total protein isolated from macrophages induced (plus) or uninduced (minus) with LPS. COX-2 was detected using a polyclonal antibody (Cayman Chemical) specific for that protein in conjunction with chemiluminescence. (+/+) or (−/−) denotes protein from a wild-type F2 animal or a homozygous *Ptgs2* disrupted animal, respectively. The approximate sizes of the glycosylated and unglycosylated COX-2 proteins are indicated on the right.

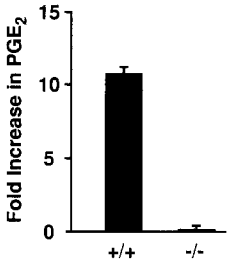


Figure 3. Synthesis of PGE_2 from Macrophages after Induction of LPS

The fold increase in PGE_2 levels from peritoneal macrophages after induction with LPS are depicted by the bars. This was calculated by dividing the levels after induction by the levels before induction. (+/+) and (−/−) denote data from wild-type and homozygous mutant F2 mice, respectively.

LPS Induction of PGE_2 Synthesis in Peritoneal Macrophages

Synthesis of PGE_2 requires prior formation of PGH_2 and thus provides a convenient measure of PGH_2 production in macrophages. Figure 3 illustrates the results obtained by measurement of PGE_2 levels from LPS-stimulated and LPS-unstimulated macrophages. Levels of PGE_2 from wild-type macrophages rose an average of 10.8 (± 0.5)-fold (n = 2) after stimulation with LPS. However, PGE_2 levels in the cultures of homozygous mutant macrophages were not significantly altered after treatment with LPS (0.05 [± 0.35]-fold increase; n = 2). This provides further evidence that *Ptgs2* has been completely inactivated by our gene disruption strategy.

Inflammatory Responses after Treatment of the Ear with Tetradecanoyl Phorbol Acetate or Arachidonic Acid

Swelling of the ear in response to tetradecanoyl phorbol acetate (TPA) or arachidonic acid (AA) are standard assays by which inflammation can be quantitated (Opas et al., 1985; Gad et al., 1986). Ear thickness is measured prior to exposure to the inflammatory agent and then at a set time after exposure. The thickness before exposure is taken to be the 100% value (baseline), and the percentage increase in thickness relative to the baseline value is then calculated. F2 wild-type mice exposed to TPA had an average increase in ear thickness of 91 ± 10% (n = 5), while homozygous mutant F2 mice had an increase of 101 ± 15% (n = 6; Figure 4). Similarly, wild-type F2 mice exposed to AA had an average increase in ear thickness of 93 ± 12% (n = 7), while their F2 homozygous mutant counterparts had an increase of 88 ± 13% (n = 7; Figure 4). A two-tailed Student's t test comparing wild-type and homozygous mutant animals demonstrates that both genotypes showed significant responses to the inflammatory mediators ($p < 0.001$). The responses to both TPA and AA did not differ between wild-type and homozygous mutant genotypes ($p > 0.5$). Thus, an active *Ptgs2* gene is not essential for these inflammatory responses.

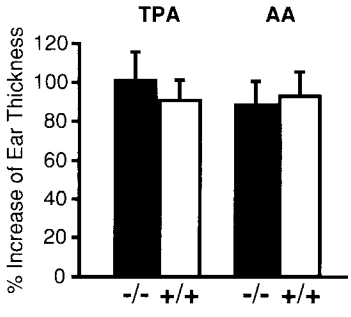


Figure 4. Response of Mice to the Inflammatory Mediators TPA and AA

Bar graph depicting the percentage increase in ear thickness after treatment with TPA and AA. (+/+) denotes data from wild-type F2 animals; (−/−) denotes data from homozygous *Ptgs2* disrupted animals.

Mortality and Renal Pathology in Homozygous Mutant Mice

Survival of weaned F2 animals heterozygous for disruption of *Ptgs2* did not differ from that of F2 wild types. However, several homozygous mutant animals were found dead at approximately 8 weeks of age. To ascertain the likely cause of their death, we sacrificed seven homozygous mutants and seven wild-type mice at 6–16 weeks of age for comparative pathological analysis.

