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Background—Cyclooxygenase-2 (COX-2) is upregulated in pulmonary artery smooth muscle cells (PASMCs) during hypoxia and may play a protective role in the response of the lung to hypoxia. Selective COX-2 inhibition may have detrimental pulmonary vascular consequences during hypoxia.

Methods and Results—To investigate the role of COX-2 in the pulmonary vascular response to hypoxia, we subjected wild-type and COX-2–deficient mice to a model of chronic normobaric hypoxia. COX-2–null mice developed severe pulmonary hypertension with exaggerated elevation of right ventricular systolic pressure, significant right ventricular hypertrophy, and striking vascular remodeling after hypoxia. Pulmonary vascular remodeling in COX-2–deficient mice was characterized by PASMC hypertrophy but not increased proliferation. Furthermore, COX-2–deficient mice had significant upregulation of the endothelin-1 receptor (ET_A) in the lung after hypoxia. Similarly, selective pharmacological inhibition of COX-2 in wild-type mice exacerbated hypoxia-induced pulmonary hypertension and resulted in PASMC hypertrophy and increased ET_A receptor expression in pulmonary arterioles. The absence of COX-2 in vascular smooth muscle cells during hypoxia in vitro augmented traction forces and enhanced contractility of an extracellular matrix. Treatment of COX-2–deficient PASMCs with iloprost, a prostaglandin I_2 analog, and prostaglandin E_2 abrogated the potent contractile response to hypoxia and restored the wild-type phenotype.

Conclusions—Our findings reveal that hypoxia-induced pulmonary hypertension and vascular remodeling are exacerbated in the absence of COX-2 with enhanced ET_A receptor expression and increased PASMC hypertrophy. COX-2–deficient PASMCs have a maladaptive response to hypoxia manifested by exaggerated contractility, which may be rescued by either COX-2–derived prostaglandin I_2 or prostaglandin E_2. (***Circulation*. 2008;117:2114-2122.**)

Key Words: hypertension, pulmonary ■ hypertrophy ■ hypoxia ■ prostaglandins ■ remodeling ■ vasculature

Pulmonary hypertension is a severe and frequently fatal disease commonly associated with chronic hypoxemia in disorders such as chronic obstructive pulmonary disease and interstitial lung disease.[1,2] The hallmark of pulmonary hypertension is the development of elevated pulmonary vascular resistance, leading to increased right ventricular (RV) afterload and ultimately progression to right heart failure and death. The mechanisms by which low-resistance arterioles in the pulmonary circulation narrow include pulmonary vasoconstriction, in situ thrombosis, and pulmonary vascular remodeling.[1] Vascular remodeling involves pathological changes in all 3 layers of the pulmonary arteries, including endothelial dysfunction, smooth muscle cell hyperplasia, and hypertrophy, as well as adventitial fibroblast proliferation, myofibroblast differentiation, and extracellular matrix deposition.[3] Endothelial injury leads to the release of potent vasoconstrictors, including thromboxane A_2 and endothelin-1 (ET-1), which can overwhelm the effects of endothelium-derived vasodilators such as prostacyclin (prostaglandin [PG] I_2) and nitric oxide, thereby promoting remodeling of the arteriolar wall.[1–3]

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Although current state-of-the art therapy with vasodilators, endothelin receptor antagonists, and phosphodiesterase inhibitors may stabilize disease and improve quality of life in patients with pulmonary hypertension,[1,2] these agents do not reverse the underlying vascular remodeling process. There is

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therefore a need to identify novel pathways and potential therapeutic targets that target vascular remodeling to halt or reverse the progression of this devastating disease.

The cyclooxygenase (COX) enzymes (COX-1 and COX-2), which catalyze conversion of arachidonic acid to a series of prostanoids, may play a key role in the development of pulmonary vascular remodeling in response to hypoxia. COX-2, the inducible isoform of COX, is upregulated by hypoxia in pulmonary artery smooth muscle cells (PASMCs), and both elevated thromboxane A_2 levels and reduced PGI_2 levels have been demonstrated in patients with idiopathic and secondary forms of pulmonary hypertension.[4,5] Overexpression of PGI_2 synthase in the lung protects against the development of hypoxia-induced pulmonary hypertension in mice,[6] and continuous administration of prostacyclin to patients with pulmonary arterial hypertension improves mortality and quality of life.[7] Furthermore, deletion of the PGI_2 receptor exacerbates vascular remodeling in a mouse model of hypobaric hypoxia–induced pulmonary hypertension.[8] However, the role of COX-2 in hypoxia-induced pulmonary vascular remodeling has not yet been elucidated.

Recent studies have demonstrated accelerated atherosclerosis[9,10] and vascular remodeling in mice lacking the PGI_2 receptor.[11] Deletion of the PGI_2 receptor or selective COX-2 inhibition enhances vascular hyperplasia and remodeling of the systemic vasculature in murine models of transplant arteriosclerosis and flow-dependent vascular remodeling.[11] In addition, recent work suggests that COX-2 inhibition enhances platelet deposition and intravascular thrombosis in a rat model of hypobaric hypoxia–induced pulmonary hypertension.[12] Selective inhibition of COX-2 also is associated with an increased incidence of adverse cardiovascular events.[13–15] These potential vascular sequelae associated with pharmacological COX-2 inhibition appear to arise from alterations in multiple vascular effectors, including PGI_2 and PGE_2, which may directly or indirectly modulate platelet function, vascular tone, and remodeling.[15] Selective COX-2 inhibition may thus perturb the complex balance of vascular mediators and promote vascular remodeling and/or a prothrombotic state in susceptible patients.[13,15]

Given the potential consequences of COX-2 inhibition on the systemic vasculature, we examined the effect of COX-2 deficiency on the development of pulmonary hypertension and vascular remodeling in a mouse model of chronic hypoxia. Mice deficient in COX-2 developed an exaggerated response to hypoxia with elevated RV systolic pressure (RVSP), striking pulmonary vascular remodeling, and severe RV hypertrophy (RVH). Interestingly, the absence of COX-2 during hypoxia led to increased PASMC hypertrophy but did not enhance smooth muscle cell proliferation under hypoxic conditions either in vivo or in vitro. In addition, deficiency of COX-2 during hypoxia resulted in significant upregulation of the ET-1 receptor (ET_A), increased traction forces, and augmented contractility of PASMCs on collagen gel matrixes. This enhanced contractility was attenuated by both exogenous iloprost, a PGI_2 analog, and PGE_2. Our findings suggest that COX-2 plays a critical protective role in the pulmonary vasculature under hypoxic conditions and that selective COX-2 inhibition may be hazardous to patients with pulmonary hypertension, particularly under conditions of hypoxemia.

