Role of cyclooxygenase isoforms in prostacyclin biosynthesis and murine prehepatic portal hypertension


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Submitted 9 January 2008; accepted in final form 20 August 2008

Skill NJ, Theodorakis NG, Wang YN, Wu JM, Redmond EM, Sitzmann JV. Role of cyclooxygenase isoforms in prostacyclin biosynthesis and murine prehepatic portal hypertension. Am J Physiol Gastrointest Liver Physiol 295: G953–G964, 2008. First published September 4, 2008; doi:10.1152/ajpgi.00013.2008.—Portal hypertension (PHT) is a common complication of liver cirrhosis and significantly increases morbidity and mortality. Abrogation of PHT using NSAIDs has demonstrated that prostacyclin (PGI2), a direct downstream metabolite product of cyclooxygenase (COX) activity, is an important mediator in the development of experimental and clinical PHT. However, the role of COX isoforms in PGI2 biosynthesis and PHT is not fully understood. Prehepatic PHT was induced by portal vein ligation (PVL) in wild-type, COX-1−/−, and COX-2−/− mice treated with and without COX-2 (NS398) or COX-1 (SC560) inhibitors. Hemodynamic measurements and PGI2 biosynthesis were determined 1–7 days after PVL or sham surgery. Gene deletion or pharmacological inhibition of COX-1 or COX-2 attenuated but did not ameliorate PGI2 biosynthesis after PVL or prevent PHT. In contrast, treatment of COX-1−/− mice with NS398 or COX-2−/− mice with SC560 restricted PGI2 biosynthesis and abrogated the development of PHT following PVL. In conclusion, either COX-1 or COX-2 can mediate elevated PGI2 biosynthesis and the development of experimental prehepatic PHT. Consequently, PGI2 rather than COX-selective drugs are indicated in the treatment of PHT. Identification of additional target sites downstream of COX may benefit the >27,000 patients whom die annually from cirrhosis in the United States alone.

IN THE UNITED STATES cirrhosis and chronic liver disease is the 12th leading cause of death and in 2005 accounted for more than 27,000 deaths (27). Portal hypertension (PHT) is a serious complication of liver cirrhosis, whereby increased resistance to portal flow is almost always the initial pathophysiological event and is followed by an increased portal venous flow through a hyperdynamic splanchnic system (47). Increased portal venous pressure promotes the formation of collateral venous circulation and esophageal and gastric varices (57). These events impart a significant increase in mortality and morbidity via a propensity for variceal hemorrhage and encephalopathy (4). About 25–40% of all cirrhotic patients have varices of which one-third will hemorrhage with a 20–30% mortality rate (34). In the absence of liver transplantation the current, recommended treatments for PHT and variceal formation are β-blockers to reduce heart rate and portal venous flow, variceal banding, and transhepatic intrajugular portal shunts (18, 28). However, these treatment schemes do not focus on the underlying etiology that increases portal venous pressure. In particular, they do not address the aberrations in systemic and splanchnic circulation that promote increased portal flow and increased portal venous pressure. A cohort of studies have shown that overexpression of prostacyclin (PGI2) reduces systemic and splanchnic resistance and the resultant increased flow is key to increased portal venous pressure (6, 22, 49, 61).

PGI2 is a potent vasodilator and antithrombotic agent and is a member of the prostanooid family. The biosynthesis of prostanooids, which include the prostaglandins and thromboxanes, occurs in three steps: 1) the mobilization of arachidonic acid, from membrane phospholipids through the action of phospholipase A2; 2) the formation of prostaglandin endoperoxide H2 (PGH2) via prostaglandin endoperoxide H synthase (PGHS); and 3) the conversion of PGH2 to specific prostanooids through the action of a synthase such as PGI2 synthase (PGIS) to form PGI2. There are two PGHS isoforms that have similar enzymological and structural properties. These are commonly referred to as cyclooxygenase (COX) enzymes or COX-1 and COX-2 (Figure 1) (51, 52). Therefore, since COX-1 and -2 catalyze the first committed step in the production of PGI2, nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit prostaglandin biosynthesis, should limit PGI2 biosynthesis and the development of PHT. Indeed, PHT can be abrogated in rats and rabbits by reducing PGI2 levels following the blockade of COX activity with the NSAID indomethacin (6, 14). Unfortunately, gastric ulceration is a serious complication associated with the use of NSAIDs.

Until recently the maintenance of gastric mucosal integrity was believed to be dependent on COX-1 derived protective PGE2 from gastric and duodenal epithelium (24, 48, 59). In contrast, under normal physiological conditions PGI2 biosynthesis is believed to be mediated by COX-2 (10, 37). This diversity argued that COX-1 and -2 have distinct functions in tissues and organs and that selective inhibition of COX isoforms may have useful therapeutic outcomes. Animal models and clinical studies have shown that COX-2 inhibitors do not induce gastric ulcers (35). Consequently, it was hoped that this new class of drug could be beneficial in the acute treatment of PHT. However, since then, COX-2 inhibitors have been further studied, leading to the withdrawal of rofecoxib (Vioxx) and valdecoxib (Bextra) (9), while celecoxib (Celebrex) remains available albeit with an FDA-mandated boxed warning.

