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Hyperoxia Increases Phosphodiesterase 5 (PDE5) Expression and Activity in Ovine Fetal Pulmonary Artery Smooth Muscle Cells

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Abstract

In the pulmonary vasculature, cGMP concentrations are regulated in part by a cGMP-dependent phosphodiesterase, 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 (FPASMC) to high levels of oxygen for 24 hours leads to decreased responsiveness to exogenous nitric oxide (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 FPASMC, hyperoxia leads to increased oxidative stress without increasing cell death. Treatment of normoxic FPASMC with H₂O₂ is sufficient to induce PDE5 expression and activity, suggesting that reactive oxygen species (ROS) mediate the effects of hyperoxia in FPASMC. 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% O₂ 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 iNO.

Keywords

pulmonary circulation; persistent pulmonary hypertension of the newborn; cyclic GMP; phosphodiesterases

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INTRODUCTION

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 live born term infants¹. 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^{2, 3}.

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% O₂ is considered a first line therapy in infants with PPHN^{1, 4}. However, data from the adult ARDS literature⁵, from the neonatal bronchopulmonary dysplasia (BPD) literature⁶, and from the neonatal resuscitation literature^{7, 8} 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 eleven 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^{9, 10}. In the fetal lung, PDE5 activity and expression is highest in pulmonary arteries. 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 birth¹³, 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 two hours^{14, 15}. There is little 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 norepinephrine⁷. Additionally, in a hyperoxic rat model of BPD, 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 hyperoxia¹⁸. 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 iNO resistance and rebound pulmonary hypertension after iNO 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 FPASMC were prepared from intrapulmonary arteries as described in the expanded materials and methods section. FPASMC were treated in incubators with 21%

O₂-5% CO₂, 50% O₂-5% CO₂, or 95% O₂-5% CO₂ ± DETANONOate (100 μM; Cayman Chemical, Ann Arbor, MI), ± sildenafil (100 nM, provided by Dr. Sharron Francis and Dr. Jackie Corbin), ± N-acetylcysteine (NAC, 500 μM, Sigma, St. Louis, MO); or with 21% O₂-5% CO₂ ± a single dose of H₂O₂ (50 μM; Sigma). FPASMC were harvested for analysis after 24 hours.

Cyclic GMP enzyme-linked immunoassay

Treated cells were lysed, and cGMP content was measured by EIA in triplicate according to the manufacturer's protocol and as described in the expanded materials and methods (Cayman Chemical). Results are shown as pmol cGMP per mg total protein.

Real-time PCR

FPASMC were harvested for RNA utilizing the Aurum Total RNA Mini Kit (Biorad, Hercules, CA), and RNA was quantified using the Quant-it RiboGreen assay (Molecular Probes/Invitrogen, Carlsbad, CA). cDNA was prepared from total RNA utilizing the iScript cDNA Synthesis Kit (BioRad). Real-time PCR was performed using the iQ SYBR Green Supermix (BioRad) with the iCycler iQ real-time PCR detection system (BioRad) 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. Relative PDE5 amounts were normalized to β-actin expression using the ΔΔCT method¹⁹.

Western Blot

After treatment, FPASMC were harvested for total protein (40 μg) which was subjected to Western blot analysis as described in the expanded materials and methods section utilizing commercially available antibodies: PDE5 (BD Transduction, San Jose, CA) and β-actin (Sigma). Bands were visualized via chemiluminescence (Pierce, Rockford, IL), using a Digital Science Image Station (Kodak, Rochester, NY). Expression for PDE5 was normalized to β-actin expression.

Immunocytochemistry

FPASMC were plated onto collagen-treated glass coverslips and treated as above. After treatment, cells were fixed, permeabilized, and stained for immunocytochemistry with anti-PDE5 utilized in conjunction with a fluorescent secondary antibody (Molecular Probes/Invitrogen) as described in the expanded materials and methods section.