Methods

Detailed methods are described in the expanded Methods in the Data Supplement available online.

Animals

Mice that were wild type (WT) or homozygous null for targeted disruption of COX-2 (B6:129S7-Ptgs2^{tm1Jed}, The Jackson Laboratory, Bar Harbor, Me) were studied.

Hypoxic Exposure and Hemodynamic Measurements

Eight- to 10-week-old COX-2$^{-/-}$ and COX-2$^{+/+}$ littermates were exposed to normobaric hypoxia (10% O_2, OxyCycler chamber, Biospherix Ltd, Redfield, NY)[16,17] or normoxia (21% O_2) for 2 weeks. Eight- to 10-week-old C57BL/6 WT mice were treated with vehicle or nimesulide (40 mg/L)[11,18] in the drinking water during a 2-week exposure to hypoxia or normoxia. After exposure, mice were anesthetized with sodium pentobarbital (60 mg/kg), and hemodynamic measurements were performed.[17,19] The hearts were excised, and the ventricles were dissected and weighed. RVH was assessed by normalizing RV weight to total body weight (RV weight/total body weight).[16,17]

Histological Analysis and Morphometry

Lungs were inflated, harvested, fixed in methyl Carnoy's solution, and embedded in paraffin. Sections were stained with hematoxylin and eosin[20] and immunostained for α-smooth muscle actin (α-SMA; 1:50).[21,22] Remodeling was quantified as described previously.[16,17,19] Percent wall thickness was calculated as follows: wall thickness (%)$=(\text{area}_{ext}-\text{area}_{int})\div\text{area}_{ext}\times 100$, where area_{ext} represents the external diameter and area_{int} represents the internal diameter of each vessel.[16,17,19] PASMC hypertrophy was calculated as vessel wall area divided by nuclei per vessel and reported as area per cell.

Western Blot Analysis

Protein extracts from lungs exposed to hypoxia or normoxia were analyzed by Western blot analysis[22,23] with a monoclonal α-SMA antibody (1:2000) and a monoclonal ET_A antibody (1:500, BD Biosciences, San Jose, Calif). Equal loading was confirmed with an anti-tubulin antibody (1:8000).

Cell Culture

Primary aortic smooth muscle cells (VSMCs) were isolated from COX-2$^{-/-}$ and COX-2$^{+/+}$ embryos at 18.5 days postconception as described.[22,24] Primary PASMCs were isolated from adult (8- to 10-week-old) COX-2$^{-/-}$ and COX-2$^{+/+}$ mice as described with modification.[25] Hypoxia experiments were performed in an Invivo2 400 Hypoxia Workstation (Biotrace International BioProducts, Bothell, Wash).[26]

Traction Force Microscopy

Contractile forces exerted by COX-2$^{-/-}$ and COX-2$^{+/+}$ VSMCs were assessed by traction force microscopy as described.[27–29] Cells were exposed to hypoxia (1%) or normoxia for 24 hours, and in certain experiments, cells were treated with ET-1 (20 nmol/L). Traction forces exerted by individual cells before and after ET-1 treatment were determined.

Collagen Matrix Contraction Assay

COX-2$^{-/-}$ and COX-2$^{+/+}$ PASMCs and VSMCs were plated on type I collagen gel matrices and exposed to hypoxia or normoxia for 24 hours. Gel size was defined as the sum of the 2 longest gel diameters; gel contraction was expressed as a percentage of the original gel size.[30] In certain experiments, cells were treated with PGE_2 (1 μmol/L), iloprost (1 μmol/L), or vehicle (30% ethanol in PBS) during hypoxic exposure. In other experiments, cells were treated

with either forskolin (10 μmol/L) or vehicle (4% ethanol in DMEM) during hypoxic exposure. Rat pulmonary artery smooth muscle (RPASMCs) were treated with NS-398 (5 μmol/L) or vehicle (25% dimethyl sulfoxide in PBS).

Statistical Analysis

Data are presented as mean±SEM. Statistical significance was determined by the Student *t* test for comparisons between 2 groups; ANOVA was used for comparisons between >2 groups or for multiple comparisons. Statistical significance was accepted at $P<0.05$.

The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Absence of COX-2 Leads to Exaggerated Elevation of RVSP and Severe RVH After Chronic Hypoxia

To determine the role of COX-2 in the pulmonary vascular response to hypoxia, COX-$2^{-/-}$ and COX-$2^{+/+}$ mice were exposed to 2 weeks of normobaric hypoxia or normoxia. COX-2 was induced 5-fold in the lungs of WT mice after hypoxia with no difference in COX-1 expression between COX-$2^{-/-}$ and COX-$2^{+/+}$ mice either at baseline or after exposure to hypoxia (Figure I of the online Data Supplement). COX-$2^{-/-}$ mice developed significant elevation in RVSP (31.3±1.3 mm Hg) compared with COX-$2^{+/+}$ mice (25.7±1.5 mm Hg; $P<0.05$) and normoxic controls (Figure 1A). In addition, COX-$2^{-/-}$ mice developed significant RVH (RV weight/body weight, 1.08±0.04 mg/g) compared with COX-$2^{+/+}$ mice (0.88±0.05 mg/g; $P<0.05$) and normoxic controls (Figure 1B). Total body weight was not different between COX-$2^{-/-}$ (24.1±0.6 g) and COX-$2^{+/+}$ (23.5±0.45 g) mice.

In addition, WT mice were treated with nimesulide, a selective COX-2 inhibitor, during exposure to hypoxia compared with normoxia. Similar to COX-$2^{-/-}$ mice, nimesulide-treated WT mice developed more severe pulmonary hypertension after exposure to hypoxia with a significant increase in RVSP (26±1 mm Hg) compared with vehicle-treated WT mice (22±1.3 mm Hg; $P<0.05$) and normoxic controls. In addition, nimesulide-treated WT mice developed exaggerated RVH in response to hypoxia with a significant percentage increase in RV weight (33%; $P<0.05$) compared with vehicle-treated WT mice.