Despite the increased risks, the biological basis for the cardiovascular consequences of COX-2 inhibition continues to be elucidated and research into COX-2 inhibitors and COX
enzymology persists (13, 15, 20, 35, 63). In particular, two recent publications into the gastrointestinal advantages of COX-2 inhibitors shows that the role of COX inhibitors are still relevant and as such the role of COX isoforms in PGI2 biosynthesis and PHT is significant (17, 29). Previous experimental models investigating PGI2 biosynthesis in PHT have been inconclusive. Increased levels of COX-1 and -2 in the aorta and mesenteric vascular bed of portal vein-ligated (PVL) rats suggest no isoform preference (43). Conversely, Tsugawa et al. (56) have shown that NS398, a COX-2-specific inhibitor, prevented PHT in PVL rats. Moreover, pharmacological studies can sometimes be misleading. For example, (1) COX-1 gene-deleted mice have no gastric pathology and are resistant to indomethacin-induced gastric ulceration (30), 2) selective inhibition of COX-1 in healthy rats with SC560 does not induce gastric ulcers (19, 58), 3) COX heterodimers are resistant to COX isoform selective inhibition (63), and 4) both COX-1 and COX-2 contribute to increased PGI2 in non-PHT vascular disorders (3). Therefore, the COX isoform profile associated with elevated PGI2 in PHT is not clear and a better understanding is required.

To address which COX isoform is essential to the development of PHT, we utilized a murine prehepatic PVL model of PHT in wild-type and COX-1/−/− and COX-2−/− mice. Within this study we investigate the effect of 1) selective inhibition of COX-2 (NS398) or COX-1 (SC560), 2) the targeted gene deletion of either COX-1 or COX-2, and 3) a combination of targeted COX isoform gene deletion with pharmacological COX inhibition (COX-1/−/− ± NS398 or SC560 or COX-2−/− ± SC560 or NS398) on PGI2 levels, abdominal aortic flow (systemic hyperemia), or splenic pulp pressure following PVL. This data will improve our understanding of PGI2 biosynthesis and therapeutic targets needed to benefit cirrhotic patients at risk of PHT and variceal hemorrhage.

**EXPERIMENTAL PROCEDURES**

**Prehepatic PHT Model; Partial Portal Vein Ligation**

All studies were approved by the Indiana University institutional animal care and use committee and adhered to American Association for Accreditation of Laboratory Animal Care and federal guidelines for the humane care and treatment of animals. Mice were maintained in sterilized isolete cages on a 12:12-h light-dark cycle and were allowed access to food and water ad libitum. Mice were anesthetized by halothane inhalation. A midline laparotomy was performed and the portal vein was exposed. A blunt-ended 27-gauge needle was placed alongside the portal vein and a 4-0 silk suture was tied around the vein and needle, after which the needle was withdrawn, producing a standardized stenosis. In sham animals the procedure consisted of dissection and visual inspection of the portal vein without ligature. The abdomen was closed and the animals were allowed to recover under a heat lamp.

**Physiological Measurements**

Physiological measurements were performed as previously described by Theodorakis et al. (55). At the indicated times after sham operation or PVL, animals were anesthetized and subjected to laparotomy to allow physiological measurements to be taken. Portal pressure was determined by measuring the splenic pulp pressure. We have previously shown that portal venous pressure and splenic pulp pressure are directly proportional (55). To measure splenic pulp pressure a microtip pressure transducer (SPR-839, Millar Instruments) was inserted in the spleen. Aortic flow was measured by placing an ultrasonic Doppler flow probe (Transonic no. 11RB) around the abdominal aorta between the diaphragm and celiac artery. Flow rates were obtained with a Transonic T206 Blood Flow Meter (Transonic Instruments). Aortic blood flows were standardized per gram of body weight.

**Gene-Deficient Mice**

Mice containing targeted mutations in prostaglandin H synthase (ptghs)-1 gene (COX-1; strain B6;129P2-Ptgs1tm1) (30) and the ptghs-2 gene (COX-2, strain B6;129P2-Ptgs2tm1) (39) were purchased from Taconic (Germantown, NY). COX-1/2 double-knockout mice were created via embryonic stem cell (ESC) technology (54). ESCs containing two targeted Ptgs2 alleles and one targeted Ptgs1 allele (COX-2 knockout) or one targeted Ptgs2 allele and one targeted Ptgs1 allele (COX-1 knockout) were injected into C57BL/6 blastocysts. The resultant preimplantation embryos were transferred into foster mothers and chimaeric embryos were generated. Diagnosed chimaeras were mated with C57BL/6 wild-type mice and the resulting offspring were intercrossed to generate homozygous knockout mice. Gene-deficient mice were backcrossed to C57BL/6 background for >10 generations. To confirm targeted gene deletion, genomic DNA was isolated from frozen tails and genotypes were determined via PCR with primers flanking the targeted mutation. The following primers were used for PCR: forward primers 5′-ATC GTG AGA AAG TGC TCT GCT-3′ and 5′-AGC TTT TTT TGT CCC TTG TCC-3′, and reverse primers 5′-AGG GTG GAG GTG CAA TGG TTT-3′ and 5′-GCT GAT GAG CAT CTC CAC GT-3′. PCR reactions were performed in 30 μl with 100 ng genomic DNA and the following cycle conditions: 35 cycles of 94° for 30 sec, 59° for 30 sec, and 72° for 1 min. PCR products were resolved on 1.5% agarose gel and stained with ethidium bromide. To determine if targeted gene deletion had occurred, the presence or absence of the expected bands was noted.