PDE5 Activity Assay

After treatment, FPASMC were harvested for total protein (5 μg), which was assayed for cGMP hydrolytic activity using a commercially available colorimetric cyclic nucleotide phosphodiesterase assay kit (Biomol, Plymouth Meeting, PA) as described in the expanded materials and methods section. Assays were performed with and without sildenafil (100 nM) to determine PDE5-specific cGMP-hydrolytic activity. Results are shown as PDE5-specific pmol cGMP hydrolyzed per mg total protein per minute.

Detection of Reactive Oxygen Species (ROS)

FPASMC were infected with 100 PFU/cell of a RoGFP adenoviral construct as described in the expanded materials and methods. RoGFP is a previously characterized ratiometric fluorescent probe sensitive to cellular oxidative stress²⁰. 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 cells²⁰. Ro-GFP infected FPASMC were exposed to 21% O₂-5% CO₂ or 95% O₂-5% CO₂ for 24 hours, and subsequently, their oxidative status was analyzed using multilaser flow cytometry as described in the expanded materials and methods.

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% O₂ for 24 hours as described in the expanded materials and methods section. Control groups included newborn lambs sacrificed approximately 24 hours after spontaneous delivery (one day nonventilated, n = 4) and fetal lambs delivered and sacrificed at 136 d 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 pulmonary arteries (inner diameters of 500 μm) were dissected and isolated⁷. Total protein was isolated from lung and pulmonary artery (PA) tissue using the commercially available PARIS kit (Ambion, Austin, TX). 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 blocs were snap frozen, cut into thin sections (8 microns), and collected on glass slides. Sections were fixed with acetone and stained with anti-PDE5, in conjunction with a fluorescent secondary antibody (Molecular Probes/Invitrogen) as described in the expanded materials and methods section.

Statistics

Data are presented as mean ± SEM. Data were analyzed by paired t-test or one-way repeated measures ANOVA with Bonferroni's multiple comparison test where appropriate, using Prism (GraphPad Software Inc., San Diego, CA). P<0.05 was considered statistically significant.

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 donor⁷, we sought to determine the effect of hyperoxia on cGMP levels in FPASMC from resistance PA. FPASMC were exposed to 21% O₂-5% CO₂, 50% O₂-5% CO₂, or 95% O₂-5% CO₂ ± DETANONOate (100 μM), a long-acting NO donor, for 24 hours (Figure 1). Treatment with exogenous NO in normoxia led to a significant increase in cGMP levels (446 ±64% of control, p<0.001). However, treatment with exogenous NO in the context of hyperoxia blunted the increase in cGMP levels (p<0.001 for 21% O₂+NO vs. 50% O₂+NO and 21% O₂+NO vs. 95% O₂+NO). Thus, exposure to hyperoxia for 24 hours makes the FPASMC less responsive to exogenous NO, the primary clinical therapy for PPHN.

Hyperoxia Increases PDE5 Expression in FPASMC

We hypothesized that the blunting of NO-mediated cGMP responsiveness in hyperoxia represents a PDE5-mediated desensitization. FPASMC were exposed to 21% O₂-5% CO₂, 50% O₂-5% CO₂, or 95% O₂-5% CO₂ for 24 hours. FPASMC 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% O₂; 185±33% of control in 95% O₂; p<0.001, Figures 2C and 2D). Thus, FPASMC exposure to hyperoxia for 24 hours leads to increased PDE5 mRNA and protein expression.

Hyperoxia Increases PDE5 Activity in FPASMC in a Dose-Dependent Fashion

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^{21–23}. Hyperoxia for 24 hours led to significantly increased PDE5 phosphorylation in FPASMC (Online Data Supplement Figure 1). Similarly, hyperoxia for 24 hours led to significantly increased PDE5 activity in FPASMC (161±26% of control in 50% O₂; 309±94% of control in 95% O₂, p<0.05, Figure 3). Furthermore, the increase in PDE5 activity in 95% O₂-5% CO₂ is significantly greater than that seen in 50% O₂-5% CO₂ (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.