Absence of COX-2 Leads to Enhanced Pulmonary Vascular Remodeling After Chronic Hypoxia

Hematoxylin and eosin staining revealed enhanced vascular remodeling in COX-$2^{-/-}$ mice after hypoxia compared with COX-$2^{+/+}$ mice (Figure 2A). COX-$2^{-/-}$ mice developed exaggerated vascular remodeling with a significant increase in wall thickness of pulmonary arterioles (51±2.4%) after hypoxia compared with COX-$2^{+/+}$ mice (33.7±2.6%; $P<0.05$) and normoxic controls (Figure 2B). Similarly, nimesulide-treated WT mice developed enhanced vascular remodeling after exposure to hypoxia with a significant increase in pulmonary arteriolar wall thickness (43±1%) compared with vehicle-treated WT mice (33.5±1.1%; $P<0.05$) and normoxic controls (Figure 2C).

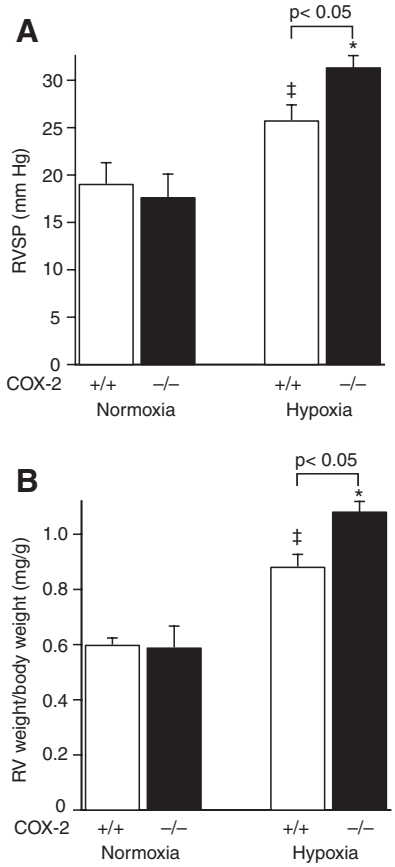

Figure 1. COX-$2^{-/-}$ mice have exaggerated elevation of RVSP and severe RVH after chronic hypoxia. A, RVSP in COX-$2^{+/+}$ (open bars) and COX-$2^{-/-}$ (solid bars) mice after hypoxia (n=18 per group) and normoxia (n=8 per group). B, RV weight (mg) normalized for body weight (g) in COX-$2^{+/+}$ and COX-$2^{-/-}$ mice after hypoxia (n=18 per group) and normoxia (n=8 per group). Error bars represent SE ($P<0.05$ for hypoxic COX-$2^{-/-}$ mice vs hypoxic COX-$2^{+/+}$ mice; *$P<0.05$ for hypoxic COX-$2^{-/-}$ mice vs normoxic controls; ‡$P<0.05$ for hypoxic COX-$2^{+/+}$ mice vs normoxic controls).

Neither Proliferation nor Migration Differ Between COX-$2^{-/-}$ and COX-$2^{+/+}$ VSMCs Exposed to Hypoxia

To elucidate the mechanisms by which COX-2 modulates pulmonary vascular remodeling, we investigated the effect of COX-2 deficiency on VSMC proliferation and migration. As shown in supplemental Figure II, during exposure to hypoxia, neither proliferation nor migration differed between COX-$2^{-/-}$ and COX-$2^{+/+}$ VSMCs in response to platelet-derived growth factor. In addition, BrdU staining demonstrated no difference in proliferation in vivo between COX-$2^{-/-}$ and COX-$2^{+/+}$ mice after hypoxia (data not shown); however, there was a clear increase in the number of BrdU-positive cells in the pulmonary vessels of both hypoxic groups compared with baseline. These results demonstrate that neither enhanced VSMC proliferation nor migration accounts for the hypoxic vascular remodeling in COX-$2^{-/-}$ mice.

COX-$2^{-/-}$ Mice Have Enhanced PASMC Hypertrophy After Hypoxia

Trichrome staining revealed minimal collagen deposition in the distal remodeled vessels with no difference between COX-$2^{-/-}$

