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Absence of COX-2 exacerbates hypoxia-induced pulmonary hypertension and enhances contractility of vascular smooth muscle cells

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Abstract

Background—Cyclooxygenase-2 (COX-2) is upregulated in pulmonary artery smooth muscle cells (PASMC) during hypoxia and may play a protective role in the lung's response 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 following 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 ET-1 receptor (ET_AR) in the lung following hypoxia. Similarly, selective pharmacologic inhibition of COX-2 in wild-type mice exacerbated hypoxia-induced pulmonary hypertension and resulted in PASMC hypertrophy and increased ET_AR expression in pulmonary arterioles. 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 PASMC with iloprost, a prostaglandin (PG) I_2 analog, as well as PGE2, 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 is exacerbated in the absence of COX-2 with enhanced ET_A receptor expression and increased PASMC hypertrophy. COX-2 deficient PASMC have a maladaptive response to hypoxia manifested by exaggerated contractility which may be rescued by either COX-2-derived PGI_2 or PGE_2.

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Keywords

hypertension; pulmonary; hypertrophy; hypoxia; prostaglandins; remodeling; vasculature

Introduction

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 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 three 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 release of potent vasoconstrictors including thromboxane A_2 (TXA_2) and endothelin-1 (ET-1) which can overwhelm the effects of endothelial-derived vasodilators such as prostacyclin (PGI_2) and nitric oxide (NO), thereby promoting remodeling of the arteriolar wall.[1–3]

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

The cyclooxygenase 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 cyclooxygenase, is upregulated by hypoxia in pulmonary artery smooth muscle cells (PASMC) and both elevated TXA_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] As well, recent work suggests that COX-2 inhibition enhances platelet deposition and intravascular thrombosis in a rat model of hypobaric hypoxia-induced pulmonary hypertension.[12] In addition, selective inhibition of COX-2 is associated with an increased incidence of adverse cardiovascular events.[13–15] These potential vascular sequelae associated with pharmacologic 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 pro-thrombotic 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 right ventricular systolic pressure (RVSP), striking pulmonary vascular remodeling, and severe right ventricular hypertrophy (RVH). Interestingly, absence of COX-2 during hypoxia led to increased PASMC hypertrophy, but did not affect 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 PASMC on collagen gel matrices. This enhanced contractility was attenuated by both exogenous iloprost, a PGI_2 analog, as well as 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 at http://circ.ahajournals.org.

Animals

Mice that were wild-type (+/+, WT) or homozygous null (−/−) for targeted disruption of COX-2 (B6:129S7-Ptgs2^{tm1Jed}, Jackson Laboratories, 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. Following exposure, mice were anesthetized with sodium pentobarbital (60 mg/kg) and hemodynamic measurements were performed.[17,19] The hearts were excised and the ventricles dissected and weighed. RVH was assessed by normalizing RV weight to total body weight (TBW) (RV weight/TBW).[16,17]

Histologic Analysis and Morphometry

Lungs were inflated, harvested, fixed in Methyl Carnoy's solution, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E)[20] and immunostained for α-smooth muscle actin (α-SMA, 1:50).[21,22] Remodeling was quantitated as described previously.[16,17,19] Percent wall thickness was calculated as: wall thickness (%) = ($area_{ext} - area_{int}$) ÷ $area_{ext} \times 100$, where $area_{ext}$ represents the external diameter and $area_{int}$ represents the internal diameter of each vessel respectively.[16,17,19] PASMC hypertrophy was calculated as: vessel wall area ÷ # 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 endothelin-1 receptor (ET_A) antibody (1:500, BD Biosciences). Equal loading was confirmed with an anti-tubulin antibody (1:8000).

Cell culture

Primary aortic smooth muscle cells (VSMC) were isolated from COX-$2^{-/-}$ and COX-$2^{+/+}$ embryos at 18.5 dpc as described.[22,24] Primary PASMC were isolated from adult (8–10 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, WA).[26]

Traction force microscopy

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

Collagen matrix contraction assay

COX-$2^{-/-}$ and COX-$2^{+/+}$ PASMC and VSMC were plated on type I collagen gel matrices and exposed to hypoxia or normoxia for 24 h. Gel size was defined as the sum of the two longest gel diameters and gel contraction expressed as a percentage of the original gel size.[30] In certain experiments, cells were treated with either PGE_2 (1 μM), iloprost (1 μM), or vehicle (30% ethanol in PBS) during hypoxic exposure. In other experiments, cells were treated with either forskolin (10 μM) or vehicle (4% ethanol in DMEM) during hypoxic exposure. RPASMC were treated with NS-398 (5 μM) or vehicle (25% DMSO in PBS).

