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

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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 thromboxane, 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
enzyromology 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 still relevant (63), and treatment 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 isolator 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 0-0 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 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.

Fig. 1. Prostaglandin synthesis pathway. A: cyclooxygenase is a heme-containing glycoprotein and is a key enzyme in prostanoid biosynthesis by catalyzing the conversion of arachidonic acid to PGH2. Subsequently, PGH2 is converted to active prostanoids via secondary prostanoid synthases. Prostacyclin has a half-life of -1 h before inactivation via a nonenzymatic hydration process 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.

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Plasma 6-keto-Prostaglandin F1α Levels

Prostaglandin (PGI₂) and thromboxane (TXA₂) have a relatively short half-life in vivo before they are converted to the biologically inactive 6-keto-PGF₁α, and thromboxane-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-PAK C-18 cartridge that had been pre-equilibrated 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 quanitate systemic PGI₂ biosynthesis in mice and humans (3, 44). In contrast, urine 6-keto-PGF₁α is predominately 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 quantitation was validated by a competitive ELISA for 2,3-dinor-6-keto-PGF₁α and 6-keto-PGF₁α. COX-1 gene-specific primers (COX1, 5' atacctgcttgtacagcaattggca 3', and 5'ctacatcatctgctgctgctagaa; COX-2, 5' gctgctgctgctgctgctg 3' gagagaaggagatggctgctg 3') were complementary to the site-specific mutations previously published (30, 39).

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 gene-knockout mice (B6;129P2-Ptgs1tm1), and COX-2 gene-knockout (B6;129P2-Ptgs2tm1) mice were subjected to either sham PVL or surgery as described above. In wild-type mice 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

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 PHT (1). The two doses will show the effects of each compound when given at selective and non-isomeric-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 after surgery, abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF₁α were determined. To determine the effect of pharmacological COX inhibition in COX isoform gene-knockout mice, COX-1⁻/⁻ and COX-2⁻/⁻ mice were given 2 mg/ml NS398 or 20 mg/kg ip SC560 12 h before and after PVL or sham operation. After surgery NS398 was given daily whereas SC560 was given every 48 h. Urine was collected...
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-PGF₁α 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 PGI₂ 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 PGI₂ 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 PGI₂ biosynthesis plasma 6-keto-PGF₁α and urine 2,3-dinor-6-keto-PGF₁α were quantitated. PGI₂ rapidly converts to the stable and biologically inert 6-keto-PGF₁α and after β-oxidation to 2,3-dinor-6-kPGF₁α 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/)

Prehepatic portal hypertension (PHT) was induced in B6;129P2 wild-type mice by PVL. Control mice received a sham operation. At 1–7 days after surgery splenic pulp pressure and abdominal aortic flow were measured and plasma was analyzed for metabolites of prostacyclin (6-keto-PGF₁α) and thromboxane (TXB₂). In addition, total urine output from the 7-day sham and PVL mice was collected and measured for 2,3-dinor-6-keto-PGF₁α (β-oxidative metabolite of 6-keto-PGF₁α). A: abdominal aortic flow increased progressively following PVL (▨) and was significantly different to shams (◇) after 4 and 7 days. B: splenic pulp pressure rapidly increased in the PVL group (▨) but was not increased following sham surgery (◇). C: plasma 6-keto-PGF₁α levels increased significantly following PVL (shaded bars) but not following sham operation (open bars). The urine 2,3-dinor-6-keto-PGF₁α 7-day level was also significantly increased in 7-day PVL mice compared with 7-day shams. D: plasma TXB₂ levels were unaltered by either PVL (shaded bars) or sham (open bars) operation. *PVL vs. sham, $P < 0.05$; means ± SE; $n = 5$ per group.
excreted in the urine. 2,3-dinor-6-keto-PGF$_{1\alpha}$ and plasma 6-keto-PGF$_{1\alpha}$ levels have previously been used to quantitate systemic PGI$_2$ biosynthesis in mice and humans (3, 44). Plasma 6-keto-PGF$_{1\alpha}$ 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-PGF$_{1\alpha}$ excretion was significantly increased 336% following PVL compared with shams (Fig. 2C). In comparison, plasma level of TXB$_2$ was not altered following PVL. TXB$_2$ is the stable in vivo hydrolyzed product of TXA$_2$, which is the unstable and bioactive metabolic product coupled to COX-1 activity. Not at any time following surgery was plasma TXB$_2$ 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-PGF$_{1\alpha}$ 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-PGF$_{1\alpha}$ 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-PGF$_{1\alpha}$ 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-PGF$_{1\alpha}$ compared with wild-type 7-day shams. Following PVL, abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF$_{1\alpha}$ were increased 35, 56, and 315%, respectively, in 7-day PVL COX-2$^{-/-}$ mice.