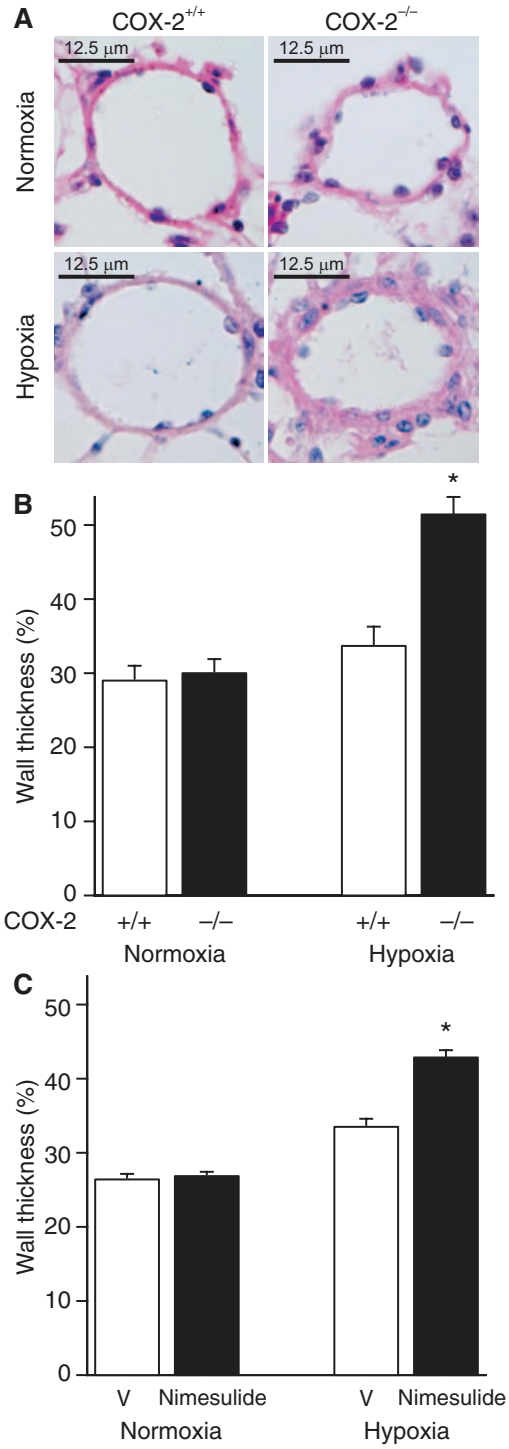

Figure 2. Absence of COX-2 results in enhanced pulmonary vascular remodeling after chronic hypoxia. A, Representative 5-μm hematoxylin and eosin–stained sections from COX-$2^{+/+}$ (left) and COX-$2^{-/-}$ (right) mice after normoxia (top) and hypoxia (bottom). Quantification of percent wall thickness of pulmonary arterioles in the lungs of (B) COX-$2^{+/+}$ (open bars) and COX-$2^{-/-}$ (solid bars) mice after normoxia (n=5 per group) and hypoxia (n=8 per group) and (C) vehicle (V) and nimesulide-treated WT mice after normoxia (n=6 per group) and hypoxia (n=10 per group). Ten vessels were analyzed per mouse. Data are expressed as mean±SE (*P<0.05 for hypoxic COX-$2^{-/-}$ mice vs hypoxic COX-$2^{+/+}$ mice and normoxic controls [B]; *P<0.05 for nimesulide- vs vehicle-treated hypoxic WT mice and normoxic controls [C]).

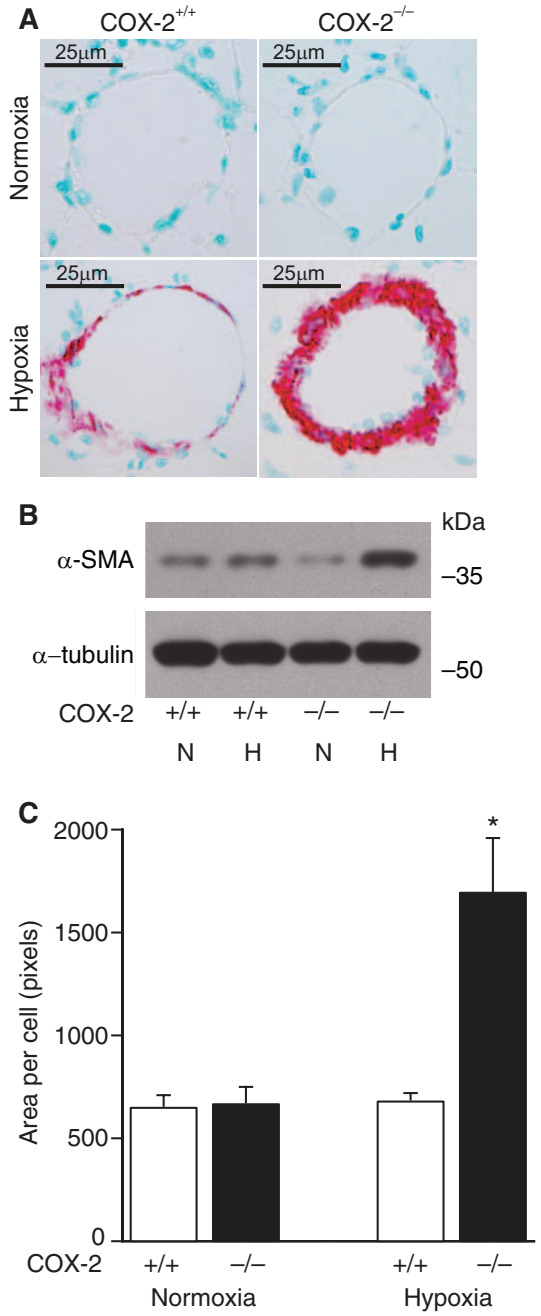

Figure 3. COX-$2^{-/-}$ mice have enhanced PASMCs hypertrophy after hypoxia. A, Immunostaining of lungs from COX-$2^{+/+}$ (left) and COX-$2^{-/-}$ (right) mice after normoxia (top) and hypoxia (bottom) for α-SMA. B, Western blot analysis for α-SMA on lungs from COX-$2^{+/+}$ and COX-$2^{-/-}$ mice after hypoxia and normoxia. C, Quantification of PASMC size in COX-$2^{+/+}$ (open bars) and COX-$2^{-/-}$ (solid bars) mice. Ten vessels per mouse were analyzed after normoxia (n=5 per group) and hypoxia (n=8 per group). Data are expressed as mean±SE (*P<0.05 for hypoxic COX-$2^{-/-}$ mice vs hypoxic COX-$2^{+/+}$ mice and normoxic controls).

and COX-$2^{+/+}$ mice (data not shown). However, immunostaining for α-SMA demonstrated that, after hypoxia, COX-$2^{-/-}$ mice developed striking vascular remodeling with neomuscularization of distal pulmonary arterioles, characterized by large neointimas containing α-SMA–positive cells (Figure 3A). In contrast, COX-$2^{+/+}$ mice developed significantly less remodeling with few α-SMA–positive cells in remodeled vessels. To

quantify α-SMA in the lungs of COX-2$^{-/-}$ and COX-2$^{+/+}$ mice after hypoxia and normoxia, Western blot analysis was performed for α-SMA. When corrected for loading, there was no difference in α-SMA protein expression between COX-2$^{-/-}$ and COX-2$^{+/+}$ mice at baseline. However, after hypoxia, lungs of COX-2$^{-/-}$ mice demonstrated a nearly 3-fold increase in α-SMA protein expression compared with only a 1.3-fold increase in lungs of COX-2$^{+/+}$ mice (Figure 3B).

These findings, in addition to our proliferation and migration results, suggested that smooth muscle cell hypertrophy may be the predominant mechanism driving hypoxic vascular remodeling in lungs of COX-2$^{-/-}$ mice. Indeed, morphometric analysis demonstrated that COX-2$^{-/-}$ mice developed significant PASMC hypertrophy after hypoxia with a significant increase in area per cell (1693±266 pixels) compared with COX-2$^{+/+}$ mice (678±41 pixels; $P<0.05$; Figure 3C). Similarly, nimesulide-treated WT mice developed exaggerated PASMC hypertrophy after exposure to hypoxia with a significant increase in area per cell (1405±62 pixels) compared with vehicle-treated WT mice (686±30 pixels; $P<0.05$) and normoxic controls (supplemental Figure III).

