Hyperoxia Increases Phosphodiesterase 5 Expression and Activity in Ovine Fetal Pulmonary Artery Smooth Muscle Cells

Kathryn N. Farrow, Beezly S. Groh, Paul T. Schumacker, Satyan Lakshminrusimha, Lyubov Czech, Sylvia F. Gugino, James A. Russell, Robin H. Steinhorn

Abstract—In the pulmonary vasculature, cGMP concentrations are regulated in part by a cGMP-dependent phosphodiesterase (PDE), PDE5. Infants with persistent pulmonary hypertension of the newborn (PPHN) are often mechanically ventilated with high oxygen concentrations. The effects of hyperoxia on the developing pulmonary vasculature and PDE5 are largely unknown. Here, we demonstrate that exposure of fetal pulmonary artery smooth muscle cells (FPASMCs) to high levels of oxygen for 24 hours leads to decreased responsiveness to exogenous NO, as determined by a decreased intracellular cGMP response, increased PDE5 mRNA and protein expression, as well as increased PDE5 cGMP hydrolytic activity. We demonstrate that inhibition of PDE5 activity with sildenafil partially rescues cGMP responsiveness to exogenous NO. In FPASMCs, hyperoxia leads to increased oxidative stress without increasing cell death. Treatment of normoxic FPASMCs with H$_2$O$_2$ is sufficient to induce PDE5 expression and activity, suggesting that reactive oxygen species mediate the effects of hyperoxia in FPASMCs. In support of this mechanism, a chemical antioxidant, N-acetyl-cysteine, is sufficient to block the hyperoxia-mediated increase in PDE5 expression and activity and rescue cGMP responsiveness to exogenous NO. Finally, ventilation of healthy neonatal sheep with 100% O$_2$ for 24 hours leads to increased PDE5 protein expression in the resistance pulmonary arteries and increased PDE5 activity in whole lung extracts. These data suggest that PDE5 expression and activity play a critical role in modulating neonatal pulmonary vascular tone in response to common clinical treatments for PPHN, such as oxygen and inhaled NO. (Circ Res. 2008;102:226-233.)

Key Words: pulmonary circulation ■ persistent pulmonary hypertension of the newborn ■ cyclic GMP ■ phosphodiesterases

Persistent pulmonary hypertension of the newborn (PPHN) is a clinical syndrome that results from a failure of the pulmonary vasculature to transition to extrauterine life. Infants typically present shortly after birth with respiratory distress and cyanosis but a structurally normal heart. The estimated incidence of PPHN is 0.2% of liveborn term infants. PPHN is a manifestation of a heterogeneous group of disorders, and the response to therapy frequently depends on the underlying disease. Despite advances in clinical therapy, including high frequency ventilation, inhaled nitric oxide (iNO), and extracorporeal membrane oxygenation, there is still significant morbidity and mortality associated with this disease. Furthermore, iNO does not improve survival, and many infants do not respond or sustain their response to iNO for reasons that are unclear.

One factor that confounds the treatment of these infants is the use of supplemental oxygen. Oxygen has long been considered a vasodilator in the pulmonary circulation, and thus, 100% O$_2$ is considered a first-line therapy in infants with PPHN. However, data from the adult acute respiratory distress syndrome literature, from the neonatal bronchopulmonary dysplasia literature, and from the neonatal resuscitation literature suggest that exposure to high levels of oxygen may cause lasting lung injury, oxidative stress, and pulmonary vascular remodeling. Thus, the rationale for high inspired oxygen concentrations has come under question, although the mechanisms by which oxygen affects PPHN therapy are not known.

Phosphodiesterases (PDEs) constitute a superfamily of enzymes comprised of 11 different PDE families. Each family of PDEs has unique enzymatic properties, as well as specific tissue and cellular distributions. The prevalent PDE within the lung is PDE5, although PDE3 and PDE4 are also expressed in pulmonary tissue. In the fetal lung, PDE5 activity and expression is highest in pulmonary arteries (PAs). In lambs, it decreases shortly after birth and then rises...
again 4 to 7 days later, suggesting developmental regulation.\(^{11,12}\) Moreover, as the primary enzyme responsible for regulating cGMP, PDE5 represents an important regulator of NO-mediated vascular relaxation.

Endogenous NO and cGMP are important in the normal pulmonary vascular transition after birth,\(^ {13}\) and PDE5 activity may be increased in a lamb model of PPHN.\(^ {14–17}\) All of the studies that have examined PDE5 expression or activity in the pulmonary vasculature have been conducted in fetal animals exposed to low oxygen tension in utero\(^ {16,17}\) or in fetal animals exposed to high levels of inspired oxygen for less than 2 hours.\(^ {14,15}\) There are few data regarding the effects of longer hyperoxia exposures on PDE5 expression and activity in the pulmonary vasculature. Recently published data demonstrate that ventilation of newborn sheep with 100% oxygen increases contractile responses to norepinephrine.\(^ {7}\) Additionally, in a hyperoxic rat model of bronchopulmonary dysplasia, treatment with sildenafil, a PDE5 inhibitor, decreased pulmonary vascular resistance and improved lung angiogenesis, suggesting that PDE5 activity plays a critical role in mediating pulmonary vascular tone in the context of hyperoxia.\(^ {18}\) We propose that increased PDE5 expression and activity in pulmonary vascular smooth muscle in response to hyperoxia may explain abnormal baseline vasoreactivity, as well as iNOS resistance and rebound pulmonary hypertension after iNOS withdrawal. A greater understanding of the mechanisms of PDE5 regulation in the pulmonary vasculature will be of critical importance to identifying improved therapies for infants with PPHN.

Materials and Methods

Cell Culture

Primary cultures of FPASMCs were prepared from intrapulmonary arteries as described in the expanded Materials and Methods section in the online data supplement, available http://circres.ahajournals.org. FPASMCs were treated in incubators with 21% O\(_2\)–5% CO\(_2\), 50% O\(_2\)–5% CO\(_2\), or 95% O\(_2\)–5% CO\(_2\) with or without DETANONOate (100 \mu mol/L; Cayman Chemical, Ann Arbor, Mich), sildenafil (100 \mu mol/L, provided by Dr Sharron Francis and Dr Jackie Corbin), or N-acetylcysteine (NAC) (500 \mu mol/L, Sigma, St Louis, Mo); or with 21% O\(_2\)–5% CO\(_2\) with or without a single dose of H\(_2\)O\(_2\) (50 \mu mol/L; Sigma). FPASMCs were harvested after analysis for 24 hours.

Cyclic GMP Enzyme-Linked Immunoassay

Treated cells were lysed, and cGMP content was measured by enzyme-linked immunosassay in triplicate according to the protocol of the manufacturer and as described in the expanded Materials and Methods section (Cayman Chemical). Results are shown as picomoles of cGMP per milligram of total protein.