Sildenafil Partially Rescues cGMP Responsiveness in FPASMC in Hyperoxia

If increased PDE5 activity leads to decreased cGMP responsiveness in FPASMC hyperoxia-exposed, these effects should be blocked by a PDE5 inhibitor, sildenafil. FPASMC were exposed to 21% O₂-5% CO₂ or 95% O₂-5% CO₂ for 24 hours ± sildenafil (100 nM). In baseline conditions, addition of sildenafil led to a significant increase in PDE5 protein expression (246±30% of control, p<0.05 vs. 21% O₂, Figure 4A and B), likely as a result of increased cGMP levels activating the PDE5 promoter, which has been previously described²⁴. Hyperoxia for 24 hours led to significantly increased PDE5 protein expression in FPASMC (192±40% of control, p<0.05 vs. 21% O₂, Figures 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 vs. 95% O₂, Figures 4A and 4B).

As expected, sildenafil significantly blocks PDE5 activity in both 21% O₂ and 95% O₂ (p<0.05 vs. 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 vs. 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 vs. 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 vs. 21% O₂, Figure 4D) and in 95% O₂ (26.2±8.8-fold relative to 95% O₂, p<0.001 vs. 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.

ROS Increase PDE5 Expression and Activity in FPASMC

The effects of hyperoxia are likely mediated via ROS, and others have shown that ROS may play an important role in the underlying pathophysiology of PPHN^{25–27}. We utilized a ratiometric fluorescent ROS-sensitive probe, Ro-GFP, to measure the oxidative state of the cell, using dual laser flow cytometry²⁰. Exposure of FPASMC to hyperoxia for 24 hours significantly increased the basal oxidation state of cytosolic proteins within the cell, as measured by the percent maximal oxidation of the Ro-GFP probe (18.8±4.6% in 21% O₂-5% CO₂ vs. 33.8±7.9% in 95% O₂-5% CO₂, p<0.05, Figure 5A), without increasing cell death (Online Data Supplement Figure 2). Thus, ROS likely represents a critical mediator for the hyperoxia effects on PDE5 expression and activity in FPASMC.

We next sought to determine if treatment of FPASMC with exogenous oxidants in the context of normoxia was sufficient to replicate the hyperoxia effects on PDE5 expression, phosphorylation, and activity. Treatment of FPASMC with a single dose of H₂O₂ (50 μM) led to a significant increase in PDE5 protein expression (311 ± 87% vs. untreated, p<0.05, Figures 5B and 5C), PDE5 phosphorylation (Online Data Supplement Figure 3), and PDE5 activity (163±32% vs. untreated, p<0.05, Figure 5D). Similarly, pretreatment with sildenafil significantly blocked PDE5 activity in both untreated and H₂O₂-treated FPASMC (Online Data Supplement Figure 4). 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.

N-Acetyl-Cysteine (NAC) Rescues cGMP Responsiveness in FPASMC in Hyperoxia

The hypothesis that increased ROS leads to decreased cGMP responsiveness in FPASMC exposed to hyperoxia would suggest that these effects can be blocked by an antioxidant, NAC. FPASMC were exposed to 21% O₂-5% CO₂ or 95% O₂-5% CO₂ for 24 hours ± NAC (500 μM). Hyperoxia for 24 hours led to significantly increased PDE5 protein expression in FPASMC (304±100% of control, p<0.01 vs. 21% O₂, Figures 6A and 6B), and this effect is completely blocked with NAC (106±46% of control, p<0.01 vs. 95% O₂, Figures 6A and 6B).

Furthermore, NAC significantly blocks PDE5 activity in 95% O₂ (p<0.05 vs. 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% O₂+NO vs. 95% O₂+NO, Figure 6D). Treatment of cells with exogenous NO and NAC in 95% O₂ restored normal cGMP responsiveness relative to 95% O₂ with exogenous NO alone (p<0.05 vs. 95% O₂ + NO, Figure 6D). Therefore, NAC, an antioxidant, is sufficient to rescue the effects of exogenous NO on cGMP levels in FPASMC in hyperoxia.