General gross anatomical examination was made of both genotypes, and microscopic examination was done on skin, brain, eye, lungs, heart, liver, spleen, lymph nodes, kidneys, reproductive organs, and gastrointestinal tract. Significant and consistent genotype-related abnormalities were observed in the kidneys of three of three 6-week-old, three of three 8-week-old, and one of one 16-week-old mice homozygous for *Ptgs2* disruption.

Gross examination of kidneys from 8-week-old male homozygous mutant mice revealed kidneys that were small and pale and that had a granular appearance on the capsular surface. Kidneys from wild-type animals were normal in appearance. Light microscopic examination of kidneys from four wild-type males and three wild-type females revealed no histologic abnormalities (Figure 5A). In contrast, the kidneys of all adult homozygous animals examined (four males and three females) had lesions of mild to marked severity. In its mildest form, the nephropathy was characterized by multifocal areas of abnormal subcapsular parenchyma comprised of small immature glomeruli and tubules, consistent with nephron hypoplasia (Figure 5B). In some cases, the cortex appeared thinned, and the number of glomeruli were reduced in comparison with wild-type kidneys. Glomeruli not within the hypoplastic zone were frequently found to be enlarged. Other pathological findings included cortical areas of tubular atrophy and regeneration, protein and cellular casts within tubular lumens, tubular dilation, interstitial inflammation and fibrosis, and papillary mineralization. In general, these changes were more severe in the male than in the female homozygous mutants, and they increased in severity with advancing age. The 8-week-old homozygous mutant mouse kidneys had a few small scattered foci of tubular

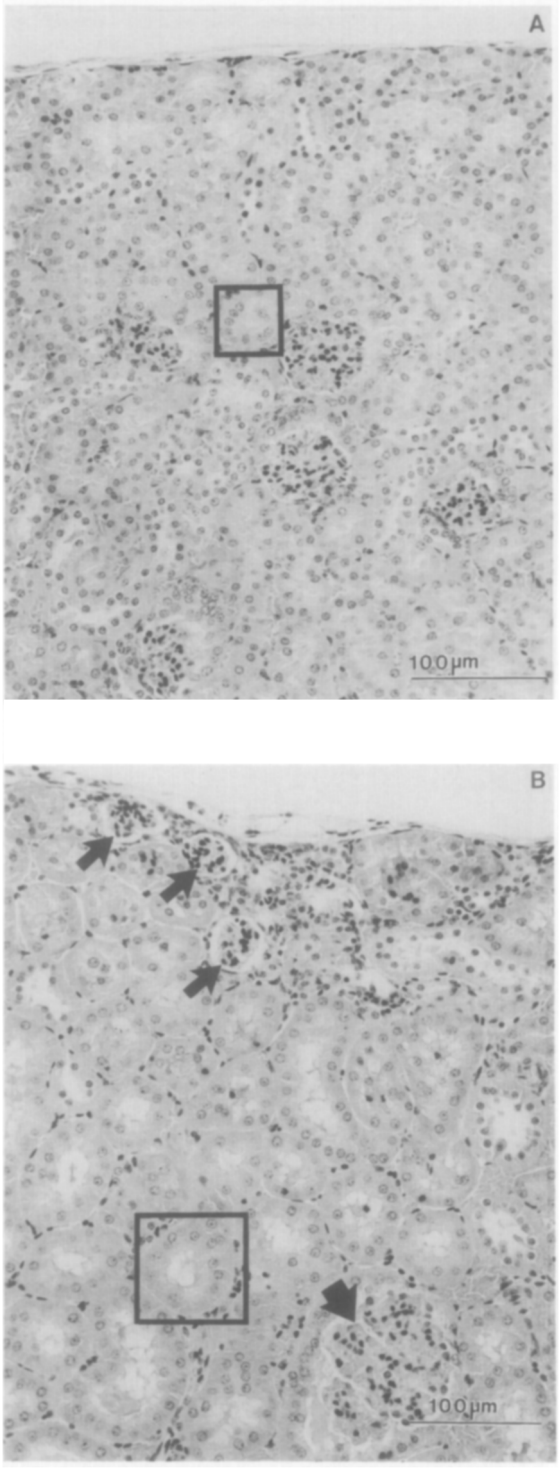


Figure 5. Comparison of Renal Cortex from Wild-Type and *Ptgs2* Disrupted Mice

(A) Renal cortex from an 8-week-old wild-type F2 male mouse. The box encloses a normal tubule.