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined by the Student's t-test for comparisons between 2 groups, and analysis of variance (ANOVA) for comparisons between more than 2 groups or for multiple comparisons. Statistical significance was accepted at $p<0.05$.

The authors had full access to the data and take responsibility for its integrity. 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 following 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 wild-type (WT) mice following hypoxia with no difference in COX-1 expression between COX-$2^{-/-}$ and COX-$2^{+/+}$ mice either at baseline or following exposure to hypoxia (Supplemental Figure 1). 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 versus normoxia. Similar to COX-$2^{-/-}$ mice, nimesulide-treated WT mice developed more severe pulmonary hypertension following 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. As well, 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 following chronic hypoxia

H&E staining revealed enhanced vascular remodeling in COX-$2^{-/-}$ mice following 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%) following 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 following 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).

Neither proliferation nor migration differ between COX-$2^{-/-}$ and COX-$2^{+/+}$ VSMC 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 2, during exposure to hypoxia, neither proliferation nor migration differed between COX-$2^{-/-}$ and COX-$2^{+/+}$ VSMC in response to PDGF. In addition, BrdU staining demonstrated no difference in proliferation *in vivo* between COX-$2^{-/-}$ and COX-$2^{+/+}$ mice following 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 following hypoxia

Trichrome staining revealed minimal collagen deposition in the distal remodeled vessels with no difference between COX-$2^{-/-}$ and COX-$2^{+/+}$ mice (data not shown). However, immunostaining for α-SMA demonstrated that, following 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 quantitate α-SMA in the lungs of COX-$2^{-/-}$ and COX-$2^{+/+}$ mice following 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 following 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 following 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 following 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 3).

COX-2$^{-/-}$ VSMC have enhanced traction forces following hypoxia

Previous studies with cultured pulmonary VSMC 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 (TFM) to measure the traction forces exerted by individual VSMC following exposure to hypoxia *in vitro* (Figure 4A). COX-2$^{+/+}$ and COX-2$^{-/-}$ VSMC had no difference in traction forces under normoxic conditions (COX-2$^{-/-}$ 102 ± 14 Pa; COX-2$^{+/+}$ 108 ± 19 Pa), however following hypoxia, COX-2$^{-/-}$ VSMC developed a significant increase in traction forces (150 ± 18 Pa) compared with COX-2$^{+/+}$ VSMC (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 VSMC.

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 contractility of vascular smooth muscle cells during hypoxia, we harvested protein from lungs of COX-2$^{-/-}$ and COX-2$^{+/+}$ mice and performed Western blot analysis for the ET_A receptor (ET_AR). COX-2$^{-/-}$ mice had dramatic induction of the ET_A receptor following hypoxia with a 5-fold induction in ET_AR protein expression compared with only a 5% increase in COX-2$^{+/+}$ mice (Figure 5A). In addition, following exposure to hypoxia, nimesulide-treated WT mice demonstrated a greater than 30-fold increase in ET_AR expression in pulmonary arterioles by immunohistochemistry compared with only a 3-fold increase in vehicle-treated WT mice (Supplemental Figure 4). Furthermore, when COX-2$^{+/+}$ and COX-2$^{-/-}$ VSMC were treated with ET-1 following hypoxia, COX-2$^{-/-}$ VSMC developed a significant increase in traction forces (202 ± 17 Pa) compared with COX-2$^{+/+}$ VSMC (160 ± 15 Pa; $p<0.05$) and normoxic controls (Figure 5B).

COX-2$^{-/-}$ PASMC have enhanced contractility on collagen gels following hypoxia

Given these findings, we investigated whether COX-2$^{-/-}$ PASMC would demonstrate enhanced contractility of a 3-dimensional collagen matrix. Consistent with our TFM results, COX-2$^{-/-}$ PASMC demonstrated enhanced contraction of collagen matrices following hypoxia (Figure 6A) compared with COX-2$^{+/+}$ PASMC. At 4 h following matrix release, hypoxic COX-2$^{-/-}$ PASMC exhibited exaggerated gel contraction (55 ± 2.2% original gel size) compared with hypoxic COX-2$^{+/+}$ PASMC (80 ± 2.3% original gel size; $p<0.05$) (Figure 6B). Similarly, COX-2$^{-/-}$ VSMC demonstrated exaggerated gel contraction (48 ± 1.9% original gel size) compared with COX-2$^{+/+}$ VSMC (73 ± 5% original gel size; $p<0.05$) following hypoxic exposure (Figure 6B). In addition, pharmacologic inhibition of COX-2 in a rat pulmonary artery smooth muscle (RPASMC) cell line resulted in increased contraction (67 ± 3.4% original gel size) during hypoxia compared with vehicle control (83 ± 1.6% original gel size; $p<0.05$) (Figure 6B).