Ventilation of Healthy Neonatal Sheep with 100% O₂ For 24 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 sacrificed the lambs to harvest lung and resistance PA tissue. Ventilation with 100% O₂ for 24 hours led to increased PDE5 protein expression in resistance pulmonary arteries as compared to both control nonventilated fetal lambs and one-day non-ventilated lambs (1.8±0.4-fold vs. fetal lambs, 3.2±0.7-fold vs. one-day lambs, p<0.05, Figure 7A). There was a trend toward decreased PDE5 protein expression in one-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 one-day lambs (3.9±0.9-fold vs. fetal lambs, 2.6±0.6-fold vs. one-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 PA (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% O₂⁷.

DISCUSSION

Our results demonstrate that in primary cultures of FPASMC 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 due, 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 FPASMC, 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. Utilizing 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 (Online Data Supplement Figure 2). The FPASMC 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 FPASMC is diminished in hyperoxia indicates that the increase in PDE5 expression and activity is biologically significant (Figure 1). However, since sildenafil only partially rescues the cGMP response to exogenous NO (Figure 4D), other key signaling mediators may also be impacted by hyperoxia in fetal PASM. Soluble guanylyl cyclase (sGC) is the primary enzyme responsible for cGMP production. sGC protein expression is decreased in nonventilated fetal lambs with PPHN, suggesting that it may also be an important regulator of neonatal vascular tone²⁸. Recent studies suggest that sGC protein expression in FPASMC is decreased by ROS, specifically H₂O₂²⁹. 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, ANP and CNP, signal through their respective natriuretic peptide receptors, NPR-A and NPR-B, to produce vasorelaxation. These receptors are coupled to particulate guanylate cyclase (pGC) activity, which can thus impact cGMP concentrations and thereby affect relaxation of the pulmonary arteries. While these molecules play a role in the intact vessels and animals³⁰, they are not likely to influence the changes seen in the isolated fetal PASM since there are no adjacent endothelial cells to produce the natriuretic peptides. Thus, while other signaling mediators such as sGC may contribute, the present study demonstrates that PDE5 represents one critical factor for the regulation of intracellular cGMP levels in the context of hyperoxia.