(B) Renal cortex from an 8-week-old homozygous mutant mouse photographed at the same magnification as (A) and showing subcapsular glomerular hypoplasia (denoted by small upward-pointing arrows) and hypertrophy of deeper cortical glomeruli (indicated by the large downward-pointing arrow). The box encloses a hypertrophied tubule. Stained with hematoxylin and eosin.

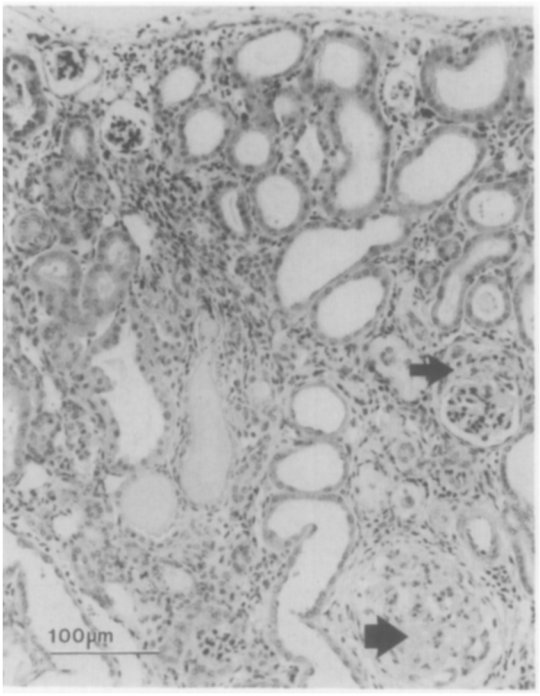


Figure 6. Severe Kidney Disease in Mature Adult Homozygous Mutant Mice

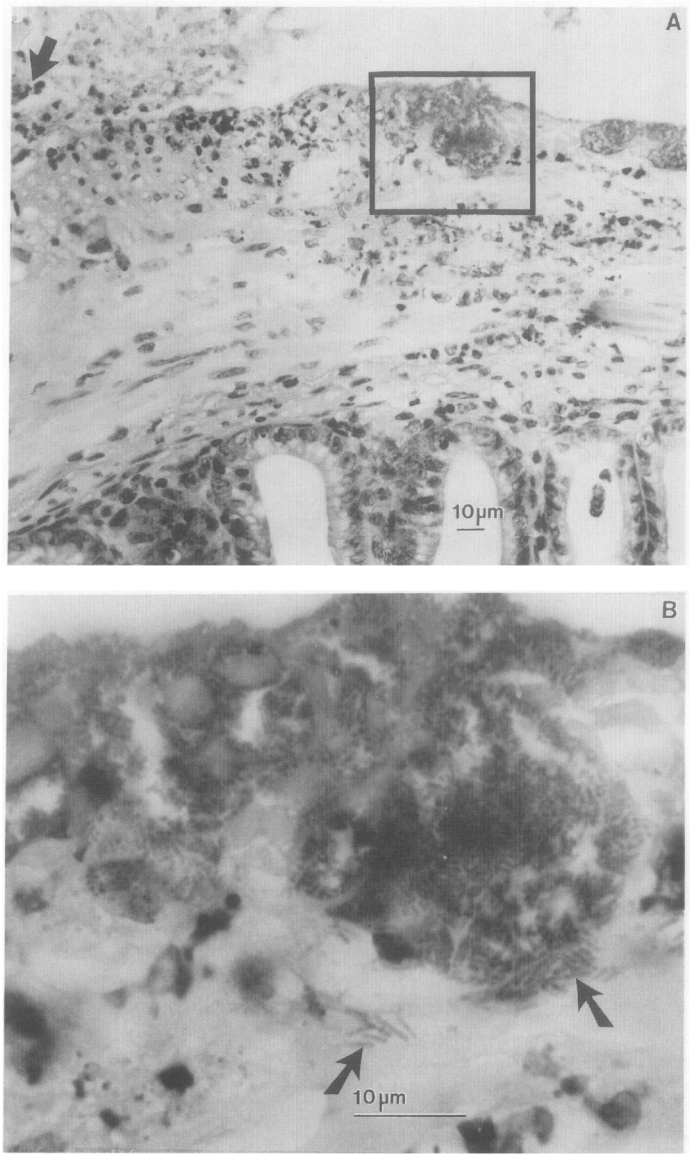
Renal cortex from a 16-week-old male homozygous mutant mouse showing segmental (small arrow) and global (large arrow) glomerular sclerosis, as well as tubule atrophy and interstitial fibrosis. Note the subcapsular hypoplastic glomeruli. Stained with hematoxylin and eosin. The capsular margin is at the top.

