Intravascular Thrombosis After Hypoxia-Induced Pulmonary Hypertension
Regulation by Cyclooxygenase-2

Graham P. Pidgeon, PhD*; Rasa Tamosiuniene, MD*; Gang Chen, PhD; Irene Leonard, MD; Orina Belton, PhD; Aidan Bradford, PhD; Desmond J. Fitzgerald, MD

Background—Pulmonary hypertension induced by chronic hypoxia is characterized by thickening of pulmonary artery walls, elevated pulmonary vascular resistance, and right-heart failure. Prostacyclin analogues reduce pulmonary pressures in this condition; raising the possibility that cyclooxygenase-2 (COX-2) modulates the response of the pulmonary vasculature to hypoxia.

Methods and Results—Sprague-Dawley rats in which pulmonary hypertension was induced by hypobaric hypoxia for 14 days were treated concurrently with the selective COX-2 inhibitor SC236 or vehicle. Mean pulmonary arterial pressure (mPAP) was elevated after hypoxia (28.1±3.2 versus 17.2±3.1 mm Hg; n=8, P<0.01), with thickening of small pulmonary arteries and increased COX-2 expression and prostacyclin formation. Selective inhibition of COX-2 aggravated the increase in mPAP (42.8±5.9 mm Hg; n=8, P<0.05), an effect that was attenuated by the thromboxane (TX) A2/prostaglandin endoperoxide receptor antagonist ifetroban. Urinary TXB2 increased during hypoxia (5.9±0.9 versus 1.2±0.2 ng/mg creatinine; n=6, P<0.01) and was further increased by COX-2 inhibition (8.5±0.7 ng/mg creatinine; n=6, P<0.05). In contrast, urinary excretion of the prostacyclin metabolite 6-ketoprostaglandin F1α decreased with COX-2 inhibition (8.6±3.0 versus 27.0±4.8 ng/mg creatinine; n=6, P<0.05). Platelet activation was enhanced after chronic hypoxia. COX-2 inhibition further reduced the PFA-100 closure time and enhanced platelet deposition in the smaller pulmonary arteries, effects that were attenuated by ifetroban. Mice with targeted disruption of the COX-2 gene exposed to chronic hypoxia had exacerbated right ventricular end-systolic pressure, whereas targeted disruption of COX-1 had no effect.

Conclusions—COX-2 expression is increased and regulates platelet activity and intravascular thrombosis in hypoxia-induced pulmonary hypertension. (Circulation. 2004;110:2701-2707.)

Key Words: thrombosis ■ hypoxia ■ hypertension, pulmonary ■ prostaglandins

Pulmonary hypertension, characterized by elevated pulmonary artery pressure and right-heart failure, most commonly arises as a consequence of hypoxia or thromboembolism.1,2 Hereditary forms of the disease arise from mutations in several genes, including the gene for bone morphogenetic protein receptor II.3,4 Vascular remodeling occurs with thickening of pulmonary artery walls and intravascular thrombosis.5

Prostaglandins (PGs) have been implicated in the development of pulmonary hypertension, and an imbalance between the generation of thromboxane A2 (TXA2) and prostacyclin (PGI2) has been reported in both primary and secondary forms of the disease.6 PGI2, the principle metabolite of arachidonic acid in vascular smooth muscle and endothelial cells and a potent vasodilator and platelet inhibitor, reduces pulmonary pressures in patients with primary pulmonary hypertension.7 In addition, transgenic mice overexpressing PGH2 synthase, which catalyzes the conversion of PGH2 to PGI2, are protected against the development of pulmonary hypertension in response to chronic hypoxia.8 There are several possible explanations for the effect of PGI2 in pulmonary hypertension other than vasodilation, including enhanced angiogenesis9 and inhibition of platelet activation.10 Indeed, intra-arterial thrombosis has been reported in patients with hypoxia-related pulmonary hypertension.2,11

PGs are generated by the enzyme cyclooxygenase (COX), of which there are 2 isoforms, the constitutively expressed COX-1 and the inducible COX-2. In healthy subjects, PGI2 is largely generated through COX-2.12 Because COX-2 is induced by hypoxia13 and oxidative stress,14 COX-2 may be the
isoform responsible for the generation of PGI₂ in pulmonary hypertension. In contrast, TXA₂ is largely derived from platelets in which the principle isoform is COX-1. Activation of TX receptors induced vasoconstriction and potentiated pulmonary hypertension in an experimental rat model. In these experiments, we have shown that COX-2 is induced in pulmonary hypertension secondary to chronic hypoxia and that inhibition of COX-2 aggravates the rise in pulmonary pressure by sensitizing to endogenous TXA₂ and enhancing platelet activation.