COX-2$^{-/-}$ VSMCs Have Enhanced Traction Forces After Hypoxia

Previous studies with cultured pulmonary VSMCs have shown that myosin light chain phosphorylation and cell contractility increase in parallel with cell area as the cells spread on the extracellular matrix.[29] Thus, to determine whether a difference in VSMC contractility may contribute to the exaggerated pulmonary hypertension and vascular remodeling in COX-2$^{-/-}$ mice, we used traction force microscopy to measure the traction forces exerted by individual VSMCs after exposure to hypoxia in vitro (Figure 4A). COX-2$^{+/+}$ and COX-2$^{-/-}$ VSMCs had no difference in traction forces under normoxic conditions (COX-2$^{-/-}$, 102±14 Pa; COX-2$^{+/+}$, 108±19 Pa); however, after hypoxia, COX-2$^{-/-}$ VSMCs developed a significant increase in traction forces (150±18 Pa) compared with COX-2$^{+/+}$ VSMCs (104±14 Pa; $P<0.05$; Figure 4B). These data suggest that deficiency of COX-2 during hypoxia dramatically alters the contractile response of individual VSMCs.

Absence of COX-2 During Hypoxia Leads to Enhanced ET_A Receptor Expression and Exaggerated Traction Forces in Response to ET-1

To investigate the mechanism by which deficiency of COX-2 augments the contractility of VSMCs during hypoxia, we harvested protein from lungs of COX-2$^{-/-}$ and COX-2$^{+/+}$ mice and performed Western blot analysis for the ET_A receptor. COX-2$^{-/-}$ mice had dramatic induction of the ET_A receptor after hypoxia with a 5-fold induction in ET_A receptor protein expression compared with only a 5% increase in COX-2$^{+/+}$ mice (Figure 5A). In addition, after exposure to hypoxia, nimesulide-treated WT mice demonstrated a >30-fold increase in ET_A receptor expression in pulmonary arterioles by immunohistochemistry compared with only a 3-fold increase in vehicle-treated WT mice (supplemental Figure IV). Furthermore, when COX-2$^{+/+}$ and COX-2$^{-/-}$ VSMCs were treated with ET-1 after hypoxia, COX-2$^{-/-}$ VSMCs developed a significant increase in traction forces (202±17 Pa) compared with COX-2$^{+/+}$ VSMCs (160±15 Pa; $P<0.05$) and normoxic controls (Figure 5B).

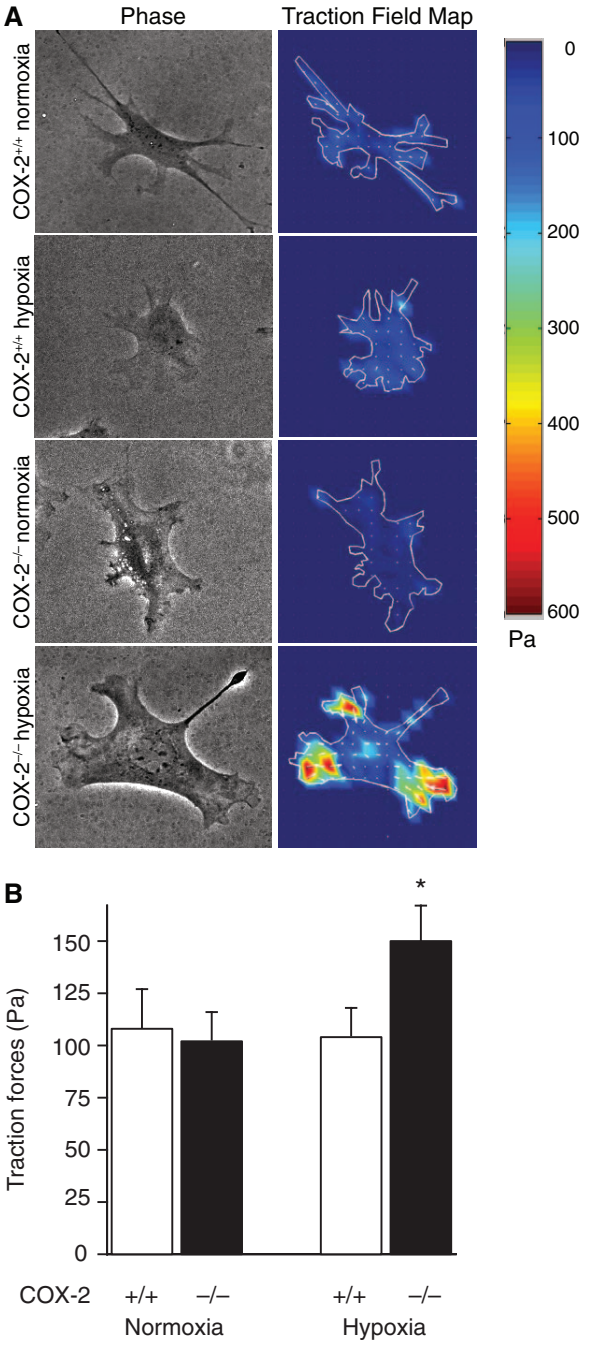

Figure 4. COX-2$^{-/-}$ VSMCs have enhanced traction forces after hypoxia. A, Representative phase-contrast views (left) and traction field maps (right) in COX-2$^{+/+}$ (top) and COX-2$^{-/-}$ (bottom) VSMCs after normoxia (COX-2$^{+/+}$, n=16 cells; COX-2$^{-/-}$, n=23 cells) and hypoxia (COX-2$^{+/+}$, n=27 cells; COX-2$^{-/-}$, n=19 cells). Color scale indicates magnitude of traction in pascals. B, Traction forces (in pascals) in COX-2$^{+/+}$ and COX-2$^{-/-}$ VSMCs after normoxia and hypoxia. Data are expressed as mean±SE (*$P<0.05$ for hypoxic COX-2$^{-/-}$ VSMCs vs COX-2$^{+/+}$ VSMCs and normoxic controls).