atrophy and interstitial fibrosis that were not present in the 6-week-old homozygous mutant mice. The single homozygous mutant male that survived to necropsy at 16 weeks of age had severe focal interstitial fibrosis and tubular atrophy associated with focal segmental and global glomerular sclerosis (Figure 6). Renal arteries and arterioles were unremarkable.

To ascertain the general timeframe in which the renal pathology developed, a litter of 3-day-old pups was sacrificed and their kidneys examined. The renal histology of the COX-2-deficient mice did not differ from that of wild type. In particular, the 3-day-old homozygous mice had a normal subcapsular zone of immature nephrogenic tissue and normal numbers of glomeruli in the kidney cortex. This is in contrast with the 6-week and older homozygous mice, which showed abnormal nephron hypoplasia in the subcapsular region and abnormally low numbers of glomeruli in the kidney cortex. Thus, COX-2-deficient mice show postnatal developmental abnormalities in their kidneys that then progressively deteriorate with increasing age.

Peritoneal Pathology in Some Homozygous Mutant Mice

Of the three *Ptgs2* homozygous mice 8 weeks of age (the age at which death in homozygous mutants was most frequently observed), two were found to have suppurative



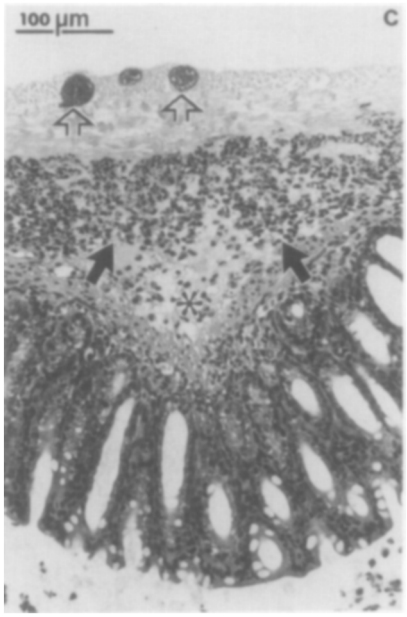


Figure 7. Marked Inflammatory Response to Bacterial Invasion of the Peritoneum

(A) Peritonitis involving the gut wall of a male 8-week-old homozygous mutant mouse. Purulent exudate (denoted by the arrow), focal acute inflammation of the muscularis, and focal accumulation of bacteria (see inset box). Stained with hematoxylin and eosin.

(B) Higher magnification of the rod-like bacteria (indicated by the arrows) from the inset box in (A). Stained with hematoxylin and eosin.

(C) Leukocytic infiltration of the bowel wall in an 8-week-old homozygous mouse. Submucosal edema (indicated by the asterisk) associated with a band of intense neutrophil infiltration (indicated by the closed arrows) and cellular degeneration. Note the bacterial colonies near the serosal surface (indicated by open arrows). Stained with hematoxylin and eosin.

peritonitis. No abnormalities were observed in any of the wild-type mice. The homozygous male had acute suppurative peritonitis, while the female had chronic suppurative peritonitis. Accompanying gross lesions of the abdominal cavity were found. The female had multiple adhesions among the abdominal organs that corresponded microscopically to abscesses and chronic fibrous inflammatory tissue bridging lobes of the liver and loops of the bowel. The peritonitis involved multiple abdominal organs and was characterized by both serosal exudation and focal necrotizing acute inflammation that had penetrated into the superficial tissues of viscera and retroperitoneum (Figure 7A). Submucosal edema of the bowel wall was observed, and inflammatory infiltrates were composed predominantly of neutrophils with conspicuous leukocytoclasia (Figure 7C). Colonies of bacteria were visible in some of the inflammatory exudate and within the inflamed tissue (Figures 7B and 7C). Both mice with peritonitis had myeloid hyperplasia in their spleens. The female also had marked lymphoplasmacytic hyperplasia of the mesenteric lymph node.