Methods

Drugs

The selective COX-2 inhibitor SC236 was a gift from Dr Peter Isakson of Pharmacia, Skokie, Ill. Iprofiban, a TX receptor antagonist, was generously provided by Dr Martin Ogletree of Bristol-Myers Squibb, Princeton, NJ. SC236 was administered orally at a dose of 3 mg · kg⁻¹ · d⁻¹. We previously optimized this dose for selective COX-2 inhibition in the rat without affecting TXB₂, which is a measure of COX-1 activity, and a similar dose has been effective in other studies of COX-2 in the rat. Iprofiban was administered at a dose of 50 mg · kg⁻¹ · d⁻¹ in 0.1% methylcellulose.

Animals

All experiments were conducted in accordance with protocols approved by the institutional Biomedical Research Committee and under a license granted by the Department of Health in Ireland. Adult, virus-free, male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) weighing 300 to 350 g were placed for 2 weeks in a ventilated Plexiglas hypobaric chamber at a barometric pressure of 450 mm Hg, an inspired P0₂ of 95 mm Hg, and a predicted alveolar P0₂ of 50 to 55 mm Hg. Age- and weight-matched control rats were maintained in 21% O₂ and subjected to the same anesthesia. Bladders were drained, and urine was drawn directly with sterile syringes. All samples were free of blood to minimize contamination by platelets. Bladders were harvested, and urinary creatinine was measured by an end-systolic pressure (RVESP) was calculated by averaging 8 peak waveforms in triplicate per animal.

Platelet Function Analysis

A platelet function analyzer (PFA-100, Dade-Behring) was used to evaluate platelet activity under high shear stress. Whole blood (0.8 mL) was drawn into the reservoir of a disposable cartridge in which the aperture membrane was coated with collagen and ADP. The time to closure is dependent on the rate of platelet aggregation in response to the high shear rate and the agonist.

Immunohistochemistry

The right lobes of lungs were fixed in formalin and embedded in paraffin for immunohistochemistry. Paraffin sections (5-μm thickness) were cut and mounted on glass slides coated with 3-amino-propyltriethoxysilane (Sigma Aldrich). After deparaffinization in xylene and rehydration through graded alcohol, the slides were rinsed in phosphate-buffered saline (PBS), and endogenous peroxidase activity was quenched with 1% H₂O₂ in PBS for 30 minutes. Thereafter, slides were rinsed in PBS and incubated with normal goat serum for 30 minutes to block nonspecific binding and then incubated with affinity-purified rabbit polyclonal antibodies against specific antigens: 1:300 dilution of anti-COX-2 antibody from Cayman Chemicals; 1:200 dilution of anti-COX-1 antibody from Santa Cruz Biotech; 1:300 dilution of anti-TX-2 antibody from Cayman Chemicals; 1:300 dilution of anti-CD41 antibody from BD Biosciences Pharmingen. After being washed with PBS, the slides were incubated for 1 hour with biotinylated secondary antibody (ABC kit, Dako). After 3 washes in PBS, the slides were incubated for 30 minutes with streptavidin-biotin complex before incubation with 0.025% 3,3'-diaminobenzidine for 5 minutes. Slides were lightly counterstained with hematoxylin before dehydration and mounting for analysis by microscopy on a Leica DNLB light microscope with color video attachment for recording. The intensity of CD41 staining was estimated with Image Pro Plus 4.0 software (MediaCybernetics). Five random high-powered fields were captured per slide (n = 5/group), and the mean intensity in units was calculated per treatment group.

PG Production

Urine was collected from rats at the time of euthanization by bladder puncture with 1-mL syringes. The bladder was exposed by midline laparotomy, and urine was drawn directly with sterile syringes. All samples were free of blood to minimize contamination by platelets. Bladders were drained, and ~300 to 1000 mL of urine was snap-frozen in LN₂ for determination of TXB₂ and 6-keto-PGF₁α (the stable hydrolysis products of TXA₂ and PGI₂, respectively) by validated enzyme immunoassay according to the manufacturer’s instructions (R&D Systems, Inc.). Cross-reactivity of related eicosanoid compounds is <0.01% for PGE₂, PGD₂, and PGI₂ for the antibody TXB₂, and 0.2% for PGD₂, <0.01% for PGE₂ and TXB₂, respectively, for the antibody to 6-keto-PGF₁α. Intra-assay and interassay coefficients of variation, respectively, were 1.6% and 6.2% for the TXB₂ assay and 2.9% and 6.0% for the 6-keto-PGF₁α assay. In addition, urine was analyzed by tandem liquid chromatography–mass spectrometry–mass spectrometry for the isoprostane 8-iso-PGF₂α with O²⁻-labeled 8-iso-PGF₂α as an internal standard. Levels are expressed per milligram of urinary creatinine.