Finally, the mechanism for the interplay between oxygen, ROS, and PDE5 is not fully understood. In corpus cavernosum, the regulation of PDE5 expression is largely cGMP-dependent³¹. However, this would seem an unlikely mediator between oxygen and PDE5, given our finding of impaired cGMP signaling in FPASMC under hyperoxic conditions. Promoter analysis shows both an Sp-1 and an AP-1 site in the human PDE5 promoter region³¹. Both of these transcription factors have previously been shown to be redox-sensitive and may represent potential downstream targets of the ROS-mediated signaling which impacts PDE5³². 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, utilizing an antibody raised to a previously characterized PKG site (Online Data Supplement Figure 1) and increased PDE5 activity (Figure 3), 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 the ability of FPASMC to respond to exogenous NO, a critical therapeutic vasodilator, is impaired when FPASMC are exposed to hyperoxia for 24 hours. We have further demonstrated that hyperoxia is sufficient to induce PDE5 expression, phosphorylation, and activity in FPASMC, which is likely one critical mediator of impaired cGMP responsiveness under these conditions. These findings are novel, as they represent the first description of redox-regulation of PDE5. PDE5 appears to be exquisitely sensitive to redox regulation, as 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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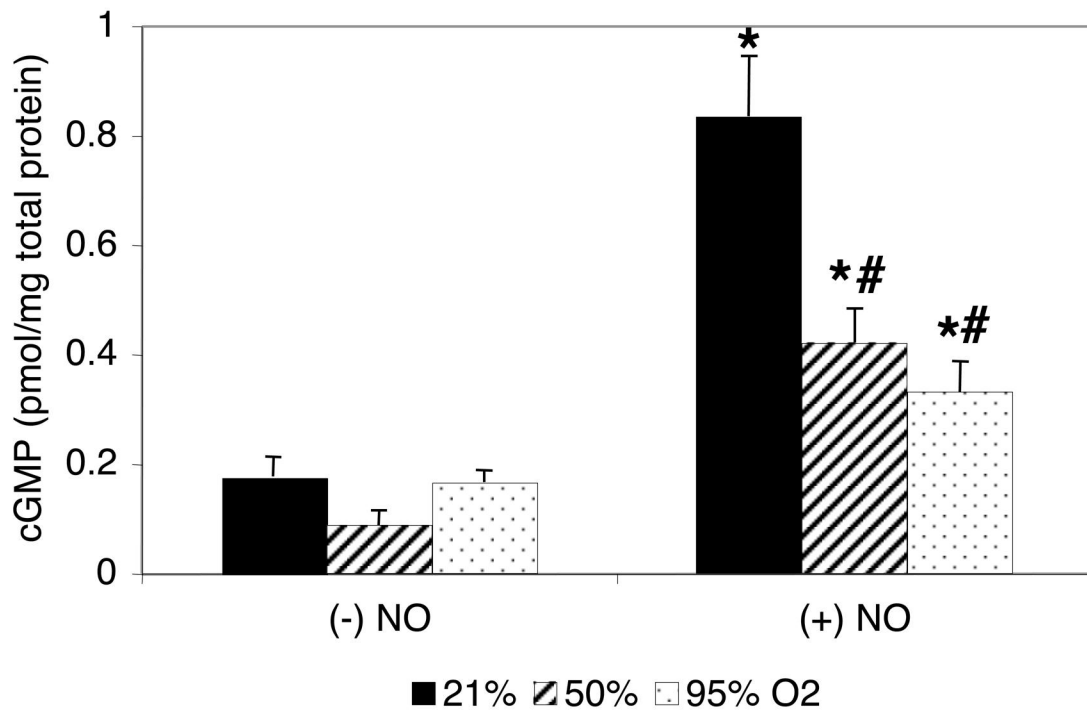


Figure 1. Exogenous NO Induces Less cGMP in FPASMC Exposed to Hyperoxia
 FPASMC were exposed to 21% O₂-5% CO₂, 50% O₂-5% CO₂, or 95% O₂-5% CO₂ ± DETANONOate (100 μM) for 24 hours. Cells were assayed for cGMP by EIA, and cGMP was normalized for mg total protein. Data are shown as mean ± SEM (n=6, read in triplicate; *p<0.05 vs. cells not treated with DETANONOate; #p<0.001 vs. 21% O₂+NO).