One qualification of these observations must be mentioned. The female mutant with chronic suppurative peritonitis had been previously treated intraperitoneally with sterile thioglycolate to harvest macrophages. None of the several wild-type mice injected at the same time displayed this pathology. In addition, the homozygous male, which had not been treated with thioglycolate, displayed a more severe peritonitis. Thus, it seems unlikely that the injection caused the peritonitis in the homozygous female.

Discussion

Abnormal Mendelian Ratios of Male F2 Progeny

The preponderance of wild-type genotypes in the male but not female progeny of heterozygous × heterozygous F1

parents suggests that the lack of functional *Ptgs2* negatively affects either the number of embryos fertilized by male sperm carrying the defect or the fitness of the embryos or neonates carrying one or more copies of the mutant allele. Some indication that the possible defect is prior to implantation is the finding that equal numbers of males (60) and females (57) reached weaning age (20 days) despite the differences in genotypic ratios of the male and female weanlings. More investigation of this anomaly will be necessary to reach any firm conclusions. It will also be necessary to establish whether later generations of mice retain the abnormal ratios of male progeny.

Normal Inflammatory Responses in *Ptgs2* Homozygous Mutant Mice

Mice homozygous for the targeted *Ptgs2* disruption completely lack the normal induction of *Ptgs2* mRNA, protein, and prostaglandin synthesis by the proinflammatory agent LPS. It is therefore surprising to find that the lack of COX-2 and PGE_2 synthesis does not affect the inflammatory response of the ear to TPA, which is known to be a potent inducer of COX-2. Similarly, AA, the substrate for COX, also induces a normal response in the mutant homozygotes. These results clearly demonstrate that COX-2 is not essential for these inflammatory responses, since its complete absence does not decrease the inflammatory response of the ear to TPA or AA.

We do not at present understand the etiology of the peritonitis in two of seven *Ptgs2* homozygous mutant mice. However, it is important to note that the affected mice homozygous for *Ptgs2* disruption showed classic inflammatory reactions to the invasion of the peritoneum by bacteria. Specifically, the COX-2-deficient mice showed necrosis and associated inflammation, fibrinous adhesions, and infiltration of leukocytes; these are conditions that would be expected in a normal inflammatory response to bacterial infection. This indicates that *Ptgs2* is not essential for these inflammatory responses, although COX-2 may be needed either for the prevention or the amelioration of the infections.

Kidney Pathology in Mice as a Result of *Ptgs2* Inactivation

The inactivation of *Ptgs2* by gene targeting is highly informative in terms of defining those organs in which COX-2 is essential. The pathology of homozygous mutant mice clearly demonstrates that the most severe effects related to disruption of *Ptgs2* are kidney abnormalities and possibly the occurrence of peritonitis in some animals.

Recent investigations have demonstrated that COX-2 levels in rat mesangial kidney cells increase in response to endothelin-1 and serotonin (Kester et al., 1994; Stroebel and Goppelt-Struebe, 1994). Salt-restricted diets also alter the intrarenal distribution of COX-2 and cause an increase in its expression (Harris et al., 1994). These observations make it less surprising that kidneys of homozygous mutant mice older than 6 weeks (both male and female) showed severe pathology.

Kidney development is not complete in mice at birth but continues postnatally for several weeks. The observation of normal renal histology in 3-day-old mice homozygous for *Ptgs2* disruption but severe abnormalities in 6-week or older homozygous mice suggests a postnatal maturation arrest in the subcapsular nephrogenic zone. This zone normally continues to generate nephrons during the postnatal period. Such an arrest would result in a subcapsular zone of hypoplastic glomeruli, which in turn would cause a compensatory hypertrophy of those glomeruli and tubules that had developed prior to the maturation arrest. The glomerular sclerosis and associated tubulointerstitial injury observed in the 16-week-old homozygous mutant could then be explained as resulting from a work overload on the reduced number of functional nephrons.