The presence of targeted gene deletion was confirmed by Southern blotting. Tails from mice were collected and genomic DNA was isolated according to manufacturer’s instructions. DNA was digested with XbaI and XmnI restriction enzymes and electrophoresed on a 0.7% agarose gel. DNA was then transferred to a nylon membrane. Hybridization was performed using a DIG probe at 42° for 16 h. The probe was generated using the DIG Probe Synthesis Kit (Roche Applied Science, Indianapolis, IN) and a primer set specific for the targeted region (forward primer: 5′-GTA CCA GAC AGT GGT GAT TCG TCT TCT GCA-3′ and reverse primer: 5′-GAC AGA AAG TGG ACC TAC CAG GGT GGG-3′). The blot was washed in 0.1% SDS and 0.1× saline-sodium citrate (SSC) at 65° for 20 min. Blots were hybridized with a DIG detection kit (Roche Applied Science, Indianapolis, IN) and detected using X-ray film.
Gene-specific primers [COX1, 5'-gagagaagagagcatgctgcgt 3'-ctgccatcgtgctgcttaga; COX-2, 5'-caacacatcatacactgcc 3'-ccactggtagaacaagacgc (cycle = 1 min each of 94°C, 60°C and 74°C × 25)] are complementary to the site-specific mutations previously published (30, 39).

**Plasma 6-keto-Prostaglandin F1α and Thromboxane B2 Levels**

Prostacyclin (PGI2) and thromboxane (TXA2) have a relatively short half-life in vivo before they are converted to the biologically inactive 6-keto-PGF1α, and thromboxone-B2 (TXB2). Both analytes were measured by commercially available competitive ELISA kits in accordance with manufacturer’s instructions (Oxford Biomedical Research). Extraction procedure was identical for 6-keto-PGF1α and TXB2. Briefly, blood was collected by cardiac puncture, injected into heparinized tubes, centrifuged and plasma stored at 4°C. Urine was collected over 24 h by use of metabolic cages and stored at 4°C prior to analysis. Urine and plasma 6-keto-PGF1α and plasma TXB2 were isolated by using SEP-PAK C-18 cartridges (Applied Separations). Plasma was diluted with methanol to a final concentration of 15% and applied to a SEP-PACK C-18 cartridge that had been preequilibrated with 2 ml of methanol and 2 ml of H2O at 1 ml/min flow rate. After application of the plasma sample, cartridges were washed with 2 ml of 15% methanol and 2 ml of petroleum ether at 1 ml/min. 6-keto-PGF1α and TXB2 were eluted from the column with 2 ml of methyl formate at 1 ml/min. Eluent was dried down by centrifugal evaporation and resuspended in extraction buffer supplied within ELISA kits (Oxford Biomedical Research).

**Urine 2,3-dinor-6-keto-Prostaglandin F1α Levels**

2,3-Dinor-6-keto-prostaglandin F1α (2,3-dinor-6-keto-PGF1α) is a stable β-oxidation metabolite of 6-keto-PGF1α, and levels have previously been used to quantify systemic PGI2 biosynthesis in mice and humans (3, 44). In contrast, urine 6-keto-PGF1α is predominantly a marker of renal COX-2 activity. Urine was collected for 7 days and 2,3-dinor-6-keto-PGF1α was extracted by a selective two-step solid-phase extraction as described previously (45). 2,3-Dinor-6-keto-PGF1α was quantitated by a competitive ELISA for 2,3-dinor-6-keto-PGF1α and 6-keto-PGF1α (Assay Designs, Ann Arbor, MI) as per manufacturer’s instructions. Briefly, urine was first acidified with 0.1 M HCl to pH 3.0 using a mini-lab low volume pH meter (IQ Scientific) before incubation overnight at room temperature. Spe-ed C-1 methyl silica 500 mg/6 ml minicolumns (Applied Separations) were equilibrated with 5 ml of methanol and 5 ml of H2O at 2 ml/min. Acidified urine was applied to the column at 0.5 ml/min and the column was washed with 5 ml of H2O and 5 ml of n-hexane at 1 ml/min. 2,3-dinor-6-keto-PGF1α was eluted with 5 ml of diethyl ether-n-hexane (85:15, vol/vol). Eluent was resuspended in prostanoid-free urine (see below for preparation of prostanoid-free urine) and pH was altered to 10 with 0.1 M sodium hydroxide and incubated for 1 h. Spe-ed C-1 methyl silica 500 mg/6 ml minicolumns were equilibrated as before, and sample was applied at 0.5 ml/min. Column was washed as before and 2,3-dinor-6-keto-PGF1α was eluted with 5 ml of chloroform at 1 ml/min, centrifugally evaporated, and resuspended in extraction buffer supplied in the urine 2,3-dinor-6-keto-PGF1α assay kit. Prostanoid-free urine is prepared by adding activated charcoal to pooled urine from unadulterated B6129P2 mice (5% wt/vol) and stirred for 1 h. After centrifugation, at 3,000 g for 15 min supernatant was taken and assayed for 2,3-dinor-6-keto-PGF1α and 6-keto-PGF1α.