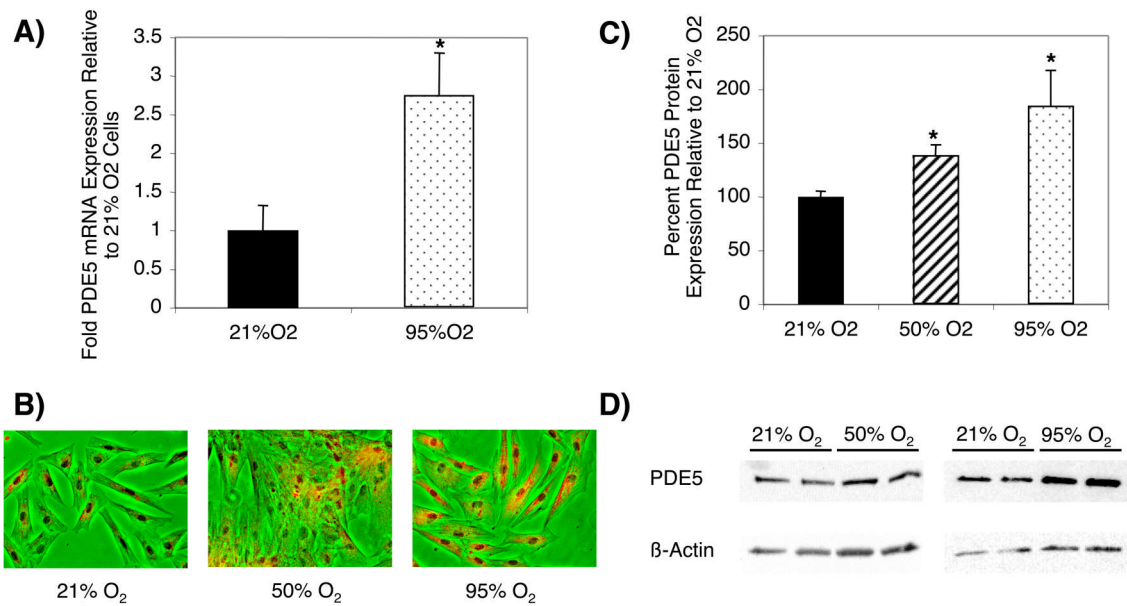


Figure 2. Hyperoxia Induces PDE5 Expression in FPASMC

FPASMC were exposed to 21% O₂-5% CO₂, 50% O₂-5% CO₂, or 95% O₂-5% CO₂ for 24 hours.

A) Cells were harvested for RNA, and RNA was subjected to real-time RT-PCR and normalized to β-actin using the ΔΔCt method. Data are shown as mean±SEM (n=9, read in duplicate, *p<0.005 vs. 21% O₂). **B)** The panels show FPASMC exposed to various oxygen concentrations for 24 hours: left - 21% O₂-5% CO₂, middle - 50% O₂-5% CO₂, right - 95% O₂-5% CO₂. Images are phase contrast pictures overlaid with PDE5 immunofluorescence in red (20X). **C)** Cells were harvested for total protein and subjected to PDE5 Western Blot analysis, with β-actin normalization. Data are shown as mean±SEM (n=12 for 21% O₂, n=9 for 50% O₂, n=12 for 95% O₂; *p<0.001 vs. 21% O₂). **D)** Representative Western blots are shown for PDE5 and β-actin in FPASMC.