Real-Time PCR

FPASMCs were harvested for RNA using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, Calif), and RNA was quantified using the Quant-it Ribogreen assay (Molecular Probes/Invitrogen, Carlsbad, Calif). cDNA was prepared from total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using the iQ SYBR Green Supermix (Bio-Rad) with the iCycler iQ real-time PCR detection system (Bio-Rad), with 50 cycles of real-time data collection using 95°C for 10 seconds and 46.1°C for 45 seconds, followed by melt curve analysis to verify the presence of a single product. Primers were designed using commercially available software and are described in the expanded Materials and Methods section. Relative PDE5 amounts were normalized to \(\beta\)-actin expression using the \(\Delta\Delta C_t\) method.\(^ {19}\)

Western Blot

After treatment, FPASMCs were harvested for total protein (40 \mu g), which was subjected to Western blot analysis as described in the expanded Materials and Methods section using commercially available antibodies: PDE5 (BD Transduction, San Jose, Calif) and \(\beta\)-actin (Sigma). Bands were visualized via chemiluminescence (Pierce, Rockford, Ill), using a Digital Science Image Station (Kodak, Rochester, NY). Expression of PDE5 was normalized to \(\beta\)-actin expression.

Immunohistochemistry

FPASMCs were plated onto collagen-treated glass coverslips and treated as above. After treatment, cells were fixed, permeabilized, and stained for immunohistochemistry with anti-PDE5 used in conjunction with a fluorescent secondary antibody (Molecular Probes/Invitrogen) as described in the expanded Materials and Methods section.

PDE5 Activity Assay

After treatment, FPASMCs were harvested for total protein (5 \mu g), which was assayed for cGMP hydrolytic activity using a commercially available colorimetric cyclic nucleotide phosphodiesterase assay kit (Biolum, Plymouth Meeting, Pa) as described in the expanded Materials and Methods section. Assays were performed with and without sildenafil (100 \mu mmol/L) to determine PDE5-specific cGMP hydrolytic activity. Results are shown as PDE5-specific picomoles of cGMP hydrolyzed per milligram of total protein per minute.

Detection of Reactive Oxygen Species

FPASMCs were infected with 100 plaque-forming units per cell of a Ro-GFP adenoviral construct as described in the expanded Materials and Methods section. Ro-GFP is a previously characterized ratiometric fluorescent probe sensitive to cellular oxidative stress.\(^ {20}\) To create this probe, surface-exposed residues in green fluorescent protein (GFP) were replaced with cysteine residues capable of forming disulfide bonds. Assessment of fluorescence ratios therefore provide a real-time measure of cysteine thiol redox status in live cells.\(^ {20}\) Ro-GFP–infected FPASMCs were exposed to 21% O\(_2\)–5% CO\(_2\) or 95% O\(_2\)–5% CO\(_2\) for 24 hours, and subsequently, their oxidative status was analyzed using multilaser flow cytometry as described in the expanded Materials and Methods section.

Ventilation Protocols for Neonatal Sheep

The Laboratory Animal Care Committees at the State University of New York at Buffalo and at Northwestern University approved this study. Near-term gestation pregnant ewes (134 days gestation) were anesthetized with pentothal and halothane, and lambs were delivered by cesarean section (n = 5). Lambs were intubated and ventilated with 100% O\(_2\) for 24 hours as described in the expanded Materials and Methods section. Control groups included newborn lambs euthanized \(\approx\)24 hours after spontaneous delivery (1 day nonventilated, n = 4) and fetal lambs delivered and euthanized at 136 days of gestation (fetal control, n = 6). For tissue harvest, all lambs were anesthetized with pentothal sodium and killed by rapid exsanguinations through a direct cardiac puncture. The heart and lungs were removed en bloc and fifth-generation PAs (inner diameters of 500 \mu m) were dissected and isolated.\(^ {7}\) Total protein was isolated from lung and PA tissue using the commercially available PARIS kit (Ambion, Austin, Tex). Total protein was then subjected to Western blot analysis for PDE5 protein expression or to measurement of PDE5-specific cGMP hydrolytic activity as described above.

Immunohistochemistry

Tissue blocks were snap frozen, cut into thin sections (8 \mu m), and collected on glass slides. Sections were fixed with acetone and
Western blots are shown for PDE5 and \( \frac{\text{NO}}{\text{O}_2} \) in normoxia led to a significant increase in cGMP levels (\( P \leq 0.001 \) for 21% \( \text{O}_2 \); 94% of control; \( P \leq 0.001 \) for 50% \( \text{O}_2 \); 26% of control; \( P \leq 0.05 \) for 95% \( \text{O}_2 \); 9% of control). Thus, exposure to hyperoxia for 24 hours makes the FPASMCs less responsive to exogenous NO, the primary clinical therapy for PPHN.

### Hyperoxia Increases PDE5 Expression in FPASMCs

We hypothesized that the blunting of NO-mediated cGMP responsiveness in hyperoxia represents a PDE5-mediated desensitization. FPASMCs were exposed to 21% \( \text{O}_2 \)–5% \( \text{CO}_2 \), 50% \( \text{O}_2 \)–5% \( \text{CO}_2 \), or 95% \( \text{O}_2 \)–5% \( \text{CO}_2 \) for 24 hours. FPASMCs exposed to hyperoxia showed increased PDE5 mRNA expression (2.7 ± 0.5-fold; \( P < 0.005 \); Figure 2A) and increased PDE5 protein expression, as determined by immunocytochemistry (Figure 2B) and Western blot (139 ± 9% of control in 50% \( \text{O}_2 \); 185 ± 33% of control in 95% \( \text{O}_2 \); \( P < 0.001 \); Figure 2C and 2D). Thus, FPASMC exposure to hyperoxia for 24 hours leads to increased PDE5 mRNA and protein expression.

### Hyperoxia Increases PDE5 Activity in FPASMCs in a Dose-Dependent Fashion

The hypothesis that increased PDE5 expression leads to decreased cGMP responsiveness in hyperoxia is valid only if increased PDE5 expression correlates with increased activity. One of the key events required for PDE5 activity is cGMP binding directly to PDE5, followed by phosphorylation by protein kinase G (PKG).21–23 Hyperoxia for 24 hours led to significantly increased PDE5 phosphorylation in FPASMCs (Figure 2 in the online data supplement). Similarly, hyperoxia for 24 hours led to significantly increased PDE5 activity in FPASMCs (161 ± 26% of control in 50% \( \text{O}_2 \); 309 ± 94% of control in 95% \( \text{O}_2 \); \( P < 0.05 \); Figure 3). Furthermore, the increase in PDE5 activity in 95% \( \text{O}_2 \)–5% \( \text{CO}_2 \) is significantly greater than that seen in 50% \( \text{O}_2 \)–5% \( \text{CO}_2 \) (\( P < 0.05 \); Figure 3). Therefore, the hyperoxia-induced increase in PDE5 protein expression correlates with increased PDE5 phosphorylation and activity, suggesting that increased PDE5 expression and activity contribute to decreased intracellular cGMP responsiveness to exogenous NO in hyperoxia.