Thus, a possible explanation for these findings is that the absence of COX-2 in the homozygous mutant mice impairs postnatal differentiation within the subcapsular nephrogenic zone. In kidneys with reduced numbers of nephrons, those nephrons that are present must increase their workload. This leads first to hypertrophy and eventually to glomerular sclerosis (Brenner, 1985). This type of glomerular compensatory hypertrophy and subsequent sclerosis occur following experimental reduction in the number of nephrons in utero (Gilbert et al., 1991) or after birth (Brenner, 1985). It is most severe when the reduction in nephrons occurs early (Celsi et al., 1986).

It is interesting to note the similarities between our findings and those seen in oligomeganephronia, a human congenital renal disease characterized by small kidneys with reduced numbers of nephrons. This disease occurs as a consequence of too few nephrons developing during nephrogenesis (McGraw et al., 1984). The glomeruli and tubules are hypertrophied and develop progressive focal segmental glomerularsclerosis, probably because of functional overload (McGraw et al., 1984).

In contrast with the severe pathology seen after disruption of both gene copies in homozygous mice, inactivating a single copy of *Ptgs2* in the heterozygous animals did not lead to any renal or peritoneal pathology.

Concluding Statements

The current findings in the animals lacking the inducible enzyme COX-2 can be summarized as follows. The animals show no innate gastrointestinal pathology, but their kidneys show abnormalities that cause a progressive deterioration as the animals age. Their inflammatory responses to TPA and AA do not differ from those of wild-type animals, and they have normal inflammatory responses to bacterial invasion of the peritoneum.

Langenbach et al. (1995 [this issue of *Cell*]) have described animals lacking the constitutive enzyme COX-1. In brief, these animals have no gastrointestinal abnormalities, but the female homozygotes have few live births when mated to male homozygotes. The animals have a decrease in AA-induced platelet aggregation, and their inflammatory responses to AA (but not to TPA) are reduced.

These joint observations suggest the need to reappraise some of the current views concerning the detailed functions of the two COX isoforms. Genetic experiments of

the type we have described are unequivocal in their ability to distinguish the cellular and physiological effects of the complete and lifelong absence of each COX isoform without any unintentional inhibition of the other. In addition, while compensatory adaptations in response to the loss of *Ptgs2* may have modified certain elements of the phenotype, the identification and study of these adaptive responses are likely to yield new insights into pathways with which COX-2 interacts. The data that can be derived from these animals should consequently be of great value for the study of the two COX isoforms and also to research directed at the development of improved NSAIDs.

Experimental Procedures

Cloning the PGH Synthase 2 Gene and Construction of the Targeting Vector

Southern blot analysis of genomic DNA from strain 129 mice indicated that a *Ptgs2*-specific probe (derived by PCR) hybridized to a 16 kb BglII fragment. Two regions in exon 10 of the murine *Ptgs2* gene (Fletcher et al., 1992), which are highly conserved between species but not between COX-1 or COX-2, were chosen for PCR primers to generate the *Ptgs2* probe. The primers used were from the 5′ region of exon 10 (GAGAAGGAAATGGCTGCACA) and the 3′ region of exon 10 coding sequence (TTACAGCTCAGTTGAACGCCT); the resulting probe corresponds to amino acid residues 450–585 (Fletcher et al., 1992). Genomic DNA was isolated from E14TG2a mouse ES cells (Hooper et al., 1987) and digested with BglII. Fragments from 10–20 kb in length were isolated and used to make a library in λ phage Charon 35. A murine *Ptgs2* cDNA probe (provided by Dr. D. Young, University of Rochester) was also used in the screening process. Clones covering the entire gene coding sequence were isolated, and two EcoRI fragments that contain all but the most 3′ portion of the gene were subcloned. A 6.0 kb EcoRI fragment (containing exon 1 through the 5′ part of exon 8) and a 1.8 Kb BstXI–EcoRI fragment (containing a 3′ portion of exon 8, exon 9, and a 5′ part of exon 10) were used to create the targeting vector (see Figures 1A and 1B).

Cell Culture, Electroporation, and Selection

BK4 cells (a subclone of E14TG2a, provided by Dr. B. Koller, University of North Carolina at Chapel Hill) were cultured on murine embryonic fibroblasts in DMEM-H (Life Technologies) supplemented with 15% fetal bovine serum (Life Technologies), 100 μM β-mercaptoethanol, and 2 mM L-glutamine. The cells were trypsinized and resuspended in 0.4 ml of DMEM. Electroporation was carried out in the presence of 2–5 nM targeting construct DNA that had been linearized with SacII; a 1 s discharge from a 200 μF capacitor charged to 300 V was used. Selection with G418 and ganciclovir was carried out as described by Mansour et al. (1988).