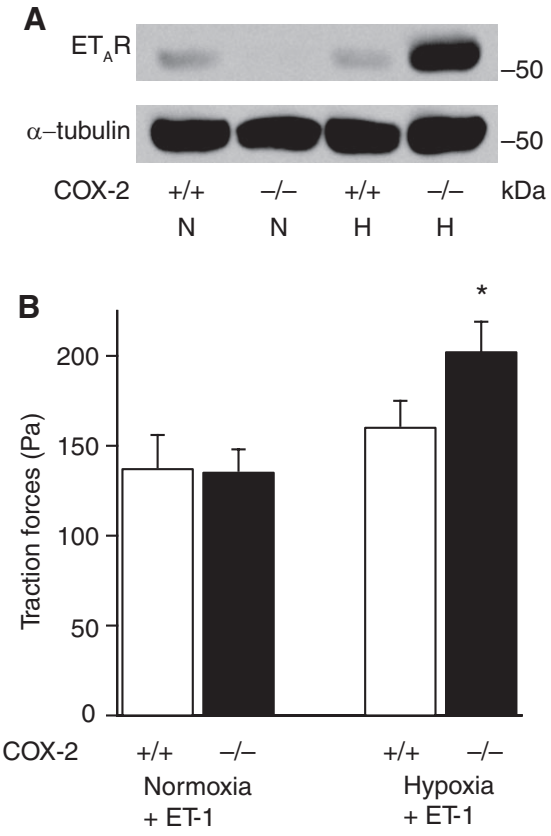

Figure 5. Absence of COX-2 during hypoxia leads to enhanced ET_A receptor expression in lungs and enhanced traction forces in response to ET-1. A, Total protein was isolated from lungs of COX-$2^{+/+}$ and COX-$2^{-/-}$ mice after hypoxia and normoxia, and Western blot analysis was performed for the ET_A receptor. Loading was quantified with an anti-tubulin antibody. A representative of 3 experiments is shown. B, Traction forces of COX-$2^{+/+}$ and COX-$2^{-/-}$ VSMCs after normoxia (COX-$2^{+/+}$, n=16 cells; COX-$2^{-/-}$, n=23 cells) and hypoxia (COX-$2^{+/+}$, n=27 cells; COX-$2^{-/-}$, n=19 cells) after stimulation with ET-1. Data are expressed as mean±SE (*P<0.05 for hypoxic COX-$2^{-/-}$ VSMCs vs COX-$2^{+/+}$ VSMCs and normoxic controls).

COX-$2^{-/-}$ PASMCs Have Enhanced Contractility on Collagen Gels After Hypoxia

Given these findings, we investigated whether COX-$2^{-/-}$ PASMCs would demonstrate enhanced contractility of a 3-dimensional collagen matrix. Consistent with our traction force microscopy results, COX-$2^{-/-}$ PASMCs demonstrated enhanced contraction of collagen matrixes after hypoxia (Figure 6A) compared with COX-$2^{+/+}$ PASMCs. At 4 hours after matrix release, hypoxic COX-$2^{-/-}$ PASMCs exhibited exaggerated gel contraction (55±2.2% of original gel size) compared with hypoxic COX-$2^{+/+}$ PASMCs (80±2.3% of original gel size; P<0.05; Figure 6B). Similarly, COX-$2^{-/-}$ VSMCs demonstrated exaggerated gel contraction (48±1.9% of original gel size) compared with COX-$2^{+/+}$ VSMCs (73±5% of original gel size; P<0.05) after hypoxic exposure (Figure 6B). In addition, pharmacological inhibition of COX-2 in an RPASMC cell line resulted in increased contraction (67±3.4% of original gel size) during hypoxia compared with vehicle control (83±1.6% of original gel size; P<0.05; Figure 6B).

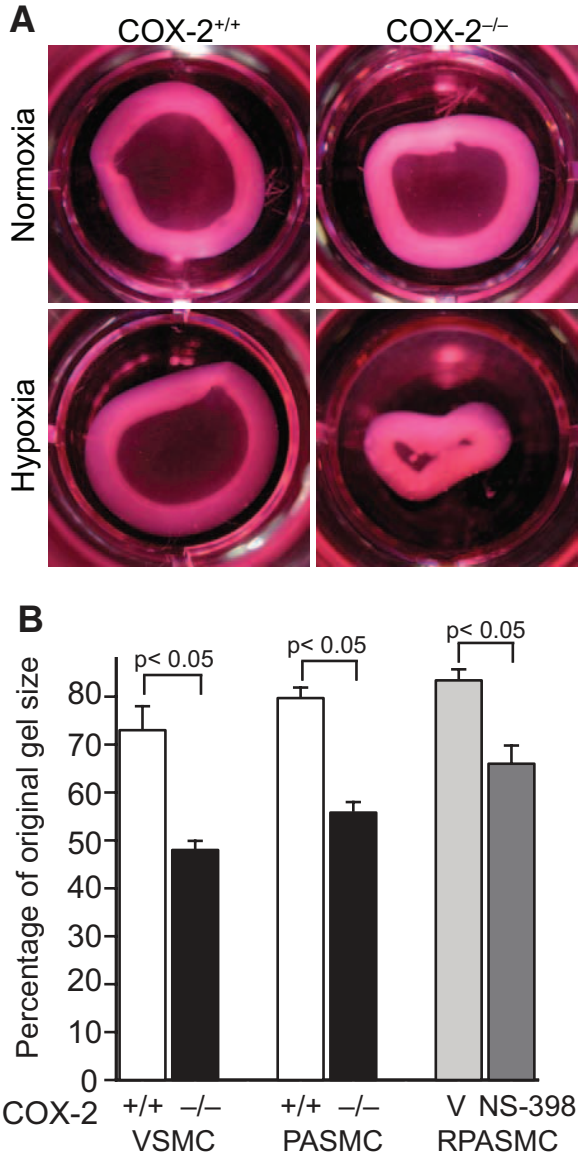

Figure 6. COX-$2^{-/-}$ PASMCs have enhanced contractility on collagen gels after hypoxia. A, Representative photographs of collagen gels from COX-$2^{+/+}$ (left) and COX-$2^{-/-}$ (right) PASMCs under normoxic (top) and hypoxic (bottom) conditions. B, Gel contraction after matrix release. Data are presented as the percentage of the original collagen gel size for VSMCs, PASMCs (COX-$2^{+/+}$, open bars; COX-$2^{-/-}$, solid bars), and RPASMCs (vehicle, light shaded bar; NS-398, darker shaded bar) exposed to hypoxia. Data are expressed as mean±SE (P<0.05 for COX-$2^{-/-}$ VSMCs vs COX-$2^{+/+}$ VSMCs; P<0.05 for COX-$2^{-/-}$ PASMCs vs COX-$2^{+/+}$ PASMCs; P<0.05 for NS-398–treated vs vehicle-treated RPASMCs).