Statistical Analysis

All data are expressed as mean ± SEM. A sample size calculation done with JavaStat for n = 3 per group, which indicated that a sample size of 6 was required for minimal detectable differences. Final data analysis was performed with a Kruskal-Wallis 1-way ANOVA, (InStat version 3.0), with Bonferroni post tests where appropriate.

Hemodynamic Measurements

After 14 days of hypoxia, the rats were anesthetized by intraperitoneal pentobarbitone injection (60 mg/kg), the trachea was cannulated, and the lungs were mechanically ventilated (SAR-830P small-animal ventilator, CWE) with warmed and humidified 5% CO₂ in air. The thoracic cavity was opened, the heart and lungs were exposed, and an incision in the right atrium. Perfusate consisting of a physiological saline solution (pH 7.4) was maintained at a constant flow rate (0.04 mL · min⁻¹ · 100 g⁻¹) with a peristaltic pump so that changes in pressure-conductance system (Millar Instruments) coupled to a PowerLab/4SP A/D converter (AD Instruments). Right ventricular end-systolic pressure (RVESP) was calculated by averaging 8 peak waveforms in triplicate per animal.
All data were examined to ensure that they were normally distributed. Results were taken as significant when \( P < 0.05 \).

**Results**

**COX Isoforms and TX Synthase Expression**

COX-1 was constitutively expressed in the normal rat lung, with little COX-2 in the tissue under normoxic conditions (Figure 1A). COX-2 expression was increased by chronic hypoxia in vascular smooth muscle, endothelial cells, and bronchial epithelial cells (Figure 1B), whereas COX-1 expression was unaltered (Figure 1D). There was marked thickening of small lung vessels after chronic hypoxia compared with normoxic controls. Hypoxia also resulted in a diffuse increase in TX synthase expression in lung tissue (Figure 1F versus 1E).

**Selective COX-2 Inhibition Aggravates Hypoxic Pulmonary Hypertension**

Chronic hypoxia increased mean pulmonary arterial pressure (mPAP) levels compared with those in normoxic controls (25.4±3.0 versus 15.8±1.2 mm Hg; \( n=8 \), \( P<0.01 \); Figure 2). Selective inhibition of COX-2 with SC236 enhanced the rise in mPAP after hypoxia (34.8±4.4 mm Hg; \( n=8 \), \( P<0.05 \)) without altering the increase in wall thickness (data not shown). Treatment with the TXA₂/PG endoperoxide receptor antagonist ifetroban attenuated the rise in mPAP induced by either hypoxia alone (18.2±2.6 versus 25.4±3.0 mm Hg; \( n=8 \), \( P<0.05 \)) or by hypoxia plus SC236 (18.6±0.8 mm Hg versus 34.8±4.4 mm Hg; \( n=8 \), \( P<0.03 \); Figure 2). As expected, hypoxia increased hematocrit (0.59±0.03 versus 0.44±0.02; \( P<0.01 \)), which was unaffected by treatment with SC236 (0.57±0.03) or ifetroban (0.58±0.03).

To confirm that the effects with the selective COX-2 inhibitor were due to the inhibition of COX-derived PGs rather than a nonspecific effect of the COX inhibitor, we examined the response to hypoxia in mice with targeted disruption of either the COX-1 or COX-2 isoform. Chronic hypoxia in wild-type mice resulted in a significant increase in RVESP (Figure 3) compared with that in normoxic mice (24.1±1.0 versus 13.8±1.9; \( n=8 \), \( P<0.01 \)). Mice lacking the COX-2 isoform had a higher RVESP after hypoxia (36.8±2.2; \( n=8 \), \( P<0.001 \)) compared with wild-type mice. Mice with targeted disruption of the COX-1 isoform also

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**Figure 1.** Expression of COX isoforms in lung tissue after chronic hypoxia. There was marked thickening of walls of small vessels after chronic hypoxia (B, D, F) compared with normoxic animals (A, C, E). COX-2 expression was increased in bronchial epithelium and vascular smooth muscle after hypoxia (B) but absent in normoxic animals (A). COX-1 was constitutively expressed in normoxic (C) and hypoxic (D) tissue. Chronic hypoxia also induced expression of TX synthase in bronchial epithelium and vascular smooth muscle cells (F) relative to control (E). Magnification X40.

Abbreviations are as defined in text.
developed a rise in RVESP, but this was similar to that in wild-type animals (22.8±2.0; n=8).