### Results

#### Hyperoxia Decreases cGMP Induced by Exogenous NO

As recent reports show that ventilation of sheep with 100% oxygen diminishes PA relaxations to an NO donor,7 we sought to determine the effect of hyperoxia on cGMP levels in FPASMCs from resistance PA. FPASMCs were exposed to 21% \( \text{O}_2 \)–5% \( \text{CO}_2 \), 50% \( \text{O}_2 \)–5% \( \text{CO}_2 \), or 95% \( \text{O}_2 \)–5% \( \text{CO}_2 \) with or without DETANONOate (100 \( \mu \text{mol/L} \)) for 24 hours. Cells were assayed for cGMP by enzyme-linked immunoassay, and cGMP was normalized for milligrams of total protein. Data are shown as means ± SEM (\( n = 6 \); read in triplicate). \( \# P < 0.001 \) vs cells not treated with DETANONOate, \( \bar{P} P < 0.05 \) vs cells not treated with DETANONOate.

Data are presented as means ± SEM. Data were analyzed by paired t test or 1-way repeated measures ANOVA with Bonferroni multiple comparison test where appropriate, using Prism (GraphPad Software Inc, San Diego, Calif). \( P < 0.05 \) was considered statistically significant.

### Statistics

Data are presented as means ± SEM. Data were analyzed by paired t test or 1-way repeated measures ANOVA with Bonferroni multiple comparison test where appropriate, using Prism (GraphPad Software Inc, San Diego, Calif). \( P < 0.05 \) was considered statistically significant.

#### Figures

**Figure 1.** Exogenous NO induces less cGMP in FPASMCs exposed to hyperoxia. FPASMCs were exposed to 21% \( \text{O}_2 \)–5% \( \text{CO}_2 \), 50% \( \text{O}_2 \)–5% \( \text{CO}_2 \), or 95% \( \text{O}_2 \)–5% \( \text{CO}_2 \) with or without DETANONOate (100 \( \mu \text{mol/L} \)) for 24 hours. Cells were assayed for cGMP by enzyme-linked immunoassay, and cGMP was normalized for milligrams of total protein. Data are shown as means ± SEM (\( n = 6 \); read in triplicate). \( \bar{P} P < 0.05 \) vs cells not treated with DETANONOate, \( \# P < 0.001 \) vs 21% \( \text{O}_2 \) + NO.

**Figure 2.** Hyperoxia induces PDE5 expression in FPASMCs. FPASMCs were exposed to 21% \( \text{O}_2 \)–5% \( \text{CO}_2 \), 50% \( \text{O}_2 \)–5% \( \text{CO}_2 \), or 95% \( \text{O}_2 \)–5% \( \text{CO}_2 \) for 24 hours. A, Cells were harvested for RNA, and RNA was subjected to real-time RT-PCR and normalized to \( \beta \)-actin using the \( \Delta \text{Ct} \) method. Data are shown as means ± SEM (\( n = 9 \); read in duplicate). \( \bar{P} P < 0.005 \) vs 21% \( \text{O}_2 \). B, FPASMCs exposed to various oxygen concentrations for 24 hours: 21% \( \text{O}_2 \)–5% \( \text{CO}_2 \) (left); 50% \( \text{O}_2 \)–5% \( \text{CO}_2 \) (middle); and 95% \( \text{O}_2 \)–5% \( \text{CO}_2 \) (right). The images are phase-contrast pictures overlaid with PDE5 immunofluorescence in red (\( > 20 \)). C, Cells were harvested for total protein and subjected to PDE5 Western blot analysis, with \( \beta \)-actin normalization. Representative Western blots are shown for PDE5 and \( \beta \)-actin in FPASMCs.

Data are shown as means ± SEM (\( n = 12 \) for 21% \( \text{O}_2 \); \( n = 9 \) for 50% \( \text{O}_2 \); \( n = 12 \) for 95% \( \text{O}_2 \)). \( \bar{P} P < 0.001 \) vs 21% \( \text{O}_2 \).
Hyperoxia Increases PDE5 Expression and Activity

Sildenafil Partially Rescues cGMP Responsiveness in FPASMCs in Hyperoxia

If increased PDE5 activity leads to decreased cGMP responsiveness in hyperoxia-exposed FPASMCs, these effects should be blocked by a PDE5 inhibitor, sildenafil. FPASMCs were exposed to 21% O2–5% CO2 or 95% O2–5% CO2 for 24 hours with or without sildenafil (100 nmol/L). In baseline conditions, addition of sildenafil led to a significant increase in PDE5 protein expression (246±30% of control; P<0.05 versus 21% O2; Figure 4A and 4B), likely as a result of increased cGMP levels activating the PDE5 promoter, which has been described previously.24 Hyperoxia for 24 hours led to significantly increased PDE5 protein expression in FPASMCs (192±40% of control; P<0.05 versus 21% O2; Figure 4A and 4B), and in contrast to the effects of sildenafil in 21% O2, this effect is blocked with sildenafil (116±21% of control; P<0.05 versus 95% O2; Figure 4A and 4B).

As expected, sildenafil significantly blocks PDE5 activity in both 21% O2 and 95% O2 (P<0.05 versus untreated cells; Figure 4C). As in Figure 1, treatment with exogenous NO in normoxia led to a significant increase in cGMP that was blunted in the context of hyperoxia (P<0.05 for 21% O2+NO versus 95% O2+NO; Figure 4D). As expected, treatment with sildenafil led to a significant increase in cGMP levels at baseline in both 21% O2 and 95% O2 (P<0.05 versus untreated cells; Figure 4D). Treatment of cells with exogenous NO and sildenafil led to a marked increase in cGMP in 21% O2 (46.2±18.2-fold relative to 21% O2; P<0.001 versus 21% O2; Figure 4D) and in 95% O2 (26.2±8.8-fold relative to 95% O2; P<0.001 versus 95% O2; Figure 4D), but the 95% O2 cGMP levels were still significantly less than those seen in 21% O2 (P<0.01; Figure 4D). Therefore, sildenafil is sufficient to partially rescue the effects of exogenous NO on FPASMC cGMP levels in hyperoxia.

Reactive Oxygen Species Increase PDE5 Expression and Activity in FPASMCs

The effects of hyperoxia are likely mediated via reactive oxygen species (ROS), and others have shown that ROS may play an important role in the underlying pathophysiology of PPHN.25–27 We used a ratiometric fluorescent ROS-sensitive probe, Ro-GFP, to measure the oxidative state of the cell, using dual-laser flow cytometry.28 Exposure of FPASMCs to hyperoxia for 24 hours significantly increased the basal oxidation state of cytosolic proteins within the cell, as measured by the percentage maximal oxidation of the Ro-GFP probe (18.8±4.6% in 21% O2–5% CO2 versus 33.8±7.9% in 95% O2–5% CO2; P<0.05; Figure 5A), without increasing cell death (supplemental Figure II). Thus, ROS likely represents a critical mediator for the hyperoxia effects on PDE5 expression and activity in FPASMCs.