Half of each clone was picked using a micropipette and placed in a 24-well tissue culture plate overnight. The next day, the colony was trypsinized and expanded for use or was frozen in 50% serum, 40% DMEM, and 10% DMSO at −80°C. The other half of the clone was utilized for genotypic PCR analysis.

Genotype Analysis by PCR and Southern Blotting

Potential recombinant clones were initially screened by recording the presence of a PCR product diagnostic for a correct homologous recombination event (Kim and Smithies, 1988). The two primers used were derived from the *Neo* gene (ACGCGTCACCTTAATATGCG) and from a 3′ region of exon 10 that was not included in the targeting construct (AGATTGTTGTCAGTATCTGCC) (see Figure 1C). DNA from clones that had undergone homologous recombination (as judged by the amplification of the correct PCR fragment) were then used for Southern blot analysis to confirm that *Ptgs2* had been disrupted.

Genotypes of mice were determined using DNA isolated from tails. Genomic DNA totaling 8–10 μg was digested with SacI overnight and applied to a 0.8% agarose gel. After separation using agarose gel electrophoresis, the DNA was transferred to Hybond nylon membrane. Membranes were probed with a 2.1 kb SacI fragment from the *Ptgs2* cDNA clone, labeled with ^{32}P. The SacI cDNA probe used is contiguous with exons 8 and 9 and the 5′ portion of exon 10. Membranes were then washed and exposed overnight to Kodak XAR film with an intensifying screen.

Microinjection and Derivation of Mutant Mice

Cells from two independent targeting events were microinjected into B6 host blastocysts and implanted in pseudopregnant female recipients to generate chimeric mice. Two male mice transmitted the 129 genome to their progeny, and both were bred to B6 females. The heterozygous F1 progeny carrying the disrupted *Ptgs2* gene were then interbred to give F2 mice, which were used for further studies.

Induction of Macrophages with LPS and Measurement of PGE_2 Levels

Macrophages were isolated by peritoneal lavage using 3 × 5 ml of sterile cold RPMI 1640 medium. Resident macrophages were seeded 1×10^7 to 1.5×10^7 cells per 60 mm dish and then placed in a humidified incubator with 5% CO_2 for 2 hr. Cells that attached to the plate were counted using an eyepiece micrometer. Medium was removed, and the cells were washed with serumless medium. Cells were then incubated in medium containing 10 μM AA for 30 min. PGE_2 production was measured using a competitive radioimmunoassay (Amersham).

Northern Blotting of Macrophages Induced with LPS

Total RNA was isolated from plated macrophages using TRIzol (Life Technologies) as recommended by the manufacturer. Each sample (5 μg) was electrophoresed in a 2.2 M formaldehyde–1% agarose gel, transferred to Hybond nylon membrane, and probed using a 2.1 kb SacI fragment from the *Ptgs2* cDNA clone, labeled with ^{32}P. The membrane was then washed and exposed overnight to Hyperfilm MP with an intensifying screen.

Western Blotting of Macrophages Induced with LPS

Cells were washed with ice-cold PBS, lysed in sample buffer (100 mM Tris–HCl [pH 6.8], 8% SDS, 6% 2-mercaptoethanol, 0.05% bromophenol blue, 20% glycerol), and boiled. Protein (5 μg) was separated by SDS–PAGE and transferred onto Hybond–ECL nitrocellulose. Membranes were blocked for 1 hr using 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBST). After washing with TBST, blots were incubated overnight with a rabbit antibody against murine COX-2 (Cayman Chemical) or COX-1 (Morita et al., 1995) in TBST with 1% nonfat milk. Blots were again washed to remove unbound anti-COX-2 or anti-COX-1 antibody and subsequently incubated with anti-rabbit IgG horseradish peroxidase–linked secondary antibody (Boehringer Mannheim) in TBST with 1% skim milk for 1 hr. Chemiluminescence was used to expose Hyperfilm–ECL according to the instructions of the manufacturer (Amersham).

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 measured after 2 hr or 7 hr for AA or TPA, respectively (Gad et al., 1986).

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