**Determination of In Vivo COX Activity Inhibition Using the COX-2 Inhibitor NS398 and COX-1 Inhibitor SC560**

NS398 [N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide] (Cayman Chemical) is marketed as a selective COX-2 inhibitor (IC50 COX-2 = 3.8 μM), but it can, at high doses, inhibit COX-1 activity (IC50 COX-1 >100 μM) (41). Titration of NS398 to selectively inhibit COX-2 in vivo with minimal effect on COX-1 was determined by measurement of serum TXB2 and 6-keto-PGF1α, and urine 6-keto-PGF1α levels 24 h following administration of 1–10 mg/ml ip NS398 or DMSO vehicle control in B6;129P2 wild-type mice (Taconic). Urine 6-keto-PGF1α is predominantly a marker of COX-2 activity whereas serum TXB2 is representative of COX-1 activity. NS398 at 2 mg/kg was chosen to selectively inhibit COX-2 activity. Please see RESULTS. To determine the duration of inhibition, 2 mg/kg NS398 was given to B6;129P2 mice and 24-h urine 6-keto-PGF1α levels were determined 0–72 h thereafter. To confirm that 2 mg/kg NS398 was COX-2 specific COX-1–/– and COX-2–/– gene-knockout mice were given 2 mg/kg ip NS398, and 24-h urine 6-keto-PGF1α levels were determined.

SC560 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole] (Cayman Chemical) is marketed as a selective COX-1 inhibitor (COX-1 IC50 is 9 nM), but it can, at high doses, inhibit COX-2 activity (COX-2 IC50 is 6.3 μM) (50). Titration of SC560 to selectively inhibit COX-1 in vivo with minimal effect on COX-2 was determined by measurement of serum TXB2 and 6-keto-PGF1α levels 24 h following administration of 10–80 mg/ml ip SC560 or DMSO vehicle control in B6129P2 wild-type mice. Urine 6-keto-PGF1α is predominantly a marker of COX-2 activity whereas serum TXB2 is representative of COX-1 activity. SC560 at 20 mg/kg was chosen to selectively inhibit COX-1 activity. Please see RESULTS. To determine the duration of inhibition 20 mg/kg ip SC560 was given to B6129P2 mice and plasma TXB2 was determined 0–72 h thereafter. To confirm that 20 mg/kg SC560 was COX-1 specific, COX-1–/– and COX-2–/– gene-knockout mice were given 20 mg/kg ip SC560 and plasma TXB2 was quantitated.

**Effects of Portal Vein Ligation on PGI2 Biosynthesis and Portal Hemodynamics in Wild-Type and COX Isoform Gene Deleted Mice**

To determine the effects of PVL on portal hemodynamics and PGI2 biosynthesis, wild-type (B6;129P2), COX-1–/– (B6;129P2-Ptg1αm1m1), and COX2α–/– (B6;129P2-Ptg2αm1m1) mice were subjected to either sham or PVL surgery as described above. In wild-type mice SC560 was given selectively to inhibit COX-2 activity (COX-2 IC50 is 6.3 nM), but it can, at high doses, inhibit COX-1 activity. SC560 at 20 mg/kg was chosen to selectively inhibit COX-2 activity. Please see RESULTS. To determine the duration of inhibition 2 mg/kg ip SC560 was given to B6129P2 mice and plasma TXB2 was determined 0–72 h thereafter. To confirm that 2 mg/kg SC560 was COX-1 specific, COX-1–/– and COX-2–/– gene-knockout mice were given 20 mg/kg ip SC560 and plasma TXB2 was quantitated.

**Effects of Pharmacological COX Inhibition in Wild-Type and COX Isoform Gene Knockout Mice on PGI2 Biosynthesis and Portal Hemodynamics**

To determine the effects of COX-1 or COX-2 selective inhibitors on PGI2 biosynthesis and portal hemodynamics following PVL wild-type mice (B6;129P2) were treated with either 2 or 10 mg/kg NS398 or 20 or 80 mg/kg SC560 12 h prior to PVL or sham surgery. Both NS398 and SC560 have previously been used as COX-selective inhibitors in the rat PVL model of PHIT (1). The two doses will show the effects of each compound when given at selective and non-isoinform-selective doses. After surgery NS398 was given daily whereas SC560 was given every 48 h. Urine was collected throughout and stored at 4°C. At 7 days following surgery, abdominal aortic flow, splenic pulp pressure, and plasma 6-keto-PGF1α and TXB2 levels were determined 1, 2, 4, and 7 days following sham or PVL (n = 5 per group). In addition, 7-day PVL and sham wild-type mice were analyzed for 7-day urine 2,3-dinor-6-keto-PGF1α levels. In COX-1–/– and COX-2–/– mice abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF1α were determined 7 days following sham or PVL surgery (n = 5 per group).

**Effects of Pharmacological COX Inhibition in Wild-Type and COX Isoform Gene Knockout Mice on PGI2 Biosynthesis and Portal Hemodynamics**

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throughout and stored at 4°C. On day 7 after PVL or sham operation, abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF$_{1\alpha}$ were determined.

Statistics

The data shown are means ± SE, with five animals per experimental group. Statistical significance was estimated by one-way ANOVA statistical analysis. A value of $P < 0.05$ was considered significant.