We next sought to determine whether treatment of FPASMCs with exogenous oxidants in the context of normoxia was sufficient to replicate the hyperoxia effects on PDE5 expression, phosphorylation, and activity. Treatment of FPASMCs with a single dose of H2O2 (50 μmol/L) led to a significant increase in PDE5 protein expression (311±87% (n=5; read in triplicate). *P<0.05 vs untreated cells, +P<0.05 vs 95% O2, #P<0.05 vs comparably treated 21% O2 cells.

Figure 3. Hyperoxia increases PDE5 activity in FPASMCs. FPASMCs were exposed to 21% O2–5% CO2, 50% O2–5% CO2, or 95% O2–5% CO2 for 24 hours, and total protein was harvested. PDE5 specific activity was measured as the sildenafil-inhibitable fraction of total cGMP hydrolysis, normalized for total milligrams of protein. Data are shown as means±SEM (n=8; read in duplicate). *P<0.05 vs 21% O2, #P<0.05 vs 50% O2.

Figure 4. Sildenafil blocks hyperoxia-induced PDE5 expression and activity and partially restores cGMP responsiveness in FPASMCs. FPASMCs were exposed to 21% O2–5% CO2 or 95% O2–5% CO2 for 24 hours with or without sildenafil (100 nmol/L). A, Cells were harvested for total protein and subjected to PDE5 Western blot analysis, with β-actin normalization. Data are shown as means±SEM (n=14). *P<0.05 vs 21% O2, #P<0.05 vs 95% O2. B, Representative Western blots are shown for PDE5 and β-actin in FPASMCs. C, PDE5-specific activity was measured as the sildenafil-inhibitable fraction of total cGMP hydrolysis, normalized for total milligrams of protein. Data are shown as means±SEM (n=12; read in duplicate). *P<0.05 vs 21% O2, #P<0.05 vs 95% O2. D, Cells were assayed for cGMP by enzyme-linked immunoassay, and cGMP was normalized for milligrams of total protein. Data are shown as means±SEM (n=8; read in duplicate). *P<0.05 vs untreated cells, +P<0.05 vs 95% O2, #P<0.05 vs comparably treated 21% O2 cells.
versus untreated; \( P < 0.05 \); Figure 5B and 5C), PDE5 phosphorylation (supplemental Figure III), and PDE5 activity (163 ± 32% versus untreated; \( P < 0.05 \); Figure 5D). Similarly, pretreatment with sildenafil significantly blocked PDE5 activity in both untreated and H2O2-treated FPASMCs (supplemental Figure IV). These data strongly suggest that ROS, in general, and H2O2, in particular, are sufficient to induce effects similar to hyperoxia on PDE5 expression and activity.

**NAC Rescues cGMP Responsiveness in FPASMCs in Hyperoxia**

The hypothesis that increased ROS leads to decreased cGMP responsiveness in FPASMCs exposed to hyperoxia would suggest that these effects can be blocked by an antioxidant, NAC. FPASMCs were exposed to 21% \( O_2 \)–5% \( CO_2 \) or 95% \( O_2 \)–5% \( CO_2 \) for 24 hours with or without NAC (500 \( \mu \)mol/L). Hyperoxia for 24 hours led to significantly increased PDE5 protein expression in FPASMCs (304 ± 100% of control; \( P < 0.01 \) versus 21% \( O_2 \); Figure 6A and 6B), and this effect is completely blocked with NAC (106 ± 46% of control; \( P < 0.01 \) versus 95% \( O_2 \); Figure 6A and 6B).

Furthermore, NAC significantly blocks PDE5 activity in 95% \( O_2 \) (\( P < 0.05 \) versus untreated cells; Figure 6C). Treatment with exogenous NO in normoxia led to a significant increase in cGMP levels that was blunted in the context of hyperoxia (\( P < 0.01 \) for 21% \( O_2 \) + NO versus 95% \( O_2 \) + NO; Figure 6D). Treatment of cells with exogenous NO and NAC in 95% \( O_2 \) restored normal cGMP responsiveness relative to 95% \( O_2 \) with exogenous NO alone (\( P < 0.05 \) versus 95% \( O_2 \) + NO; Figure 6D). Therefore, NAC, an antioxidant, is
sufficient to rescue the effects of exogenous NO on cGMP levels in FPASMCs in hyperoxia.

**Ventilation of Healthy Neonatal Sheep With 100% O2 for Twenty-Four Hours Increases PDE5 Expression and Activity**

To verify that the effects of hyperoxia on PDE5 were not confined to isolated FPASMCs, we mechanically ventilated healthy near-term sheep with 100% O2 for 24 hours and then euthanized the lambs to harvest lung and resistance PA tissue. Ventilation with 100% O2 for 24 hours led to increased PDE5 protein expression in resistance PAs as compared with both control nonventilated fetal lambs and 1-day nonventilated lambs (1.8±0.4-fold versus fetal lambs; 3.2±0.7-fold versus 1-day lambs; \(P<0.05\); Figure 7A). There was a trend toward decreased PDE5 protein expression in 1-day lambs versus fetal lambs, but this did not reach statistical significance. Similarly, ventilation with 100% O2 for 24 hours significantly increased lung PDE5 activity relative to fetal and 1-day lambs (3.9±0.9-fold versus fetal lambs; 2.6±0.6-fold versus 1-day lambs; \(P<0.05\); Figure 7B). Immunohistochemistry confirmed that ventilation with 100% O2 for 24 hours leads to increased PDE5 expression, which is primarily seen in the smooth muscle of the resistance PAs (Figure 7C). Thus, the increase in PDE5 expression and activity in neonatal sheep ventilated with 100% O2, confirms that our findings are physiologically valid and may explain the impaired vasoreactivity previously reported in sheep after exposure to 100% O2.7

**Discussion**

Our results demonstrate that in primary cultures of FPASMCs exposed to hyperoxia, there is decreased cGMP responsiveness to exogenous NO, which is an important therapeutic vasodilator for infants with pulmonary hypertension. We propose that this is attributable, in part, to a PDE5-mediated increase in cGMP degradation. In support of that theory, we demonstrate that exposure to hyperoxia for 24 hours increases PDE5 mRNA and protein expression, as well as phosphorylation and activity. Inhibition of the hyperoxia-induced PDE5 activity with sildenafil, a PDE5 inhibitor, was sufficient to partially rescue the cGMP response to exogenous NO, indicating that PDE5 is a critical regulator of cGMP in the context of hyperoxia.