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**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-PGF$_{1\alpha}$ levels following partial PVL or sham surgery. B6;129P2-Ptgs1$^{tm1}$ (COX-1$^{-/-}$) (A–C) and B6;129P2-Ptgs2$^{tm1}$ (COX-2$^{-/-}$) (D–F) mice were subjected to either a partial PVL or sham surgery, and urine was collected for 7 days. After this time the abdominal aortic flow and splenic pulp pressure were measured and the urine was analyzed for the prostacyclin metabolite 2,3-dinor-6-keto-PGF$_{1\alpha}$. Abdominal aortic flow (A and D), splenic pulp pressure (B and E), and urine 7-day 2,3-dinor-6-keto-PGF$_{1\alpha}$ (C and F) were all significantly increased 7 days following PVL compared with sham controls. Data represents means ± SE, n = 5 per group.
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.

**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

**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 SC560 but were not significantly altered compared with DMSO vehicle controls (data not shown).

**COX-1−/− and COX-2−/− mice.** To confirm COX isozyme selective inhibition 2 mg/kg NS398 or 20 mg/kg SC560 were given to COX-2−/− and COX-1−/− mice. 6-keto-PGF1α and TXB2 levels were determined in urine and plasma, respectively. NS398 at 2 mg/kg reduced urine 6-keto-PGF1α levels by 42.3% in COX-1−/− mice but had no effect in COX-2−/− gene-deleted mice compared with 0 h controls (Fig. 4C). Conversely, 20 mg/kg SC560 had no effect on plasma TXB2 levels in COX-1−/− mice, whereas in COX-2−/− mice plasma TXB2 was reduced 45.1% (Fig. 4D).

**Pharmacological Inhibition of COX With NS398 and SC560 To Prevent PHT Is Dose Dependent**

To determine the effectiveness of COX inhibitors to limit PGI2 biosynthesis and the development of PHT, wild-type B6;129P2 mice were treated with 2 and 10 mg/kg NS398 or 20 and 80 mg/kg SC560 prior to and following PVL or sham surgery.

**Effect of NS398.** In DMSO-treated vehicle controls, abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF1α were significantly increased by 59, 279, and 336%, respectively, 7 days following PVL compared with 7-day sham controls. These increases were not significantly altered by daily administration of 2 mg/kg NS398 and remained increased by 70.6, 213, and 291%, respectively. However, when NS398 was given at 10 mg/kg, which is not COX-2 selective, abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF1α were not significantly increased in PVL mice compared with either DMSO shams or 10 mg/kg NS398 shams (Fig. 5, A–C). There were no differences in abdominal aortic flow or splenic pulp pressure among DMSO shams, 2 mg/kg NS398 shams, and 10 mg/kg NS398 shams.