RESULTS

Portal Vein Ligation Increases PGI$_2$ Biosynthesis and Portal Hemodynamics in Wild-Type and COX-1$^{-/-}$ and -2$^{-/-}$ Gene-Deficient Mice

Mortality. Sham and PVL surgery was associated with a 0 and 29% mortality rate, respectively, 7 days following surgery. There was no significant difference in 7-day mortality rates between B6;129P2 wild-type and COX-1$^{-/-}$ or COX-2$^{-/-}$ mice following either sham or PVL surgery.

Wild-type mice. In B6;129P2 wild-type mice portal hemodynamics and PGI$_2$ biosynthesis were significantly increased following PVL compared with sham-operated controls. The abdominal aortic flow increased steadily 1 and 2 days following PVL but was not significantly different from shams. After 4 and 7 days the abdominal aortic flow was significantly raised 23.5 and 58.8%, respectively, in PVL-treated mice compared with sham-operated controls (Fig. 2A). The splenic pulp pressure progressively increased immediately following PVL and was elevated 64.2, 189.3, 266.3, and 281.5% at 1, 2, 4, and 7 days post-PVL, respectively, compared with sham-operated controls (Fig. 2B). To determine PGI$_2$ biosynthesis plasma 6-keto-PGF$_{1\alpha}$ and urine 2,3-dinor-6-keto-PGF$_{1\alpha}$ were quantitated. PGI$_2$ rapidly converts to the stable and biologically inert 6-keto-PGF$_{1\alpha}$ and after β-oxidation to 2,3-dinor-6-kPGF$_{1\alpha}$ is

![Fig. 2. Portal vein ligation (PVL) increases splenic pulp pressure, abdominal aortic flow, and prostacyclin levels but does not increase thromboxane levels.](http://ajpgi.physiology.org/)
excreted in the urine. 2,3-dinor-6-keto-PGF₁α and plasma 6-keto-PGF₁α levels have previously been used to quantitate systemic PGI₂ biosynthesis in mice and humans (3, 44). Plasma 6-keto-PGF₁α was increased twofold 2 days after PVL and was maximally increased by 2.5-fold after 7 days compared with shams (Fig. 2C). Correspondingly, 7-day urine 2,3-dinor-6-keto-PGF₁α excretion was significantly increased 336% following PVL compared with shams (Fig. 2C). In comparison, plasma level of TXB₂ was not altered following PVL. TXB₂ is the stable in vivo hydrolyzed product of TXA₂, which is the unstable and bioactive metabolic product coupled to COX-1 activity. Not at any time following surgery was plasma TXB₂ level significantly different between sham and PVL mice (Fig. 2D).

**COX-1⁻/⁻ and -2⁻/⁻ gene-deleted mice.** Although COX-1⁻/⁻ and COX-2⁻/⁻ mice were screened by the supplier (Taconic Laboratories), COX gene deficiency was confirmed by PCR. Wild-type genomic DNA used as a PCR template generated products corresponding to COX-1- and COX-2-specific sequences. When genomic DNA from COX-1⁻/⁻ and COX-2⁻/⁻ mice were used as a template, PCR products for COX-1 and COX-2, respectively, were absent (data not shown).

**COX-1⁻/⁻ mice.** In sham-operated COX-1⁻/⁻ mice abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF₁α were not statistically different from those observed in wild-type shams. Following PVL abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF₁α were increased 32.4, 70, and 265%, respectively, in 7-day PVL COX-1⁻/⁻ mice compared with 7-day sham-operated COX-1⁻/⁻ controls (Fig. 3, A–C). With the exception of 2,3-dinor-6-keto-PGF₁α these increases were significantly less than those observed between wild-type 7-day shams and 7-day PVL-operated mice.

**COX-2⁻/⁻ mice.** In 7-day sham-operated COX-2⁻/⁻ mice, there was no significant difference in abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF₁α compared with wild-type 7-day shams. Following PVL, abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF₁α were increased 35, 56, and 315%, respectively, in 7-day PVL COX-2⁻/⁻ mice.

![Fig. 3. Effect of COX-1 or COX-2 gene deficiency on abdominal aortic flow, splenic pulp pressure, and urine 2,3-dinor-6-keto-PGF₁α levels following partial PVL or sham surgery.](http://ajpgi.physiology.org/)
compared with 7-day sham-operated COX-2/−/− controls (Fig. 3, D–F). With the exception of 2,3-dinor-6-keto-PGF1α, these increases were significantly less than those observed between wild-type 7-day shams and 7-day PVL-operated mice but were not when compared with those between COX-1/−/− 7-day shams and COX-1/−/− 7-day PVL-operated mice.

**COX Isoform Inhibition Is Dose Dependent in Wild-Type and COX Gene-Deficient Mice**

Plasma 6-keto-PGF1α levels were not statistically different among unadulterated B6;129P2, COX-1/−/−, and COX-2/−/− mice (11.1 ± 0.9, 9.1 ± 1.8, and 12.4 ± 1.2 ng/ml, respectively). In contrast, plasma TXB2 levels were significantly lower in unadulterated COX-1/−/− mice compared with B6;129P2 and COX-2/−/− mice (1.01 ± 0.3, 4.8 ± 0.4, and 4.1 ± 0.9 ng/ml, respectively) (*P* = 0.003 and 0.44, B6;129P2 vs. COX-1/−/− and COX-2/−/−, respectively).