As might be expected, exposure to hyperoxia for 24 hours leads to increased oxidative stress with the FPASMCs, as detected using a novel ratiometric fluorescent probe sensitive to changes in oxidation. This probe represents a valuable new tool to investigate the oxidation state of cells without relying on fluorescent dyes with their accompanying technical limitations. Using this tool, it is not surprising that 24 hours of hyperoxia increased the oxidation of cellular proteins. However, this oxidative stress was not sufficiently severe to cause an increase in cell death (supplemental Figure II). The FPASMCs show significant changes in cellular signaling pathways during hyperoxia, as evidenced by the increase in PDE5 expression, phosphorylation, and activity. As such, we hypothesized that ROS may serve as critical mediators in the crosstalk between oxygen and PDE5. In support of that, a single dose of exogenous H2O2 was sufficient to induce long-lasting changes in PDE5 expression, phosphorylation, and activity, which mirror those seen after exposure to hyperoxia. Similarly, the changes in PDE5 expression and activity, as well as the decreased cGMP responsiveness in hyperoxia, are all reversed with a chemical antioxidant, NAC. This confirms that ROS, in general, and H2O2, in particular, are sufficient to induce significant changes in PDE5 expression and activity, which negatively impact the pulmonary vasculature. Finally, we demonstrate that these results are recapitulated in an in vivo model, where ventilation of healthy neonatal sheep with 100% O2 for 24 hours produced an increase in PDE5 expression and activity.

PDE5 is the key enzyme for cGMP hydrolysis in the pulmonary vasculature. The observation that the response to exogenous NO in FPASMCs is diminished in hyperoxia indicates that the increase in PDE5 expression and activity is biologically significant (Figure 1). However, because silde-
It is a likely candidate for the intermediary between ROS and muscle as well as the pulmonary vasculature, confirming that is regulated by cellular redox status within aortic smooth muscle. Unlike PDE5, there is a growing body of literature that PKG activity is increased by ROS, specifically H2O2.29 Little is known regarding the impact of hyperoxia on sGC, although it is possible that changes in sGC further contribute to the decreased cGMP responsiveness to exogenous NO after exposure to hyperoxia. Similarly, atrial natriuretic polypeptide and C-type natriuretic peptide signal via their respective natriuretic peptide receptors, natriuretic peptide receptor-A and -B, to produce vasorelaxation. These receptors are coupled to particulate guanylate cyclase activity, which can thus impact cGMP concentrations and thereby affect relaxation of the PAs. Although these molecules play a role in the intact vessels and animals,50 they are not likely to influence the changes seen in the isolated fetal PASMCs because there are no adjacent endothelial cells to produce the natriuretic peptides. Thus, although other signaling mediators such as sGC may contribute, the present study demonstrates that PDE5 represents a critical factor for the regulation of intracellular cGMP levels in the context of hyperoxia.

Finally, the mechanism for the interplay among oxygen, ROS, and PDE5 is not fully understood. In corpus cavernosum, the regulation of PDE5 expression is largely cGMP dependent.31 However, this would seem an unlikely mediator between oxygen and PDE5, given our finding of impaired cGMP signaling in FPASMCs under hyperoxic conditions. Promoter analysis shows both an Sp-1 and an activator protein-1 site in the human PDE5 promoter region.31 Both of these transcription factors have previously been shown to be redox sensitive and may represent potential downstream targets of the ROS-mediated signaling that impacts PDE5.32 In contrast, studies of PDE5 activity in gastric and aortic smooth muscle demonstrate that increased cGMP causes activation of PKG, which in turn phosphorylates and activates PDE5.21,22 Consistent with these data, we demonstrate that oxygen leads to increased PDE5 phosphorylation, using an antibody raised to a previously characterized PKG site (supplemental Figure I) and increased PDE5 activity (Figure 8), suggesting that a likely mechanism for PDE5 activation by oxygen is via PKG-mediated phosphorylation (Figure 8). Unlike PDE5, there is a growing body of literature that PKG is regulated by cellular redox status within aortic smooth muscle as well as the pulmonary vasculature, confirming that it is a likely candidate for the intermediary between ROS and PDE5.33,34

In conclusion, we have demonstrated that the ability of FPASMCs to respond to exogenous NO, a critical therapeutic vasodilator, is impaired when PASMCs are exposed to hyperoxia for 24 hours. We have further demonstrated that hyperoxia is sufficient to induce PDE5 expression, phosphorylation, and activity in FPASMCs, which is likely a critical mediator of impaired cGMP responsiveness under these conditions. These findings are novel, because they represent the first description of redox regulation of PDE5. PDE5 appears to be exquisitely sensitive to redox regulation, because even a short burst of oxidative stress such as a single dose of H2O2 is sufficient to produce changes in PDE5. These studies provide key insights into the basic regulation of PDE5 in the pulmonary vasculature and its crosstalk with oxygen, as well as provide a potential mechanism for the impaired vasoreactivity seen in the oxygen-exposed pulmonary vasculature.

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Disclosures
None.

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EXPANDED MATERIALS AND METHODS:

**Cell Culture:** Primary cultures of fetal pulmonary artery smooth muscle cells (FPASMC) were prepared from intrapulmonary arteries isolated from 136-day-old fetal lambs. Adventitia was gently removed under sterile conditions, and the vessel was washed in sterile phosphate-buffered saline (PBS; Mediatech, Herndon, VA). The vessel was longitudinally cut and placed endothelial side down in a sterile Petri dish in a pool of M199 media containing collagenase type A at 37°C for 15 minutes. The endothelial surface of the vessel was gently scraped with a cell scraper, and any residual adventitia was removed. After washing with PBS, the artery was minced into small (1-2mm) pieces and placed into M199 media with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), antibiotics (Mediatech), and glutamine supplements in a 37°C, 95% air-5% CO₂ humidified incubator. Cells were maintained in culture until they reached confluence. FPASMC identity was confirmed by immunostaining with antibodies against alpha smooth muscle actin, calponin, caldesmon, and desmin. These studies demonstrated an absence of contamination with fibroblasts or endothelial cells.

All cultures were maintained in DMEM supplemented with 1 gm/L glucose (Mediatech), 10% FBS (Hyclone), antibiotics (Mediatech) and antimycotics (Mediatech) at 37°C in a humidified atmosphere with 5% CO₂-95% air. Cells were synchronized prior to experiments by transfer to serum-free DMEM with antibiotics and antimycotics twelve hours prior to the start of the experiment. All experiments were carried out using 500,000 cells per condition at between passage 3 and 8.

**Cyclic GMP enzyme-linked immunoassay:** Treated cells were lysed with 0.1N HCl, and extracts were dried and resuspended in EIA buffer (Cayman Chemical). Sample
purification and acetylation was performed according to the manufacturer’s protocol (Cayman Chemical). cGMP content of acetylated samples was measured by EIA in triplicate, relative to an acetylated cGMP standard curve, using a Labsystems Multiskan EX automated plate reader (Thermo Electron, Milford, MA) at 420 nm wavelength. Total protein in the acetylated sample was quantified using the Bradford assay \(^1\). Results are shown as pmol cGMP per mg total protein.