Iloprost and PGE_2 attenuate enhanced contractility of COX-2$^{-/-}$ PASMC on collagen gels following hypoxia

To determine if the administration of prostanoids could rescue COX-2$^{-/-}$ PASMC 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 following hypoxia. COX-2$^{-/-}$ and COX-2$^{+/+}$ PASMC and VSMC were exposed to hypoxia for 24 h and supernatants analyzed for PGE_2 and 6-keto-$PGF_{1\alpha}$, a stable PGI_2 metabolite. Levels of PGE_2 were significantly higher in WT VSMC compared with WT PASMC following hypoxia. In addition, 6-keto-$PGF_{1\alpha}$ was equally as abundant as PGE_2 in WT VSMC following

hypoxia, while almost 8-fold more abundant than PGE_2 in hypoxia-exposed WT PASMC (Supplemental Figure 5). As expected, PGE_2 and 6-keto-$PGF_{1\alpha}$ levels were markedly lower in COX-$2^{-/-}$ PASMC and VSMC.

To investigate whether repletion of these COX-2-derived prostanoids would alter the contractile phenotype of COX-$2^{-/-}$ PASMC and VSMC during hypoxia, we performed collagen matrix contraction assays in the presence of exogenous PGE_2 or iloprost, a PGI_2 analog. Vehicle-treated COX-$2^{-/-}$ PASMC and VSMC both demonstrated exaggerated gel contraction following hypoxia compared with WT controls. Contraction by hypoxic COX-$2^{-/-}$ PASMC was significantly attenuated by either iloprost (84 ± 2.6% original gel size; $p<0.05$) or PGE_2 (81 ± 2.3% original gel size; $p<0.05$) (Figure 7A). However, exaggerated contraction by COX-$2^{-/-}$ VSMC was attenuated only by PGE_2 (79 ± 2.4% original gel size; $p<0.05$), not iloprost (61 ± 2.7% 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-$2^{-/-}$ PASMC and VSMC following exposure to hypoxia (Supplemental Figure 6).

Discussion

This study highlights three important new concepts. First, deficiency or pharmacologic 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 vascular smooth muscle cells 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-$2^{-/-}$ PASMC attenuates their potent contractility in response to hypoxia, thus restoring the wild-type 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 PASMC under hypoxic conditions[12,31] and our data provide evidence that it plays a protective role in response to hypoxia. Pharmacologic 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*. suggests that pharmacologic inhibition of COX-2 in a rat model of hypobaric hypoxia enhances platelet activation and intravascular thrombosis that was partially attenuated by a TX receptor antagonist.[12] However, the effect of COX-2 deficiency on remodeling and contractility of PASMC 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 following only two weeks of hypoxia. In contrast, WT mice develop less severe pulmonary hypertension and minimal vascular remodeling in response to two weeks of hypoxia. In addition, selective pharmacologic 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 following 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, in part, be due to enhanced expression of the ET_A receptor during hypoxia, as ET-1 has been linked to VSMC hypertrophy.[32,33] Interestingly, while PGI_2 has been shown to have

inhibitory effects on proliferation of human PASMC,[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 also increase as a result of intravascular thrombosis following 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 absence of COX-2 during hypoxia enhances traction forces generated in individual vascular smooth muscle cells and augments contractility of PASMC on an extracellular matrix. Previous work has illustrated a direct correlation between cellular traction forces and myosin light chain (MLC) phosphorylation in PASMC under normoxic conditions.[29] As MLC 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 PASMC 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 VSMC. These findings expand upon prior work demonstrating that prostacyclin analogs can inhibit ET-1 release in human PASMC[35] and 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. While attenuation of contractility with PGI_2 was selective for PASMC, both PGE_2 and forskolin, an activator of adenylate cyclase, rescued the contractile phenotype in both COX-2 null PASMC and VSMC, suggesting a cAMP-dependent mechanism.

Taken together, our results demonstrate that under hypoxic conditions, COX-2 deficient PASMC have an enhanced hypertrophic and contractile response to ET-1 due, in part, to upregulation of the ET_A receptor. Our findings suggest that COX-2 induction during hypoxia attenuates expression of the ET_A receptor via 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 MLC kinase.[37,38] Potential downstream signaling mechanisms by which COX-2 may modulate ET_AR expression and mediate protection against hypoxia-induced pulmonary vascular remodeling include the protein kinase A [33] and Epac (exchange protein directly activated by cAMP) [39] signaling pathways and will be the subject of future investigations.