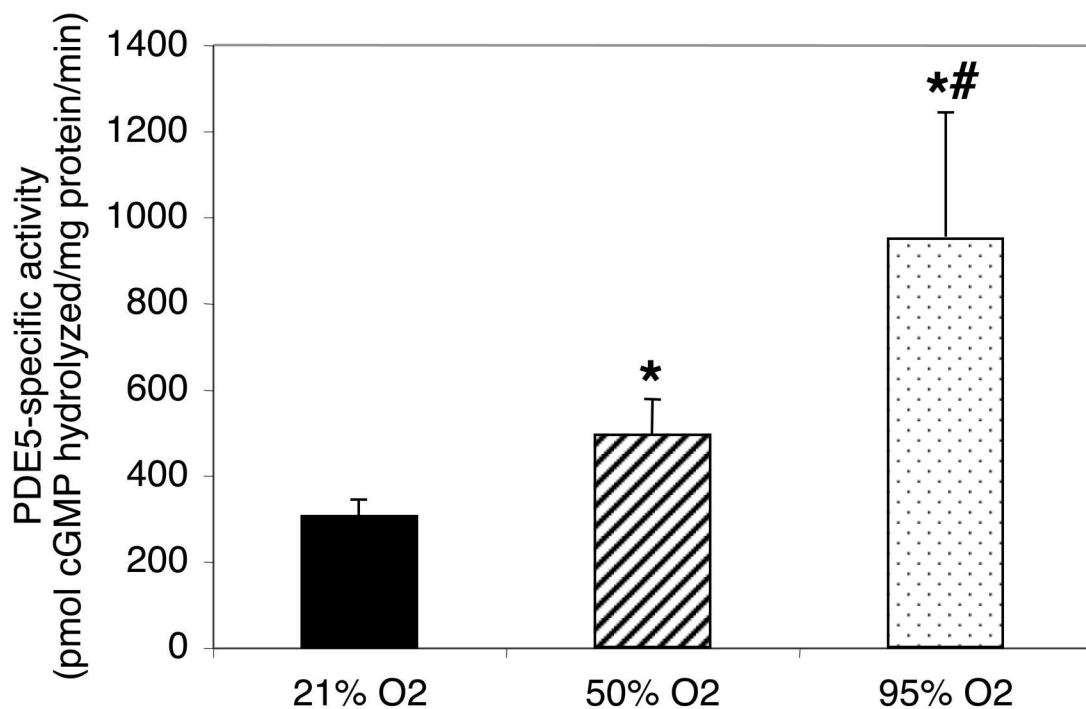


Figure 3. Hyperoxia Increases PDE5 Activity in FPASMC

FPASMC 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 mg of protein. Data are shown as mean±SEM (n=8, read in duplicate; *p<0.05 vs. 21% O₂; #p<0.05 vs. 50% O₂).

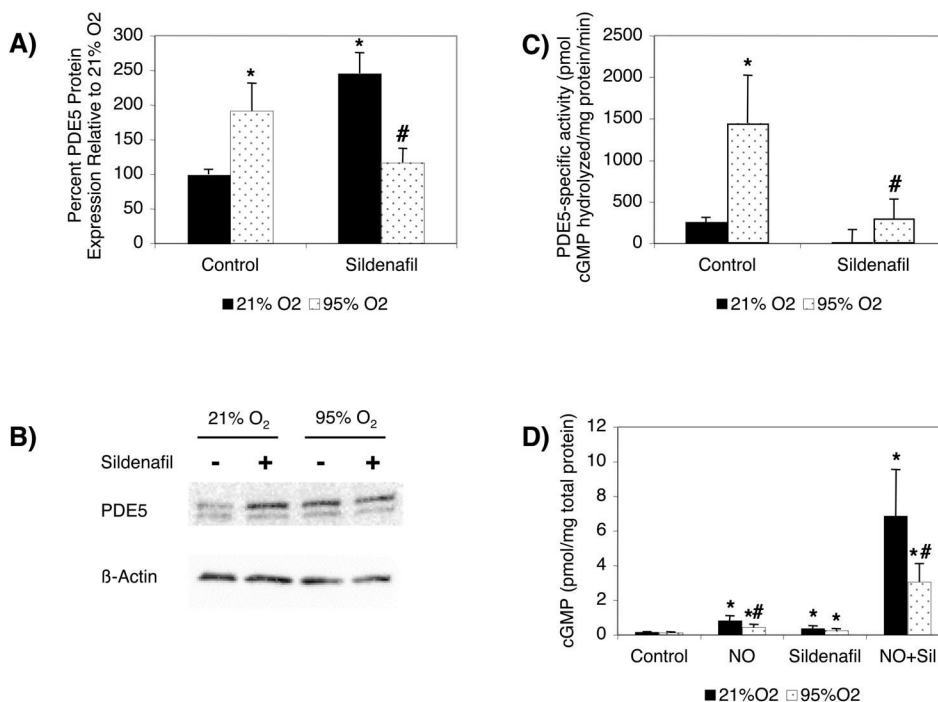


Figure 4. Sildenafil Blocks Hyperoxia-Induced PDE5 Expression and Activity and Partially Restores cGMP Responsiveness in FPASMC

FPASMC were exposed to 21% O₂-5% CO₂ or 95% O₂-5% CO₂ for 24 hours ± sildenafil (100 nM). **A)** Cells were harvested for total protein and subjected to PDE5 Western Blot analysis, with β-actin normalization. Data are shown as mean±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 FPASMC. **C)** 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=12, read in duplicate; *p<0.05 vs. 21% O₂; #p<0.05 vs. 95% O₂). **D)** Cells were assayed for cGMP by EIA, and cGMP was normalized for mg total protein. Data are shown as mean±SEM (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).