**Mortality.** There was no mortality associated with 2–10 mg/kg NS398, 10–80 mg/kg SC560, or DMSO vehicle control in unadulterated B6;129P2, COX-1/−/−, or COX-2/−/− mice.

**Wild-type mice.** Urine 6-keto-PGF1α and plasma TXB2 levels were dose dependently reduced by NS-398 and SC560 compared with DMSO controls.

**NS398.** Urine 6-keto-PGF1α (marker of COX-2 activity) was reduced 41% by 2 mg/kg NS398 and was maximally reduced 75.8% at 10 mg/kg (Fig. 4A). Plasma TXB2 (marker of COX-1 activity) was not altered at 1–4 mg/kg but was reduced 75.1% by 10 mg/ml NS398 (Fig. 4A). From this data, 2 mg/kg NS398 was determined as a COX-2-specific inhibitory dose. To determine the length of inhibition, 2 mg/kg NS398 was given to B6;129P2 mice and 24-h urine 6-keto-PGF1α levels were determined for 72 h. NS398 at 2 mg/kg reduced urine 6-keto-PGF1α 24 h following administration. After 24 h levels were not significantly different compared with 0 mg/ml DMSO vehicle control levels; therefore it was administered daily (Fig. 4C).

**SC560.** Plasma TXB2 was reduced by 26% by 20 mg/kg SC560 and was maximally reduced by 71.9% at 80 mg/kg. In contrast, urine 6-keto-PGF1α was not altered at 20 mg/kg but was reduced 44% by 80 mg/ml SC560 (Fig. 4B). To

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**Fig. 4.** In wild-type and COX-knockout mice, NS398 and SC560 are dose-dependent COX-selective inhibitors. To confirm the selectivity of commercially available COX inhibitors, B6;129P2 or COX gene-knockout mice were given either the COX-2 inhibitor NS398 (A and C) or the COX-1 inhibitor SC560 (B and D). In vivo COX inhibition was determined by quantitating plasma TXB2 (marker of COX-1 activity) or urine 6-keto-PGF1α (marker of COX-2 activity) levels. A: 2 mg/kg NS398 dose dependently reduced urine 6-keto-PGF1α levels (shaded bars) with no significant change in plasma TXB2 levels (open bars). At 10 mg/kg NS398 decreased plasma TXB2 levels also. B: 20 mg/kg SC560 reduced plasma TXB2 levels (open bars) with no significant change in urine 6-keto-PGF1α (shaded bars); 80 mg/kg SC560 decreased plasma TXB2 levels also, and 2 mg/kg NS398 and 20 mg/kg SC560 were determined to achieve selective inhibition of COX-2 and COX-1 isoforms, respectively. C: 2 mg/kg NS398 reduced urine 6-keto-PGF1α level (open bars) for only 24 h. Urine 6-keto-PGF1α was significantly reduced by 2 mg/kg NS398 in COX-1/−/− (shaded bars) but not in COX-2/−/− mice (hatched bars). D: 20 mg/kg SC560 reduced plasma 6-keto-PGF1α (open bars) for 24 and 48 h. Plasma 6-keto-PGF1α was not altered by 20 mg/kg SC560 in COX-1/−/− (shaded bars) but was significantly reduced in COX-2/−/− mice (hatched bars) (A–D). Data represents means ± SE, n = 5 per group.
determine the length of inhibition, 20 mg/kg SC560 was given to B6;129P2 mice and plasma TXB₂ levels were determined every 24 h for 72 h thereafter; 20 mg/kg SC560 reduced plasma TxB₂ levels 26 and 23% at 24 and 48 h, respectively. After 48 h there was no difference compared with 0 mg/kg DMSO vehicle controls; therefore it was administered every second day (Fig. 4D).

**Hemodynamic measurements.** No significant hemodynamic changes were observed following administration of either NS398 or SC560. Abdominal aortic flow and splenic pulp pressure trended downward with NS398 and high doses of NS398 or SC560. Abdominal aortic flow and splenic pulp changes were observed following administration of either 2 mg/kg SC560 shams (Fig. 5, A–C). NS398 at 2 mg/kg significantly reduced any increase. Conversely, 20 mg/kg SC560 had no effect on plasma TXB₂ levels in COX-2 mice. In COX-1 mice treated with 20 mg/kg SC560, abdominal aortic flow, splenic pulp pressure, and 2,3-dinor-6-keto-PGF₁α were increased 45, 70, and 295%, respectively, in 7-day sham-NS398 COX-1 mice compared with 7-day sham-SC560 mice (Fig. 6, A–C). NS398 at 2 mg/kg significantly reduced these increases, whereas 20 mg SC560 did not. Abdominal aortic flow, splenic pulp pressure, and 2,3-dinor-6-keto-PGF₁α were minimally increased 7, 14, and 9%, respectively, in 7-day PVL-NS398 COX-1 mice compared with 7-day sham-NS398 COX-1 mice. In COX-1 mice treated with 20 mg/kg SC560, abdominal aortic flow, splenic pulp pressure, and 2,3-dinor-6-keto-PGF₁α were increased 30, 58, and 265%, respectively, in 7-day PVL-SC560 mice compared with 7-days sham-SC560 mice (Fig. 6, A–C).