In summary, 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. Pharmacologic 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 pro-thrombotic 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 pre-existing pulmonary hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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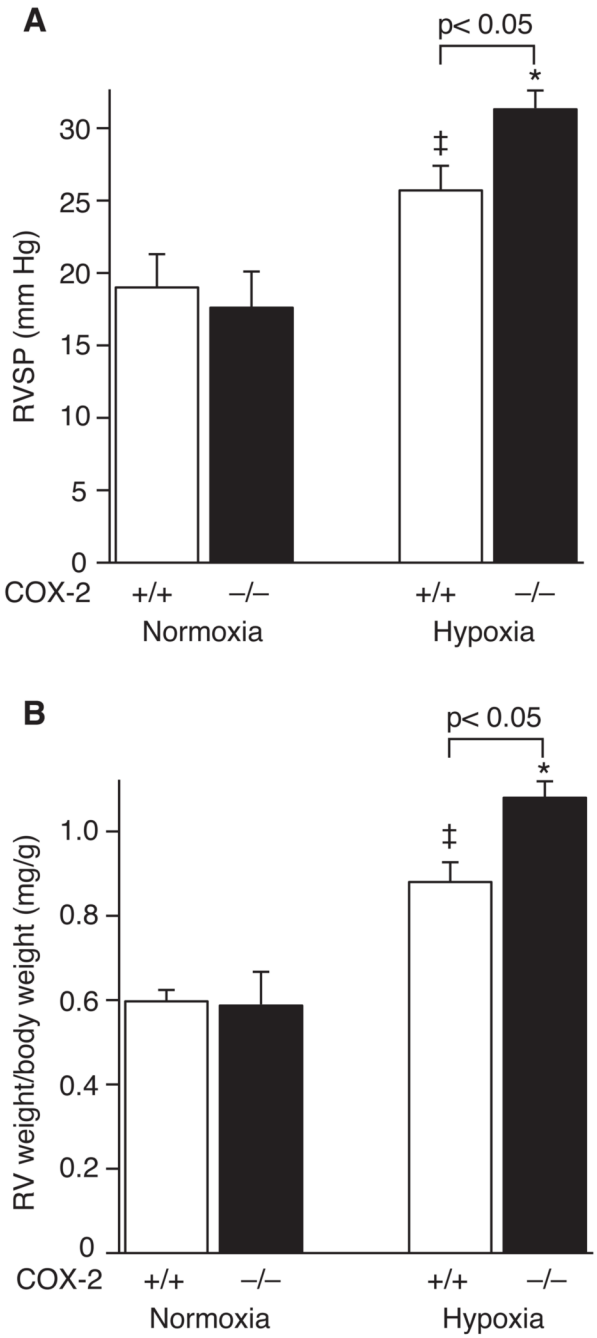

Figure 1. COX-2$^{-/-}$ mice have exaggerated elevation of RVSP and severe RVH following chronic hypoxia

A) RVSP in COX-2$^{+/+}$ (□) and COX-2$^{-/-}$ (■) mice following 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, and ‡$p<0.05$ for hypoxic COX-2$^{+/+}$ mice vs. normoxic controls). **B)** RV weight (mg) normalized for body weight (g) in COX-2$^{+/+}$ (□) and COX-2$^{-/-}$ (■) mice following 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, and ‡$p<0.05$ for hypoxic COX-2$^{+/+}$ mice vs. normoxic controls).

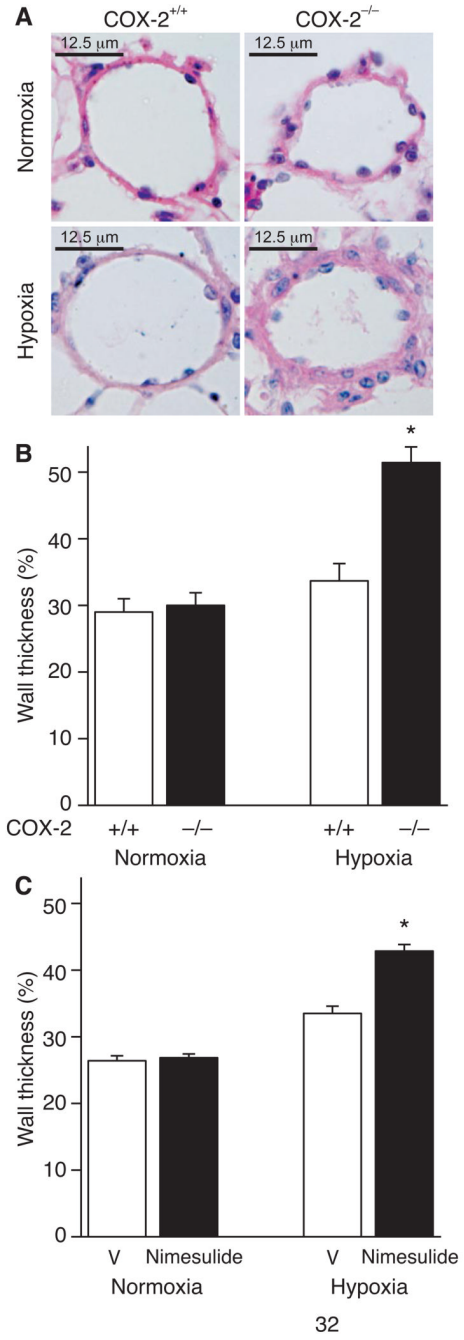

Figure 2. Absence of COX-2 results in enhanced pulmonary vascular remodeling following chronic hypoxia