Iloprost and PGE_2 Attenuate Enhanced Contractility of COX-$2^{-/-}$ PASMCs on Collagen Gels After Hypoxia

To determine whether the administration of prostanoids could rescue COX-$2^{-/-}$ PASMCs from this enhanced contractile response during hypoxia, we first analyzed the abundance and relative contribution of COX-2–derived prostanoids in the pulmonary versus the systemic circulation after hypoxia. COX-$2^{-/-}$ and COX-$2^{+/+}$ PASMCs and VSMCs were exposed to hypoxia for 24 hours, and supernatants were ana-

lyzed for PGE_2 and 6-keto-$PGF_{1\alpha}$, a stable PGI_2 metabolite. Levels of PGE_2 were significantly higher in WT VSMCs compared with WT PASMCs after hypoxia. In addition, 6-keto-$PGF_{1\alpha}$ was as abundant as PGE_2 in WT VSMCs after hypoxia but almost 8-fold more abundant than PGE_2 in hypoxia-exposed WT PASMCs (supplemental Figure V). As expected, PGE_2 and 6-keto-$PGF_{1\alpha}$ levels were markedly lower in COX-$2^{-/-}$ PASMCs and VSMCs.

To investigate whether repletion of these COX-2–derived prostanoids would alter the contractile phenotype of COX-$2^{-/-}$ PASMCs and VSMCs during hypoxia, we performed collagen matrix contraction assays in the presence of exogenous PGE_2 or iloprost, a PGI_2 analog. Vehicle-treated COX-$2^{-/-}$ PASMCs and VSMCs demonstrated exaggerated gel contraction after hypoxia compared with WT controls. Contraction by hypoxic COX-$2^{-/-}$ PASMCs was significantly attenuated by either iloprost (84±2.6% of original gel size; $P<0.05$) or PGE_2 (81±2.3% of original gel size; $P<0.05$; Figure 7A). However, exaggerated contraction by COX-$2^{-/-}$ VSMCs was attenuated only by PGE_2 (79±2.4% of original gel size; $P<0.05$), not iloprost (61±2.7% of original gel size; Figure 7B). In addition, to determine whether rescue of this phenotype is cAMP mediated, we performed collagen contraction assays in the presence of forskolin, an activator of adenylate cyclase. Similar to PGE_2, forskolin attenuated contraction of both COX-$2^{-/-}$ PASMCs and VSMCs after exposure to hypoxia (supplemental Figure VI).

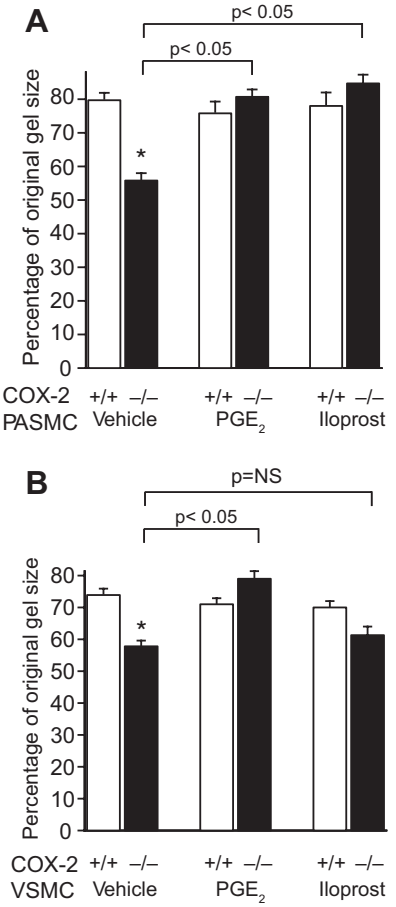

Figure 7. Iloprost and PGE_2 attenuate contractility of COX-$2^{-/-}$ PASMCs on collagen gels after hypoxia. Gel contraction by COX-$2^{+/+}$ and COX-$2^{-/-}$ PASMCs (A) and VSMCs (B) after treatment with prostanoid analogs (PGE_2 1 μmol/L, iloprost 1 μmol/L) under hypoxic conditions. Data are presented as the percentage of the original collagen gel size for hypoxic PASMCs and VSMCs (COX-$2^{+/+}$, open bar; COX-$2^{-/-}$, solid bar) treated with PGE_2, iloprost, or vehicle. Data are expressed as mean±SE (*$P<0.05$ for vehicle-treated COX-$2^{-/-}$ PASMCs vs vehicle-treated COX-$2^{+/+}$ PASMCs, $P<0.05$ for PGE_2-treated and iloprost-treated COX-$2^{-/-}$ PASMCs vs vehicle [A]; *$P<0.05$ for vehicle-treated COX-$2^{-/-}$ VSMCs vs vehicle-treated COX-$2^{+/+}$ VSMCs, $P<0.05$ for PGE_2-treated COX-$2^{-/-}$ VSMCs vs vehicle [B]).

Discussion

This study highlights 3 important new concepts. First, deficiency or pharmacological inhibition of COX-2 is detrimental during exposure to hypoxia, leading to exacerbation of pulmonary hypertension, accelerated vascular remodeling characterized by PASMC hypertrophy, and significant upregulation of the ET_A receptor. Second, deficiency of COX-2 in VSMCs during hypoxia enhances contractile forces both at a cellular level and in their interactions with the extracellular matrix. Third, repletion of either COX-2–derived PGI_2 or PGE_2 to COX-$2^{-/-}$ PASMCs attenuates their potent contractility in response to hypoxia, thus restoring the WT phenotype.

In this study, we examined the role of COX-2 in pulmonary vascular remodeling using a murine model of chronic hypoxia-induced pulmonary hypertension. COX-2 is upregulated in PASMCs under hypoxic conditions,[12,31] and our data provide evidence that it plays a protective role in response to hypoxia. Pharmacological inhibition of COX-2 is associated with remodeling of the systemic vasculature in murine models[11]; however, the effect of COX-2 deficiency on the pulmonary vasculature, particularly under conditions of hypoxemia, has not been fully defined. Recent work by Pidgeon et al[12] suggests that pharmacological inhibition of COX-2 in a rat model of hypobaric hypoxia enhances platelet activation and intravascular thrombosis, which were partially attenuated by a thromboxane receptor antagonist. However, the effect of COX-2 deficiency on remodeling and contractility of PASMCs in response to hypoxia has not yet been elucidated.