**Effect of SC560.** In DMSO-treated vehicle controls abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF1α were significantly increased by 80, 141, and 188%, respectively, 7 days following PVL compared with 7-day sham controls. These increases were not significantly altered by daily administration of 20 mg/kg SC560 and remained increased by 70, 132, and 160%, respectively. However, when SC560 was given at 80 mg/kg, which is not COX-1 selective, abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF1α were not significantly increased in PVL mice compared with either DMSO shams or 80 mg/kg SC560 shams (Fig. 5, D–F). There were no significant differences in abdominal aortic flow or splenic pulp pressure among DMSO shams, 20 mg/kg SC560 shams, and 80 mg/kg SC560 shams.

**COX Targeted Gene Deletion in Combination With Pharmacological COX Inhibition Inhibits PGI2 Biosynthesis and PHT**

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−/− or 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 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).

**COX-2−/− mice.** There were no significant differences in abdominal aortic flow, splenic pulp pressure, or 2,3-dinor-6-keto-PGF1α among 7 days 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, 58, 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.

Fig. 5. Non-isoform-selective inhibition of COX-1 and COX-2 with NS398 and SC560 prevents PHT, hyperemia, and elevated prostacyclin in wild-type mice. B6;129P2 mice were given either DMSO (vehicle control) 2–10 mg·kg−1·day−1 NS-398 or 20–80 mg·kg ip SC560 24 h prior to partial PVL or sham surgery. DMSO and 2–10 mg/kg NS398 were given daily thereafter for 7 days, and SC560 was given at 20–80 mg/kg every 48 h thereafter for 7 days. During this time total urine output was collected. After 7 days the abdominal aortic flow (A and D) and splenic pulp pressure (B and E) were measured and the urine was analyzed for the prostacyclin metabolite 2,3-dinor-6-keto-PGF1α (C and F). Histograms show data for 7-day sham-DMSO since there was no effect of 2–10 mg/kg NS398 or 20–80 mg/kg SC560 in shams. A–C: abdominal aortic flow, splenic pulp pressure, and 7-day urine 2,3-dinor-6-keto-PGF1α, were increased 7 days following PVL (shaded bars) compared with 7-day shams (open bars). Treatment with 2 mg/kg NS398 (hatched bars) did not prevent these increases, whereas treatment with 10 mg/kg NS398 (cross-hatched bars) significantly reduced all three. D–F: similarly, abdominal aortic flow, splenic pulp pressure, and urine 7-day 2,3-dinor-6-keto-PGF1α remained elevated in 7-day PVL mice treated with the low, 20 mg/kg, dose of SC560 (hatched bars) compared with PVL-DMSO and sham-DMSO controls, whereas 80 mg/kg SC560 (cross-hatched bars) prevented an increase in abdominal aortic flow, splenic pulp pressure, and 2,3-dinor-6-keto-PGF1α. Data represents means ± SE; n = 5 per group. BW, body weight.
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, PGI2 synthesis 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
prostacyclin receptor (IP). Activation of IP stimulates adenyl cyclase, leading to increased cAMP, vasodilation, inhibition of cell proliferation, and release of inflammatory mediators (60). Alternatively, PGI₂ 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 PGI₂ 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 isoforms 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). PGI₂ regulates blood flow and therefore filtration rate of the kidney (2, 16). In PGIS-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 PGI₂ 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 PGI₂ 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). Salvenini 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 PGI₂ 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 PGI₂ inhibition in any PHT treatment paradigm.

In conclusion, PGI₂ is very important to the development of systemic hyperemia and elevated portal pressure in the PVL model of prehepatic PHT. Moreover, PGI₂ biosynthesis is not COX-1 or COX-2 dependent. These findings direct further research toward other aspects of PHT vasculopathy. Targeting of PGI₂ 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 PGI₂ should not interfere with the gastric protective PGE₂ but cardiovascular complication risk may increase. Recent studies have shown that increased PGI₂ 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 PGI₂ inhibition reduce splanchnic hyperemia, 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 PGI₂ 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 PGE₂ 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 prostanooids in disease pathology, additional studies to better understand the role of PGI₂ 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 PGI₂, trigger, signaling, and NO cross talk within PHT models is needed and may bear fruit for novel targets for clinical therapy.

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