A) Representative 5 μm H&E-stained sections from COX-$2^{+/+}$ (left) and COX-$2^{-/-}$ (right) mice following normoxia (top) and hypoxia (bottom). Quantitation of percent wall thickness of pulmonary arterioles in the lungs of **(B)** COX-$2^{+/+}$ (□) and COX-$2^{-/-}$ (■) mice following normoxia (n=5 per group) and hypoxia (n=8 per group) and **(C)** vehicle (V) and nimesulide-treated WT mice following 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)**).

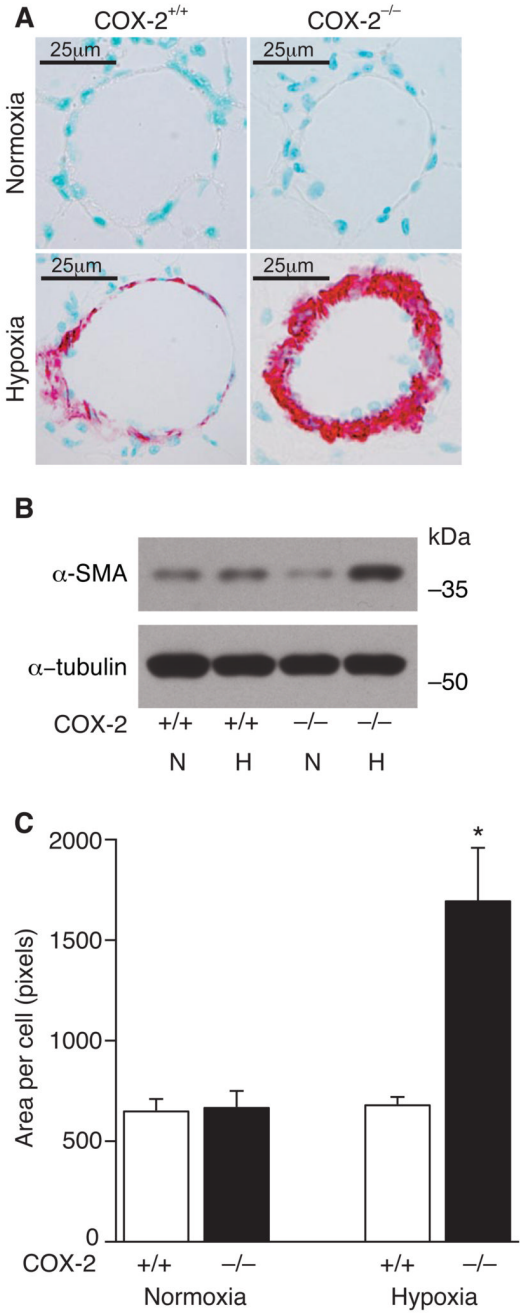

Figure 3. COX-2$^{-/-}$ mice have enhanced PASMC hypertrophy following hypoxia

A) Immunostaining of lungs from COX-2$^{+/+}$ (left) and COX-2$^{-/-}$ (right) mice following normoxia (top) and hypoxia (bottom) for α-SMA. **B)** Western blot analysis for α-SMA on lungs from COX-2$^{+/+}$ and COX-2$^{-/-}$ mice following hypoxia and normoxia. **C)** Quantitation of PASMC size in COX-2$^{+/+}$ (□) and COX-2$^{-/-}$ (■) mice. Ten vessels were analyzed per mouse following 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).