**Urinary Excretion of PG Metabolites**

Urinary 6-keto-PGF₁α was increased after hypoxia (27.0±4.8 versus 1.8±0.2 ng/mg creatinine; n=6, P<0.01; Figure 4A). The selective COX-2 inhibitor SC236, alone or in combination with the selective TXA₂/PG endoperoxide receptor antagonist ifetroban, prevented the increase in urinary 6-keto-PGF₁α (8.6±3.0 and 7.4±4.1 ng/mg creatinine, respectively; n=6, both P<0.05 compared with hypoxia), whereas ifetroban alone had no effect (25.0±3.0 ng/mg creatinine).

Urinary TXB₂ excretion also increased during chronic hypoxia (5.9±0.9 versus 1.2±0.2 ng/mg creatinine; n=6, P<0.01; Figure 4B), and this was further increased by selective inhibition of COX-2 with SC236 (8.5±0.7 ng/mg creatinine; n=6, P<0.05). Ifetroban attenuated the increase in urinary TXB₂ excretion in animals exposed to chronic hypoxia alone (2.3±0.4 ng/mg creatinine; n=6, P<0.05) or combined with SC236 (3.5±0.6 ng/mL; n=6, P<0.01).

Hypoxia increased the generation of the isoprostane 8-iso-PGF₂α (104±4.3 versus 54.1±5.9 ng/mg creatinine; n=5, P<0.001), a product generated by free-radical attack of arachidonic acid that can mimic TXA₂ by activating the TXA₂/PG endoperoxide receptor.²² COX-2 inhibition attenuated the increase after hypoxia (83.6±5.4 ng/mg creatinine; n=5, P<0.05).

**Platelet Deposition and Activity**

Platelet function under high shear stress was evaluated for each group (n=6) in whole blood with the PFA-100. Normal closure time ranged from 110 to 140 seconds. Hypoxia resulted in a shorter closure time (76±5 versus 124±12 seconds; P<0.005), which was further reduced in animals treated with the selective COX-2 inhibitor SC-236 (59±9 seconds; P<0.05; Figure 5). Treatment with the selective TXA₂/PG endoperoxide receptor antagonist ifetroban attenuated the platelet response to hypoxia and hypoxia plus SC236, as indicated by relatively prolonged closure times (97±12 and 96±14 seconds, respectively; both P<0.05).
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Platelet deposition in the pulmonary vasculature was detected by quantitative immunohistochemistry of the platelet-specific antigen CD41 (Figure 6). Chronic hypoxia resulted in adhesion of platelets in small pulmonary vessels, whereas in normoxic animals, platelet deposition was infrequent (59 ± 8 versus 27 ± 4 U; n = 5, P < 0.05; Figure 6B and 6A, respectively). The selective COX-2 inhibitor SC236 (Figure 6C) further enhanced platelet deposition (98 ± 14 U; n = 5, P < 0.05). Consistent with the platelet activity assay, the TXA2/PG endoperoxide receptor antagonist ifetroban inhibited platelet deposition in animals exposed to hypoxia alone or hypoxia plus SC236 (37 ± 6 U; n = 5, P < 0.05 versus hypoxia plus SC236; Figure 6D).

Discussion

Exogenous administration of the PG12 analogue iloprost results in clinical and hemodynamic improvement and better survival in patients with pulmonary hypertension.7 raising the possibility that endogenous PG12 is involved in its pathogenesis. Here we show that both PG12 and TXA2 modulate the pulmonary hypertension that occurs in response to chronic hypoxia in the rat. Generation of these eicosanoids increased after 14 days of hypoxia. Staining for COX isoforms demonstrated that COX-2 protein expression, largely absent in healthy lung tissue, occurred widely after hypoxia, including the smooth muscle cell layer of pulmonary vessels, whereas COX-1 expression was unchanged. The findings are consistent with in vitro evidence that hypoxia induces COX-2 expression in human pulmonary artery smooth muscle cells13 and umbilical vein endothelial cells.23 SC236, at a dose that selectively inhibits COX-2,24 prevented the increase in PG12 but not TXA2 generation, confirming its selectivity in this model. Thus, much of the TXA2 formed was through COX-1, whereas COX-2 was responsible for the increase in PG12.