**Real-time PCR Primer Sets:** PDE5 Primers for real-time PCR were designed using Beacon Designer software (Premier Biosoft International, Palo Alto, CA) and were as follows: PDE5 sense 5’-GCAAGGAAGGTATCAGAGG-3’, PDE5 antisense 5’-ACAACAATGGGTCTAAGAGG-3’, ß-actin sense 5’-AACTACCTTCAACTCCATC-3’, ß-actin antisense 5’-TGATCTTGATCTTCATTGTG-3’. For both the PDE5 and ß-actin primers, there was a single product on melt-curve analysis and good correlation for efficiency and standard curves (\(r^2 \geq 0.98\)). PCR product size was verified by agarose gel electrophoresis, and all samples were analyzed in duplicate. For each reaction, negative controls containing reaction mix and primers without cDNA were performed to verify that primers and reaction mixtures were free of template contamination.

**Western Blot Conditions:** FPASMC were harvested using 1X Mg-lysis buffer (Upstate, Charlottesville, VA) supplemented with a protease inhibitor cocktail (Sigma) and a phosphatase inhibitor cocktail (EMD Biosciences, San Diego, CA). Cell extracts were sonicated, and protein concentration was determined using the Bradford assay \(^1\). Total cell protein (40 µg) was separated on a 4-20% SDS-polyacrylamide gel (Biorad), and the protein was then transferred from the gel to a nitrocellulose membrane (Amersham, Arlington Heights, IL). Membranes were blocked with 5% nonfat dry milk
in Tris-buffered saline containing 0.1% Tween 20 (1X TBST) for one hour at room temperature. Membranes were then incubated with primary antibody at 4°C overnight at the appropriate dilution in 5% milk in 1XTBST (1:333 for PDE5 [BD Transduction, San Jose, CA], 1:500 for PhosphoPDE5 [Fabgennix, Frisco, TX], 1:2000 for ß-actin [Sigma]). After being washed, the membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) at a 1:1000 dilution in 5% milk + 1XTBST. Membranes were then washed, and the bands were visualized and quantitated via chemiluminescence (Pierce, Rockford, IL), using a Digital Science Image Station (Kodak, Rochester, NY). Expression for PDE5 and PhosphoPDE5 was normalized to ß-actin expression.

**Immunocytochemistry:** FPASMC were plated onto collagen-treated glass coverslips and treated with 21%O2-5%CO2 versus 95%O2-5%CO2 or 21% O2 versus 21% O2+H2O2. Twenty four hours after initiation of treatment, cells on coverslips were fixed with 4% formaldehyde (Fisher, Hampton, NH), permeabilized with 0.2% Triton-X (Fisher), and stained for immunocytochemistry with either anti-PDE5 or anti-phosphoPDE5 antibodies at a 1:50 dilution in 5% bovine serum albumin (BSA; Sigma). Utilizing an Alexa Fluor 488 anti-mouse antibody (Molecular Probes/Invitrogen) or a rhodamine-red labeled anti-rabbit antibody (Molecular Probes/Invitrogen) at a 1:200 dilution in 5%BSA and a Nikon Eclipse TE-300 fluorescent microscope, the intracellular localization and expression of PDE5 or phosphoPDE5 at baseline and in response to exposure to hyperoxia or H2O2 was visualized by fluorescence microscopy with excitation at 495 nm and emission at 519 nm for Alexa Fluor 488 and with excitation at 518 nm and emission at 605 nm for rhodamine red. Fluorescent images were captured
with a CoolSnap digital camera, and the average fluorescent intensities were quantified
with Metamorph imaging software (Fryer, Huntley, IL). All images are set to the same
threshold level, and average fluorescent intensity is calculated as the total fluorescent
intensity per field divided by the number of pixels per field, thereby correcting for
differences in cell number per field 2.

_**PDE5 Activity Assay:**_ After treatment, FPASMC were harvested for total protein
in lysis buffer supplemented with protease and phosphatase inhibitors as described above.
FPASMC samples were immediately placed on ice and assayed the same day. For the
ventilated sheep lung tissue, samples were snap-frozen at the time of harvest from the
animal. Protein was prepared fresh from snap-frozen tissue using the commercially
available PARIS kit (Ambion) with the lysis buffer supplemented with protease and
phosphatase inhibitors. The total lung protein was immediately placed on ice and assayed
the same day. The protein was purified over a Centri-Spin 10 column to remove any
phosphate contamination (Princeton Separations, Adelphia, NJ), since the assay
measurement is dependent on free phosphate. Protein concentration was determined as
described above. Total protein (5 µg) was assayed for cGMP hydrolytic activity using a
commercially available colorimetric cyclic nucleotide phosphodiesterase assay kit
(Biomol, Plymouth Meeting, PA). The basis for the assay is the cleavage of cGMP by a
cyclic nucleotide phosphodiesterase, such as PDE5, followed by the release of free
phosphate by a 5’ nucleotidase from *Crotalus atrox* venom. The free phosphate is
quantified using the Biomol Green reagent (Biomol) in a modified Malachite Green assay
3,4. The reactions are mixed in a 96-well plate, and reactions are started in a timed fashion
by the addition of the 5’-nucleotidase (Biomol). Each sample is read in four wells – two
without sildenafil and two with sildenafil (100 nM), to determine PDE5 specific cGMP-hydrolytic activity. The samples are then incubated at 30°C for 30 minutes and stopped in a timed fashion by the addition of the Biomol Green reagent (Biomol). The samples are incubated with the Biomol Green reagent with shaking at room temperature for 20 minutes. Results were measured using a Labsystems Multiskan EX automated plate reader at 620 nM wavelength and compared to a standard curve generated with GMP and 5’-nucleotidase. Reaction incubation times, input total protein, and sildenafil dose were all optimized with control FPASMC protein prior to proceeding with test samples. The difference between the pmol cGMP hydrolyzed per mg total protein per minute without sildenafil and the pmol cGMP hydrolyzed per mg total protein per minute with sildenafil represents the PDE5-specific cGMP-hydrolytic activity. Results are shown as the PDE5-specific pmol cGMP hydrolyzed per mg total protein per minute for each sample.

**Cell proliferation and Cell death Assays:** For cell proliferation assays, FPASMC were seeded onto 96 well plates and were treated as described above. After treatment, the cells were returned to normoxia, and cell proliferation was determined using the Cell Titer 96 AQncous One Solution kit (Promega, Madison, WI), measuring the absorbance at 490 nm using a Labsystems Multiskan EX automated plate reader according to the manufacturer’s instructions.

To measure cell death after hyperoxia treatment as described above, FPASMC were returned to normoxia, and cell death was determined using a commercially available LDH cytotoxicity assay (Biovision, Mountain View, CA), measuring the absorbance at 500 nm using a Labsystems Multiskan EX automated plate reader according to the manufacturer’s protocol. Propidium iodide (PI, Sigma) is a fluorochrome that has been
used previously to assess cell viability. PI is excluded from viable cells, but binds to chromatin after the loss of cell membrane integrity, becoming highly fluorescent (excitation at 540 nm, emission at 590 nm). FPASMC were loaded with PI (5 µM) for 10 minutes after hyperoxia treatment. Measurement was done for three random fields on each well, and readings were averaged. After reading, all cells were permeabilized with digitonin (300 µM, Calbiochem, San Diego, CA) for 10 minutes. Viability (% cell death) was calculated as PI fluorescence relative to maximal fluorescence after digitonin exposure. For both death assays, a separate well of FPASMC was treated in 21%O2-5%CO2 with staurosporine (10 µM, Sigma), as a positive control for cell death.