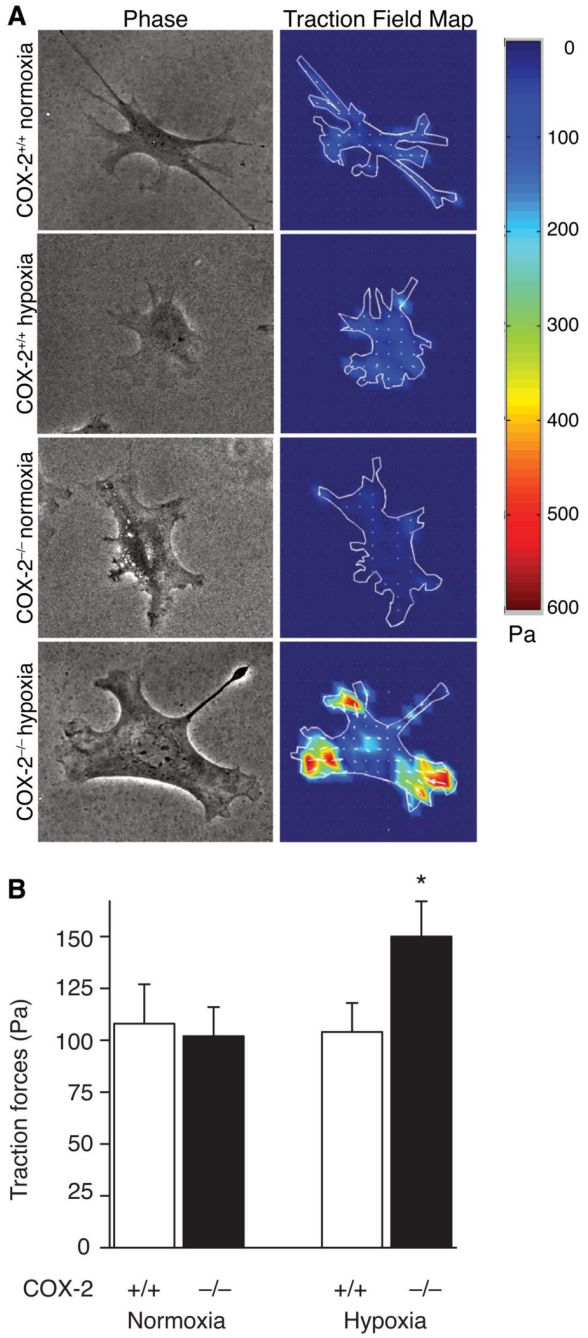

Figure 4. COX-2$^{-/-}$ VSMC have enhanced traction forces following hypoxia

A) Representative phase contrast views (left) and traction field maps (right) in COX-2$^{+/+}$ (top) and COX-2$^{-/-}$ (bottom) VSMC following 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 (Pa). **B)** Traction forces (Pa) in COX-2$^{+/+}$ and COX-2$^{-/-}$ VSMC following normoxia and hypoxia. Data are expressed as mean ± SE (*p<0.05 for hypoxic COX-2$^{-/-}$ VSMC vs. COX-2$^{+/+}$ VSMC and normoxic controls).