**To Prevent PHT Is Dose Dependent**

To determine the effect of COX-1 or COX-2 activity inhibition in combination with COX gene deletion on PGI₂ biosynthesis and PHT development, COX-1−/− and COX-2−/− mice were treated with 20 mg/kg SC560 or 2 mg/kg NS398 prior to and following PVL or sham surgery. Unfortunately, COX-1/2 double knockouts are not viable (33).

**Mortality.** Mortality rates following administration of NS398, SC560, or DMSO to sham- or PVL-operated COX-1−/− or COX-2−/− mice was 0 and 29%, respectively. There was no difference in mortality rates among B6;129P2, COX-1−/−, and COX-2−/− mice. However, PVL-COX-2−/− mice treated with 20 mg/kg SC560 were distressed compared with B6;129P2 or COX-1−/− PVL mice treated with 20 mg/kg SC560. Mice were less active, hunched, and had largely distended stomachs 7 days following PVL. This observation is in agreement with Akahoshi et al. (1), who have previously reported that SC560 (10–40 mg/kg) causes gastric mucosal damage in PHT rats but not in shams.

**COX-1−/− mice.** There were no significant differences in abdominal aortic flow, splenic pulp pressure, or 2,3-dinor-6-keto-PGF₁α among 7-day DMSO shams, 2 mg/kg NS398 shams, and 20 mg/kg SC560 shams. At 7 days following PVL surgery, abdominal aortic flow, splenic pulp pressure, and 2,3-dinor-6-keto-PGF₁α were increased 45, 70, and 295%, respectively, in PVL-DMSO mice compared with 7-day sham-DMSO controls (Fig. 6, A–C). NS398 at 2 mg/kg significantly reduced these increases, whereas 20 mg SC560 did not. Abdominal aortic flow, splenic pulp pressure, and 2,3-dinor-6-keto-PGF₁α were increased 30, 58, and 265%, respectively, in 7-day PVL-SC560 mice compared with 7-days sham-SC560 mice (Fig. 6, A–C).

**COX-2−/− mice.** There were no significant differences in abdominal aortic flow, splenic pulp pressure, or 2,3-dinor-6-keto-PGF₁α among 7-day DMSO shams, 2 mg/kg NS398 shams, and 20 mg/kg SC560 shams. In contrast, 7-day urine 2,3-dinor-6-keto-PGF₁α was reduced 32.8% in 20 mg/kg SC560 sham mice compared with DMSO sham controls. At 7 days following PVL surgery, abdominal aortic flow, splenic pulp pressure, and 2,3-dinor-6-keto-PGF₁α were increased 35.3, 53, and 295%, respectively, in PVL-DMSO mice compared with 7-day sham-DMSO controls (Fig. 6, D and E). NS398 2 mg/kg did not significantly reduce these increases, whereas 20 mg/kg SC560 significantly reduced any increase. Abdominal aortic flow, splenic pulp pressure, and 2,3-dinor-6-keto-PGF₁α were increased 40, 32, and 254%, respectively, in 7-day PVL-NS398 COX-2−/− mice compared with 7-day sham-NS398 COX-2−/− mice. In COX-2−/− mice treated with 20 mg/kg SC560 abdominal aortic flow and splenic pulp pressure were reduced 14 and 16%, respectively, whereas 2,3-dinor-6-
keto-PGF1α was increased 36.4% in 7-day PVL-SC560 mice compared with 7-day sham-SC560 mice (Fig. 6, D–F).

DISCUSSION

To investigate the role of COX isoforms in PGI2 biosynthesis and PHT we utilized commercially available COX gene-deficient mice and COX isoform-selective inhibitors and documented changes in PGI2 biosynthesis and the development of PHT following PVL. We found no COX isoform predominance in PGI2 biosynthesis or PHT development, which argues that COX-selective inhibitors would not be beneficial in the treatment of PHT. PGI2 biosynthesis, abdominal aortic flow, and splenic pulp pressure were increased in COX-1−/− and COX-2−/− mice and in wild-type mice treated with COX-1 (20 mg/kg SC560)- or COX-2 (2 mg/kg NS398)-selective inhibitors, whereas when both COX isoforms were inhibited PGI2 biosynthesis was reduced, thus preventing vasodilatation and the formation of a hyperdynamic circulation. This prevents the development of PHT in the PVL model because in the absence of hyperemia portal systemic shunting negates the resistance caused by the mechanical stenosis.
There are a couple of reasons why no COX isoform predominates in this model: 1) COX-1 and COX-2 coexist in the vasculature and may be compensating for each other. Typical of most studies using gene-knockout mice, data must be interpreted with caution because redundant and/or compensatory mechanisms can appear. Indeed, this may explain why PGI2 levels were not reduced in COX-2$^{-/-}$ mice until they were treated with the COX-1 inhibitor SC560. Moreover, PGH2 can be synthesized in platelets via COX-1 and transferred to endothelial cells and converted to PGI2 (35). 2) COX heterodimers are resistant to COX isoform selective inhibition (63). In COX gene-deficient mice heterodimers cannot form but PGI2 biosynthesis was unaltered, showing that both COX-1 and COX-2 homodimers can mediate PGI2 biosynthesis in both basal and elevated PGI2 biosynthesis. These findings argue that additional targets, downstream of COX, need to be considered and investigated. Alternative targets for PGI2 include 1) inhibition of PGIS and 2) antagonism of prostacyclin signaling.