**Detection of Reactive Oxygen Species (ROS):** FPASMC in serum-free DMEM without phenol red and with antibiotics and antifungicotics were infected in 60 mm culture dishes with 100 PFU/cell of a RoGFP adenoviral construct. RoGFP is a previously characterized ratiometric fluorescent probe sensitive to cellular oxidative stress. Twelve hours after infection, cells were returned to normal culture medium, and forty-eight hours after infection, the FPASMC were exposed to 21%O2-5%CO2 or 95%O2-5%CO2 for 24 hours. After exposure, FPASMC were lifted from the plates, and divided into three aliquots. The first aliquot was used to assess redox status while the other two were used to calibrate the probe. One aliquot was fully reduced with dithiothreitol (1 mM, Sigma), while the second was fully oxidized using T-butyl hydroperoxide (1 mM, Sigma). All aliquots were analyzed with a DakoCytomation CyAn multilaser flow cytometer using 400 nm and 485 nm excitation wavelengths, while emission was assessed at 535 nm. An uninfected control was also assessed in order to provide a signal for background subtraction. For each condition, the cysteine thiol redox status was calculated as percent
oxidized, by comparison to the values obtained for the fully reduced and fully oxidized conditions.

**Ventilation Protocols for Neonatal Sheep:** The Laboratory Animal Care Committees at the State University of New York at Buffalo and at Northwestern University approved this study. Near term gestation pregnant ewes (134 days gestation) were obtained from the Swartz family farm (Attica, NY). After 12 h of fasting, ewes were anesthetized with Pentothal and halothane, and lambs were delivered by cesarean section (n=5). Lambs were placed under servo-controlled radiant warmers, intubated, given calfactant 3 mL/kg (Infasurf, ONY Inc., Amherst, NY), and ventilated with Servo 300 ventilators (Siemens, Mississauga, ON, Canada) with the following settings: positive end-expiratory pressure, 4 cm of water; rate, 60/min; peak inspiratory pressure (PIP), approximately 25 cm of water (adjusted to deliver 10 mL/kg tidal volume using a BiCore CP-100 Monitor, BiCore Monitoring systems, Irvine, CA), and FiO2 1.0. Umbilical arterial and venous lines were placed, and ventilator settings were adjusted to maintain paCO2 between 35 and 50 mm Hg. Intravenous (IV) fluids (dextrose 10% solution with 25 mEq of sodium chloride, 20 mEq of potassium chloride and 10 mEq of sodium bicarbonate per liter) were administered continuously at 100 mL/kg/d. Fluid composition and rate was adjusted based on serum electrolyte values. Lambs were sedated with fentanyl (2 µg/kg/dose IV every 2 hours as needed) based on agitation and paralyzed with pancuronium bromide (0.1 mg/kg/dose IV every 4 hours as needed) for vigorous spontaneous movement. Arterial blood gases were monitored every 5–15 minutes during initial stabilization. Thoracotomy and instrumentation of the PA were not performed and every attempt was made to simulate management in a neonatal intensive care unit. A
neonatologist, neonatal fellow, neonatal nurse practitioner, or neonatal nurses from the Women and Children’s Hospital of Buffalo provided continuous care for the lambs.

**Immunohistochemistry:** The lambs were euthanized according to institutional policies formulated by the IACUC. A cardiac puncture was performed to exsanguinate the animal, the chest was opened, and the heart and lungs were removed *en bloc*. The right middle lobe of the lung was removed and a suction catheter (7-10 french) was inserted into the bronchus and tied securely. OCT compound was pushed gently into the deflated lobe until it expanded to normal air filled dimensions. The catheter was removed, and the tie was well secured around the lobe base so the OCT remained in the lung. The lobe was wrapped and placed in ice for 15-20 minutes to solidify the OCT. Blocks were prepared by cutting the cold lung into 3-4 mm slices and quickly freezing each slice as a cross section in additional OCT. The blocks were cut into 8-10 micron sections that were mounted onto charged slides for staining.

Sections were subsequently fixed with acetone (Sigma) for 10 minutes at 4°C, allowed to air dry, and then washed for 5 minutes with 1XPBS (Mediatech). Sections were blocked with 5% BSA at room temperature for one hour and then stained overnight at 4°C for immunohistochemistry with a commercially available anti-PDE5 antibody (BD Transduction) at a 1:50 dilution in 5% BSA. Utilizing an Alexa Fluor 488 anti-mouse antibody (Molecular Probes/Invitrogen) at a 1:200 dilution in 5% BSA and a Nikon Eclipse TE-300 fluorescent microscope, the tissue localization and expression of at baseline and in response to ventilation with 100% O₂ was visualized by fluorescence microscopy with excitation at 495 nm and emission at 519 nm for Alexa Fluor 488.
RESULTS:

Hyperoxia Increases PDE5 Phosphorylation in FPASMC:

The hypothesis that increased PDE5 expression leads to decreased cGMP responsiveness in FPASMC exposed to hyperoxia is only valid if increased PDE5 expression correlates with increased activity. One of the key events required for PDE5 activity is cGMP binding directly to PDE5, followed by phosphorylation by PKG\(^9\)-\(^{11}\). We sought to determine if FPASMC exposure to hyperoxia resulted in the required PKG-mediated phosphorylation necessary for increased PDE5 activity. In order to do this, we utilized a commercially available phospho-PDE5 antibody that was raised to bovine serine 92, which has been shown to be phosphorylated by PKG and is conserved across multiple species\(^{10,12}\). Hyperoxia for 24 hours led to significantly increased PDE5 phosphorylation in FPASMC by Western blot (457±205%, \(p<0.05\), Online Data Supplement Figures 1A and 1B) and by immunocytochemistry (224±24%, \(p<0.05\), Online Data Supplement Figures 1C and 1D). FPASMC exposure to hyperoxia also significantly increased PDE5 activity in FPASMC as shown in Figure 3. Therefore, the hyperoxia-induced increases in PDE5 protein expression correlate with increased PDE5 phosphorylation and activity, suggesting that increased PDE5 expression and activity contribute to decreased intracellular cGMP after exposure to exogenous NO under hyperoxic conditions.

Hyperoxia Does Not Increase FPASMC Death:

While others have reported that PASMC can proliferate in hyperoxia\(^{13,14}\), lung epithelial cells undergo apoptosis in response to hyperoxia\(^{15}\). In FPASMC, a colorimetric cell proliferation assay demonstrated that 24 hours of hyperoxia mildly decreased basal
Hyperoxia Increases PDE5 Expression and Activity

FPASMC proliferation (72 ± 4% of the normoxia-exposed cells, p<0.05, Online Data Supplement Figure 2A). However, utilizing either an LDH cytotoxicity assay (Online Date Supplement Figure 2B) or propidium iodide staining (Online Date Supplement Figure 2C and 2D), there was no significant difference in cell death between the normoxia- and hyperoxia-exposed FPASMC. Therefore, the decreased cGMP responsiveness to exogenous NO in FPASMC exposed to hyperoxia cannot be attributed to cell death, and hyperoxia is not inducing a cell death pathway.