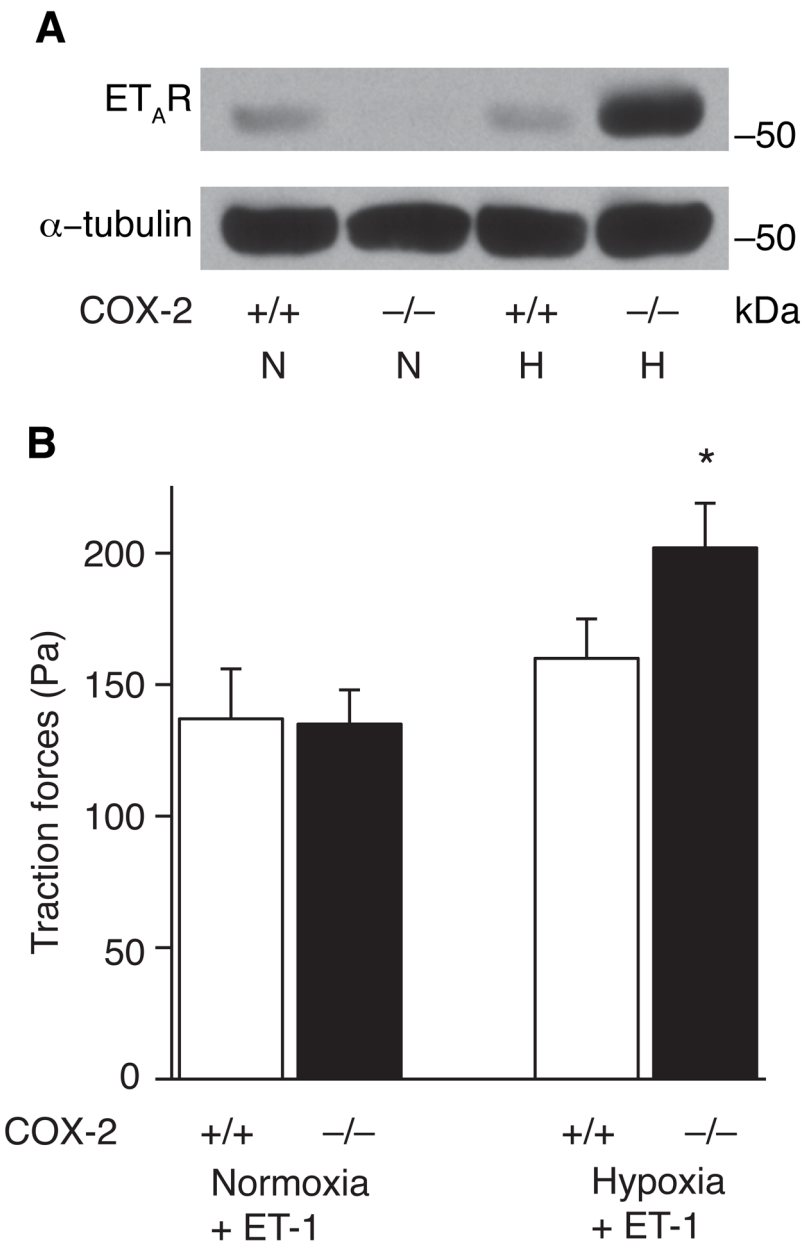

Figure 5. Absence of COX-2 during hypoxia leads to enhanced ET_A receptor expression in lungs and enhanced traction forces in response to ET-1

A) Total protein was isolated from lungs of COX-$2^{+/+}$ and COX-$2^{-/-}$ mice following hypoxia and normoxia and Western blot analysis performed for the ET_A receptor. Loading was quantitated with an anti-tubulin antibody. A representative of three experiments is shown. **B)** Traction forces of COX-$2^{+/+}$ and COX-$2^{-/-}$ VSMC following normoxia (COX-$2^{+/+}$, n=16 cells; COX-$2^{-/-}$, n=23 cells) and hypoxia (COX-$2^{+/+}$, n=27 cells; COX-$2^{-/-}$, n=19 cells) following stimulation with ET-1. Data are expressed as mean ± SE (*$p<0.05$ for hypoxic COX-$2^{-/-}$ VSMC vs. COX-$2^{+/+}$ VSMC and normoxic controls).

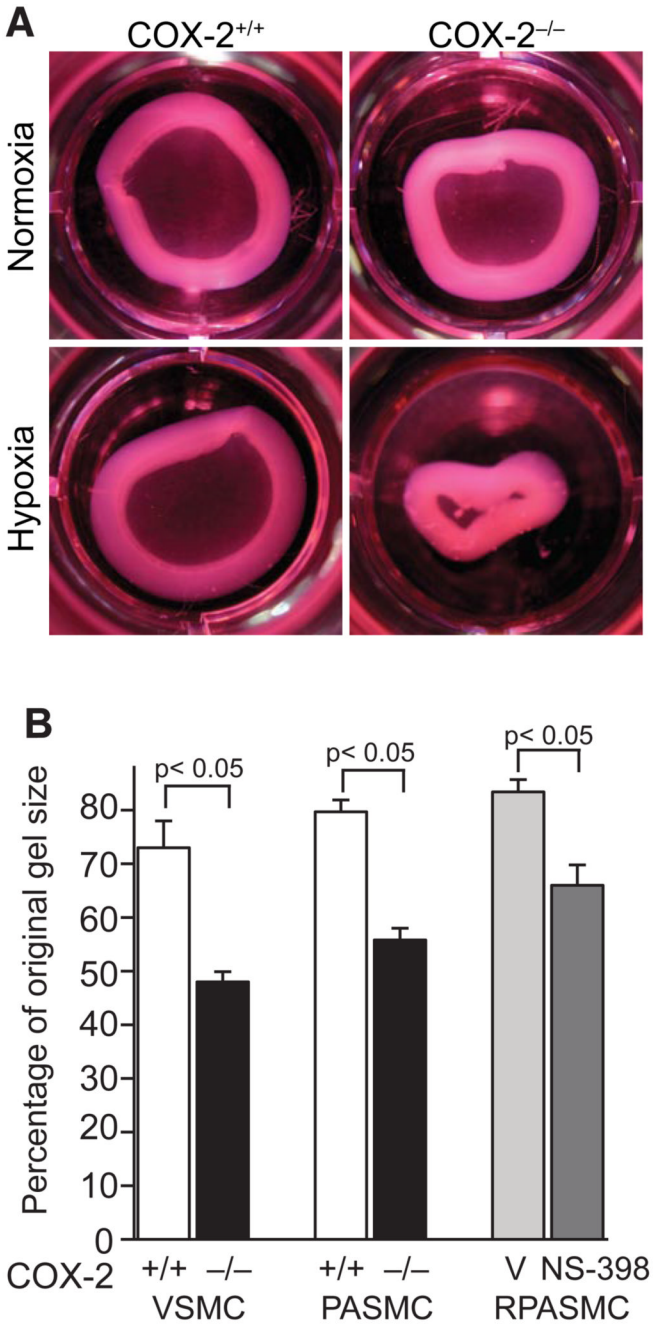

Figure 6. COX-2$^{-/-}$ PASMC have enhanced contractility on collagen gels following hypoxia