Our findings illustrate that COX-2–deficient mice develop severe pulmonary hypertension characterized by exaggerated elevation of RVSP, significant RVH, and striking vascular remodeling after only 2 weeks of hypoxia. In contrast, WT mice develop less severe pulmonary hypertension and minimal vascular remodeling in response to 2 weeks of hypoxia. In addition, selective pharmacological COX-2 inhibition during exposure to chronic hypoxia led to an exaggerated response to hypoxia, similar to COX-2–null mice, with severe pulmonary hypertension and profound pulmonary vascular remodeling compared with vehicle-treated controls. We observed the same extent of cellular proliferation in COX-$2^{-/-}$ and COX-$2^{+/+}$ mice after hypoxia, but COX-$2^{-/-}$ mice developed significant PASMC hypertrophy, accounting for the dramatic vascular remodeling. Our findings suggest that this enhanced hypertrophic response of the pulmonary vasculature to hypoxia in COX-2–null mice may be due in part to enhanced expression of the ET_A receptor during hypoxia because ET-1 has been linked to VSMC hypertrophy.[32,33] Interestingly, although PGI_2 has been shown to have inhibi-

tory effects on the proliferation of human PASMCs,[31,34] our data demonstrate that genetic deficiency of COX-2 does not alter the proliferative response of the pulmonary arteriolar vasculature to hypoxia but rather promotes a hypertrophic remodeling response.

In addition to vascular remodeling, pulmonary vascular resistance may increase as a result of intravascular thrombosis after chronic hypoxia.[1] We did not observe significant intravascular thrombosis in our mouse model, as had been previously observed in a rat model of hypobaric hypoxia–induced pulmonary hypertension.[12] Our findings now provide evidence that, in addition to vascular thrombosis,[12] COX-2 deficiency results in enhanced vascular remodeling, which exacerbates the rise in pulmonary vascular resistance in response to hypoxia.

The present study also extends our understanding of how chronic hypoxia alters PASMC contractility at a cellular level. We have demonstrated that the absence of COX-2 during hypoxia enhances traction forces generated in individual VSMCs and augments contractility of PASMCs on an extracellular matrix. Previous work has illustrated a direct correlation between cellular traction forces and myosin light chain phosphorylation in PASMCs under normoxic conditions.[29] Because myosin light chain phosphorylation and cell contractility have been shown to increase as cells enlarge by spreading on an extracellular matrix,[29] our findings suggest that hypertrophy may explain the increased contractility of COX-2–deficient PASMCs under hypoxic conditions. Upregulation of the ET_A receptor in COX-2–null mice during hypoxia likely accounts for this enhanced contractile phenotype during hypoxia, as we found an exaggerated contractile response to ET-1 in COX-2–deficient VSMCs. These findings expand on prior work demonstrating that prostacyclin analogs can inhibit ET-1 release in human PASMCs[35] and that intravenous prostacyclin may either increase ET-1 clearance or decrease its release in patients with idiopathic pulmonary hypertension.[36] Furthermore, we have shown that this phenotype can be reversed with exogenous iloprost and, interestingly, PGE_2 treatment. Although attenuation of contractility with PGI_2 was selective for PASMCs, both PGE_2 and forskolin, an activator of adenylate cyclase, rescued the contractile phenotype in COX-2–null PASMCs and VSMCs, suggesting a cAMP-dependent mechanism.

Taken together, our results demonstrate that under hypoxic conditions, COX-2–deficient PASMCs have an enhanced hypertrophic and contractile response to ET-1 resulting in part from upregulation of the ET_A receptor. Our findings suggest that COX-2 induction during hypoxia attenuates expression of the ET_A receptor through a cAMP-dependent signaling pathway, thereby modulating the contractile and growth-promoting effects of ET-1. We cannot, however, exclude other potential mechanisms of enhanced contractility that COX-2 may modulate. For example, both acute and chronic hypoxia may regulate activity or expression of voltage-gated potassium (Kv) channels, which could alter Ca^{2+} influx and activate myosin light chain kinase.[37,38] Potential downstream signaling mechanisms by which COX-2 may modulate ET_A receptor expression and mediate protection against hypoxia-induced pulmonary vascular remodeling include the protein kinase A[33] and the exchange protein directly activated by cAMP (Epac)[39] signaling pathways and will be the subject of future investigations.

Conclusions

Our findings have revealed a novel role for COX-2 in mediating protection against hypoxia-induced pulmonary hypertension and vascular remodeling, as well as modulating PASMC contractility. Pharmacological inhibition of COX-2 with selective COX-2 inhibitors has received significant attention in the literature recently. We now report that, in addition to well-recognized prothrombotic cardiovascular risks, selective COX-2 inhibition may have detrimental pulmonary vascular consequences. These findings may have significant clinical implications in patients with hypoxemic lung diseases or preexisting pulmonary hypertension.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Pulmonary hypertension is a severe disease commonly associated with chronic hypoxemia in disorders such as chronic obstructive pulmonary disease and interstitial lung disease. It is characterized by the development of pulmonary vascular remodeling, leading to elevated pulmonary vascular resistance and ultimately progression to right ventricular dysfunction and often death. Despite state-of-the-art therapy, morbidity and mortality rates remain high as a result of irreversible vascular remodeling. Cyclooxygenase-2 (COX-2) is upregulated in pulmonary artery smooth muscle cells during hypoxia and may play a protective role in the vascular response of the lung to hypoxia. In the present study, we investigated the role of COX-2 in a mouse model of hypoxia-induced pulmonary hypertension. The absence of COX-2 or pharmacological inhibition of COX-2 led to severe pulmonary hypertension after hypoxia with exaggerated elevation of right ventricular systolic pressure, significant right ventricular hypertrophy, and striking vascular remodeling. Vascular remodeling was characterized by pulmonary artery smooth muscle cell hypertrophy and significant upregulation of the endothelin-1 (ET_A) receptor in the lung during hypoxia. Our findings also demonstrate that COX-2–deficient pulmonary artery smooth muscle cells have enhanced contractility after exposure to hypoxia that can be rescued by COX-2–derived prostaglandin E_2 or prostaglandin I_2. The results of our study suggest that COX-2 plays an important protective role in the lung under hypoxic conditions and that selective COX-2 inhibition may have detrimental pulmonary vascular consequences in patients with hypoxemic lung diseases or preexisting pulmonary hypertension. These findings have significant clinical implications and raise the possibility that selective COX-2 inhibitors might worsen symptoms in patients with pulmonary hypertension.