H$_2$O$_2$ Increases PDE5 Phosphorylation in FPASMC:

We sought to determine if treatment of FPASMC with exogenous oxidants in the context of normoxia was sufficient to replicate the effects of hyperoxia on PDE5 phosphorylation. Treatment of FPASMC with a single dose of H$_2$O$_2$ (50 µM) led to a significant increase in PDE5 phosphorylation by Western Blot (184±8% vs. untreated, p<0.05, Online Data Supplement Figures 3A and 3B) and immunocytochemistry (197±10% vs. untreated, p<0.05, Online Data Supplement Figures 3C and 3D). These data strongly suggest that ROS, in general, and H$_2$O$_2$ in particular, are sufficient to induce effects similar to hyperoxia on PDE5 phosphorylation, likely via a PKG-mediated mechanism.

Sildenafil Blocks H$_2$O$_2$-Induced PDE5 Activity in FPASMC:

Treatment of FPASMC with a single dose of H$_2$O$_2$ (50 µM) led to a significant increase in PDE5 activity (163±32% vs. untreated, p<0.05, Figure 5D). Furthermore, as expected, pretreatment with sildenafil significantly blocked PDE5 activity in both untreated and H$_2$O$_2$-treated FPASMC (p<0.05 vs. cells not treated with sildenafil, Online Data Supplement Figure 4). These data strongly suggest that ROS, in general, and H$_2$O$_2$
in particular, are sufficient to induce effects similar to hyperoxia on PDE5 activity.
REFERENCES:


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FIGURE LEGENDS:

**Online Figure 1 - Hyperoxia Induces PDE5 Protein Phosphorylation in FPASMC.**

FPASMC were exposed to 21%O₂-5%CO₂ or 95%O₂-5%CO₂ for 24 hours. A) Cells were harvested for total protein and subjected to Western Blot analysis for phosphoPDE5, with β-actin normalization. Data are shown as mean±SEM (n=7, *p<0.05 vs. 21% O₂). B) Representative Western blots are shown for phosphoPDE5 and β-actin in FPASMC. C) FPASMC were plated on cover slips and exposed to hyperoxia as previously described. Cells were stained for PhosphoPDE5 and imaged using fluorescent microscopy. Fluorescence was quantified using MetaMorph. Data are shown as mean±SEM (n=6 cover slips, *p<0.05 vs. 21% O₂). D) The left panel shows FPASMC in 21%O₂-5%CO₂, and the right panel shows FPASMC in 95%O₂-5%CO₂ for 24 hours. Images are phase contrast pictures overlaid with PhosphoPDE5 immunofluorescence in red (20X).

**Online Figure 2 – Hyperoxia Does Not Increase FPASMC Death.** FPASMC were exposed to 21%O₂-5%CO₂ or 95%O₂-5%CO₂ for 24 hours. A) Cell proliferation was assayed using a Cell Titer 96 AQueous One Solution kit, measuring the absorbance at 490 nm. Results are shown as percent relative to control 21%O₂-5%CO₂ cells ± SEM (n=6, read in 8 replicates, *p<0.05 versus control cells). B) Cytotoxicity measured by LDH release from cells. Results are shown as percent cytotoxicity ± SEM (n=6, read in duplicate, *p<0.05 versus 21% and 95% cells). C) Cell death was assessed by propidium iodide immunofluorescence. Results are shown as percent cell death ± SEM (n=6, read in triplicate, *p<0.05 versus 21% and 95% cells). D) Representative propidium iodide immunofluorescence images of FPASMC exposed to 21%O₂-5%CO₂, 95%O₂-5%CO₂, or
staurosporine (1 µM) are shown. The first column of images are phase contrast images of the cells in the various conditions, the second column of images are propidium iodide immunofluorescent images at baseline, and the third column of images are propidium iodide immunofluorescent images after cell permeabilization with digitonin (300 µM).

**Online Figure 3 - H₂O₂ Induces PDE5 Protein Phosphorylation in FPASMC.**

FPASMC were treated with a single dose of H₂O₂ (50 µM) in 21%O₂-5%CO₂. Cells were harvested 24 hours later for total protein. A) Cell protein was subjected to Western Blot analysis for phosphoPDE5, with β-actin normalization. Data are shown as mean±SEM (n=5, *p<0.05 versus 21% O₂). B) Representative Western blots are shown for phosphoPDE5 and β-actin in FPASMC. C) FPASMC were plated on cover slips and exposed to H₂O₂ as previously described. Cells were stained for PhosphoPDE5 and imaged using fluorescent microscopy. Fluorescence was quantified using MetaMorph. Data are shown as mean±SEM (n=6 cover slips, *p<0.05 versus 21% O₂). D) The left panel shows FPASMC in 21%O₂, and the right panel shows FPASMC in 21%O₂+H₂O₂ for 24 hours. Images are phase contrast pictures overlaid with PhosphoPDE5 immunofluorescence in red (40X).

**Online Figure 4 – Sildenafil Blocks H₂O₂-Induced PDE5 Activity in FPASMC.**

FPASMC were treated with a single dose of H₂O₂ (50 µM) in normoxia ± sildenafil (100 nM) and were harvested 24 hours later for total protein. PDE5 specific activity was measured as the sildenafil-inhibitable fraction of total cGMP hydrolysis, normalized for total mg of protein. Data are shown as mean±SEM (n=6, read in duplicate, *p<0.05 vs. untreated cells, #p<0.05 vs. H₂O₂-treated cells).
Online Data Supplement Figure 1:

A) Bar graph showing percent PhosphoPDE5 Protein Expression Relative to 21% O₂ Cells.

B) Western blot images comparing PhosphoPDE5 and β-Actin expression under 21% O₂ and 95% O₂ conditions.

C) Bar graph showing percent PhosphoPDE5 Immunofluorescence Relative to 21% O₂ Cells.

D) Immunofluorescence images comparing PhosphoPDE5 expression under 21% O₂ and 95% O₂ conditions.
Online Data Supplement Figure 2:

A) Percent Cell Proliferation Relative to 21% Cells

B) % Cytotoxicity by LDH

C) % Cell Death by PI

D) Images showing Phase, PI, and PI+Digitonin under different conditions.

* indicates statistically significant differences.
Online Data Supplement Figure 3:

A) Percent PhosphoPDE5 Protein Expression Relative to 21% O₂ Cells

B) PhosphoPDE5

C) Percent PhosphoPDE5 Immunofluorescence Relative to 21% O₂ Cells

D) 21% O₂ 21% O₂+H₂O₂
Online Data Supplement Figure 4:

PDE5-specific activity (pmol cGMP hydrolyzed/mg protein/min)