A) Representative photographs of collagen gels from COX-2$^{+/+}$ (left) and COX-2$^{-/-}$ (right) PASMC under normoxic (top) and hypoxic (bottom) conditions. **B)** Gel contraction following matrix release. Data are presented as the percentage of the original collagen gel size for VSMC, PASMC (COX-2$^{+/+}$ □ and COX-2$^{-/-}$ ■), and RPASMC (vehicle ▫ and NS-398 ▪) exposed to hypoxia. Data are expressed as mean ± SE ($p<0.05$ for COX-2$^{-/-}$ VSMC vs. COX-2$^{+/+}$ VSMC; $p<0.05$ for COX-2$^{-/-}$ PASMC vs. COX-2$^{+/+}$ PASMC; $p<0.05$ for NS-398-treated vs. vehicle-treated RPASMC).

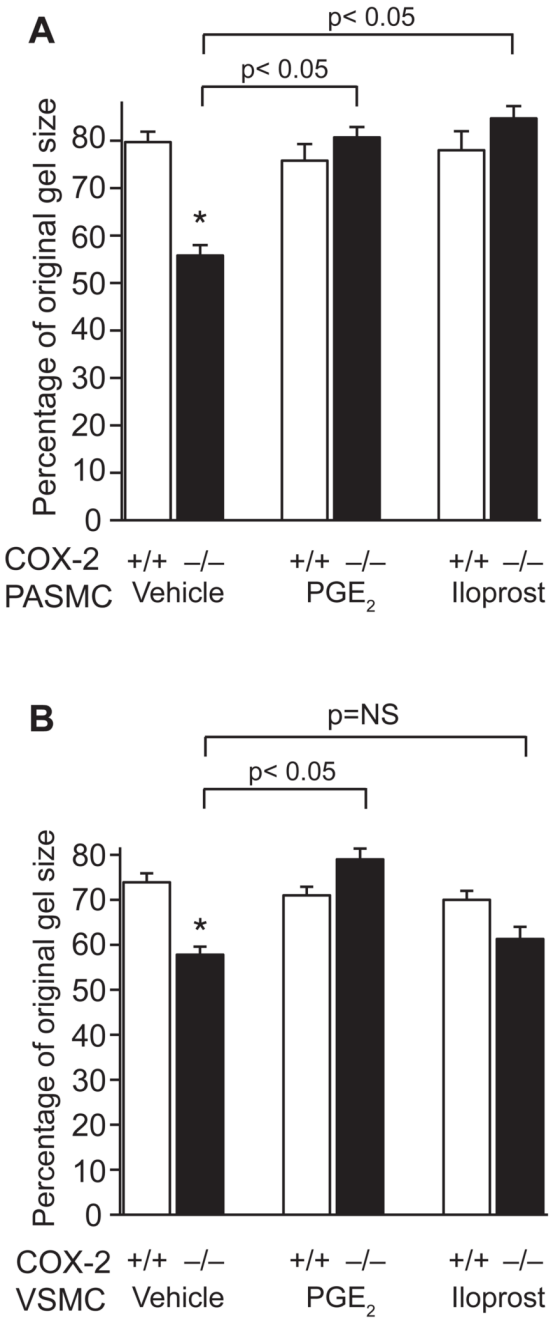

Figure 7. Iloprost and PGE_2 attenuate contractility of COX-$2^{-/-}$ PASMC on collagen gels following hypoxia

Gel contraction by COX-$2^{+/+}$ and COX-$2^{-/-}$ PASMC **(A)** and VSMC **(B)** following treatment with prostanoid analogs (PGE_2 1 μM, iloprost 1 μM) under hypoxic conditions. Data are presented as the percentage of the original collagen gel size for hypoxic PASMC and VSMC (COX-$2^{+/+}$ □ and COX-$2^{-/-}$ ■) treated with PGE_2, iloprost, or vehicle. Data are expressed as mean ± SE (*$p<0.05$ for vehicle-treated COX-$2^{-/-}$ PASMC vs. vehicle-treated COX-$2^{+/+}$ PASMC, $p<0.05$ for PGE_2-treated and iloprost-treated COX-$2^{-/-}$ PASMC vs. vehicle **(A);** *$p<0.05$ for vehicle-treated COX-$2^{-/-}$ VSMC vs. vehicle-treated COX-$2^{+/+}$ VSMC, $p<0.05$ for PGE_2-treated COX-$2^{-/-}$ VSMC vs. vehicle **(B)**).