Hypoxia resulted in a marked rise in pulmonary vascular resistance in the rat, measured as perfusion pressure, and an increase in the vessel wall thickness of small pulmonary arteries, largely reflecting an increase in vascular smooth muscle cells. Selective inhibition of COX-2 with resulting inhibition of PG12 generation aggravated the rise in pulmonary pressure. Similarly, in a murine model, targeted disruption of the COX-2 gene exacerbated RVESPs compared with those in wild-type mice after hypoxia, precluding a nonspecific effect as an explanation for the response to the COX-2 inhibitor. In combination, the findings suggest that COX-2–dependent PG12 generation protects against the development of pulmonary hypertension and are consistent with the finding that transgenic expression of PG12 synthase protects against hypoxia-induced pulmonary hypertension.8

PG12 may modify the response to hypoxia by acting as a vasodilator through remodeling of the pulmonary vascular bed or by inhibiting platelet activity.10,25,26 Extensive washing of the tissues to remove residual SC236 failed to overcome the increase in pulmonary pressure, arguing against a vasodilator effect (data not shown). On the other hand, staining for CD41 showed extensive platelet deposition in the pulmonary vasculature of hypoxic animals and particularly in those treated with the COX-2 inhibitor. There was also evidence of systemic platelet activation based on the PFA 100 assay, which is an assay of platelet aggregation at high shear. The thrombotic occlusion of small vessels by platelet aggregates may have contributed to the increase in pulmonary pressures.

The observed platelet activation may explain the enhanced generation of TXA2, because platelets are a major source of COX-1 and TXA2. There may also have been a contribution from lung tissue, where there was increased expression of TX synthase. Indeed, we observed an increased generation of TXB2 ex vivo in lung tissue after hypoxia (2.52 ± 0.2 versus 1.27 ± 0.3 pg/μg protein). Whatever the source, our experiments suggest a major role for TXA2, consistent with previous experiments showing that prolonged infusion of a TX analogue induces irreversible pulmonary hypertension.27 TXA2 may have contributed to hypoxia-induced pulmonary hypertension through platelet activation. Thus, in our experiments, antagonism of the TX receptor with ifetroban partly reversed the platelet activation and deposition induced by hypoxia. TXA2 also suppresses angiogenesis28 and may have impaired vascular remodeling, a major determinant of the pulmonary response to hypoxia, where several angiogenic factors have been implicated.11

TX receptor antagonism was particularly effective in animals where PG12 formation was selectively inhibited in the face of continued TXA2 generation. The findings suggest that the exaggerated responses in animals treated with the selective COX-2 inhibitor reflected enhanced sensitivity to TXA2. The findings highlight the role of PG12 and TXA2 in regulating platelet function in vivo and in particular, the role of PG12 in limiting the platelet response to TXA2. A similar interaction between TXA2 and PG12 was demonstrated in a mouse model, in which the prostacyclin receptor was disrupted.29 Arterial injury resulted in a proliferative response and platelet deposition, both of which were offset by disruption of the TP (thromboxane) receptor gene.

Regulation of platelet function by PG12 and TXA2 may also be relevant to human pulmonary hypertension. Intravascular thrombosis occurs in 60% of patients with hypoxia-related pulmonary hypertension.11 Plasma and urinary levels of TX...
metabolites are also increased in patients with the disease,\textsuperscript{30} perhaps reflecting enhanced platelet activation.\textsuperscript{31} The abnormal platelet activity can be normalized by administration of PGI\textsubscript{2}.\textsuperscript{10,25} Thus, it is possible that the impaired generation of PGI\textsubscript{2} reported in patients with inherited primary pulmonary hypertension contributes to the disease through enhanced platelet activity.\textsuperscript{6}

Alternative mechanisms may explain the observations. COXs and their products influence vascular remodeling through their effects on the generation of growth factors such as vascular endothelial growth factor,\textsuperscript{32} on angiogenesis,\textsuperscript{33} and on the growth of vascular smooth muscle cells.\textsuperscript{34} For example, TXA\textsubscript{2} inhibits angiogenesis and endothelial cell growth\textsuperscript{28,35} and could therefore limit the vascular remodeling that occurs in the model, an issue that was not addressed in this work. However, we did observe a decrease in vessel wall thickness after TXA\textsubscript{2} receptor antagonism. Hypoxia also induced generation of the isoprostane 8-iso-PGF\textsubscript{2α}. However, inhibition of COX-2 aggravated the rise in mPAP while significantly attenuating the rise in 8-iso-PGF\textsubscript{2α}, suggesting that the isoprostane was not involved.

In conclusion, COX-2–dependent PGI\textsubscript{2} formation limits the pulmonary hypertensive response to hypoxia, in part by suppressing TX-dependent platelet activation and deposition. The reduced PGI\textsubscript{2} formation and increased TXA\textsubscript{2} generation reported in patients with primary pulmonary hypertension\textsuperscript{6} may contribute to the disease through similar mechanisms.

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