The synthesis of stable PGH2 analogs has generated a potent inhibitor for PGIS that has been used experimentally to investigate the role of PGI2 in multiple pathophysiological events (5, 21, 42). To our knowledge, PGIS inhibitors have not been used to reduce PGI2 biosynthesis and PHT in vivo. Following synthesis, PGI2 activates the G protein-coupled cell surface...
prostaglandin receptor (IP). Activation of IP stimulates adenylyl cyclase, leading to increased cAMP, vasodilation, inhibition of cell proliferation, and release of inflammatory mediators (60). Alternatively, PG12 is also capable of activating peroxisome proliferator activated receptor β (PPARβ) (32), which is reported to have anti-inflammatory properties (31). Targeted gene-deficient mice for both IP and PPARβ have been generated (7, 54). To our knowledge, the development of PHT has not been investigated in these animals. With that said, these mice have multiple pathologies associated with a lack of normal PG12 signaling and as such any investigation would be problematic. Specifically, deletion of IP elevates blood pressure, modulates vascular remodeling, and was found to promote atherosclerosis in mice via impaired activation of neutrophils and platelets (7, 12, 25).

COX isozymes are also important in regulating renal function. In the kidney PGIS and IP are mainly found in the mesangial cells and afferent arterial endothelial cells (26, 40). PG12 regulates blood flow and therefore filtration rate of the kidney (2, 16). In PG12-deficient mice, progressive morphological abnormalities develop in the kidney and mice have increased blood pressure and elevated plasma urea, nitrogen, and creatinine levels. However, these abnormalities have not been reported in IP-deficient mice (62). In this study we found that urine output is significantly reduced immediately following PVL in wild-type and COX-1 and -2−/− mice, after which urine output increases but does not exceed presurgical levels (data not shown). This is counter to an increase in PG12 increasing renal output. The initial reduction is probably linked to the temporary drop in systemic blood flow that follows PVL. Therefore, serious consideration is required to evaluate the renal complications associated with PG12 inhibition. Such an evaluation was not included in this study and would need to be covered separately. Inasmuch, the interaction between COX and nitric oxide (NO) synthase (NOS) has also not been focused on in this study. The family of NOS enzymes also produces a potent vasodilator, NO, that has been even more significantly linked with the development of PHT (38, 55). Data suggests that NOS and COX are significantly linked and both influence and compensate for one another (11, 38). Salvemini and coworkers (46) initially demonstrated that enhanced release of prostaglandins was nearly entirely driven by NO. Subsequently, additional mechanistic studies that have investigated how NO switches on/off the COX pathway have shown pathways through which NO modulates prostaglandin production. We did not study this NO/COX cross talk specifically but we did observe that endothelial NOS (eNOS) mRNA was significantly upregulated in both arteriolar and venous tissues of COX-1−/− mice but not COX-2−/− mice (data not shown). Moreover, preliminary studies investigating PG12 in eNOS−/− mice show that plasma 6-keto-PGF1α was not increased following PVL (data not shown). Consequently, NO/COX cross talk is highly pertinent in both normal and disease pathophysiology and needs to be considered when considering the potential of PG12 inhibition in any PHT treatment paradigm.

In conclusion, PG12 is very important to the development of systemic hyperemia and elevated portal pressure in the PVL model of prehepatic PHT. Moreover, PG12 biosynthesis is not COX-1 or COX-2 dependent. These findings direct further research toward other aspects of PHT vasculopathy. Targeting of PG12 directly via either its synthesis or signaling might be an improvement but may have the same caveats as COX-2 inhibitors and may also affect renal function. Inhibition of PG12 should not interfere with the gastric protective PGE2 but cardiovascular complication risk may increase. Recent studies have shown that increased PG12 in the rat PVL model corresponds with a decreased in vivo platelet activity resulting in reduced laser-induced thrombus formation (13). Although this protects against cardiovascular accidents it exacerbates variceal bleeding. Therefore, not only would PG12 inhibition reduce splanchic hypervolemia, it would also reduce bleeding complications associated with PHT. Any increased cardiovascular risk may be reduced by utilizing a multitarget approach. Targeted inhibition of both thromboxane and PG12 synthesis would be one option. In addition to PGIS inhibitors thromboxane synthase inhibitors have been identified (U44069) as have IP antagonists (8, 21). Alternatively, given the resistance of COX heterodimers to isoform selective inhibitors and the discovery of COX-1 inhibitors that do not induce gastrointestinal complications, it may be possible to reduce systemic thromboxane via COX-1 inhibition without disturbing PG12 gastrointestinal protection. However, evidence in this article and others suggests that COX-1 inhibitors are not neutral under pathological conditions (1). Nevertheless, given the renewed interest in COX and prostanoids in disease pathology, additional studies to better understand the role of PG12 in PHT are warranted and relevant to develop new treatments to reduce PHT and its associated mortality and morbidity. In particular, a better understanding of PG12, trigger, signaling, and NO cross talk within PHT models is needed and may bear fruit for novel targets for clinical therapy.

GRANTS

The research was funded by a grant awarded to J. V. Sitzmann through the National Institute of Diabetes and Digestive and Kidney Diseases (08RO1DK47067).

REFERENCES


15. F1 alpha for determination with radioimmunoassay.