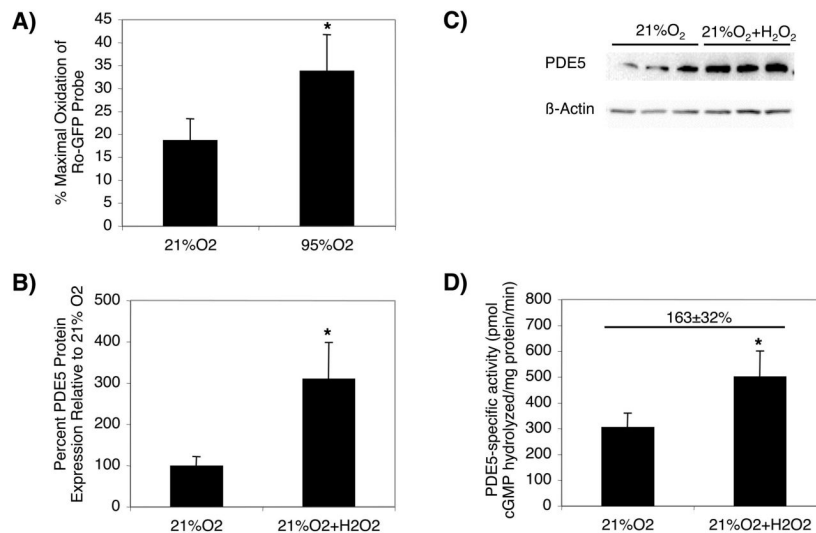


Figure 5. ROS Increase PDE5 Protein Expression and Activity in FPASMC

A) FPASMC were infected with adenovirus containing the H₂O₂-sensitive Ro-GFP probe for 48 hours. Infected cells were exposed to 21% O₂-5% CO₂ or 95% O₂-5% CO₂ for 24 hours and then subjected to dual-excitation flow cytometry to quantitate the probe's oxidative state. Data are expressed as % maximal probe oxidation ± SEM (n=6, *p<0.05 vs. 21% O₂). **B)** FPASMC were treated with a single dose of H₂O₂ (50 μM) in 21% O₂-5% CO₂. Total protein was harvested 24 hours later and subjected to PDE5 Western Blot analysis, with β-actin normalization. Data are shown as mean±SEM (n=5, *p<0.05 vs. untreated cells). **C)** Representative Western blots are shown for PDE5 and β-actin in FPASMC. **D)** 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=5, read in duplicate, *p<0.05 vs. untreated cells).

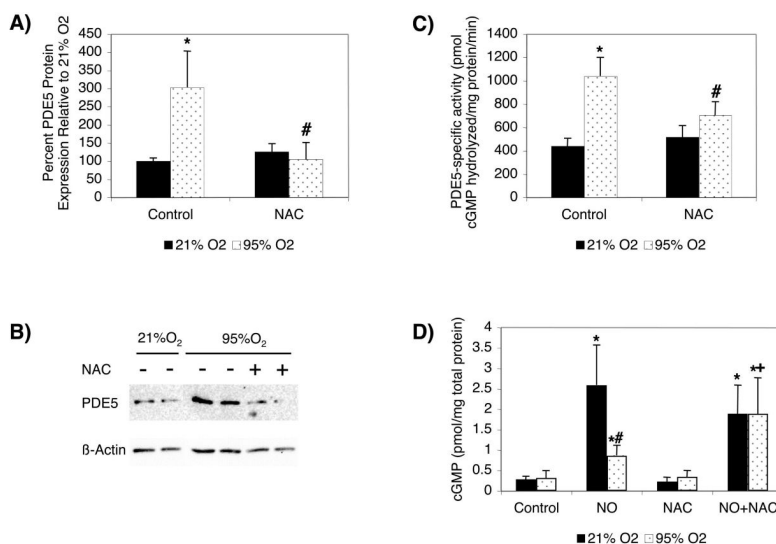


Figure 6. NAC Blocks Hyperoxia-Induced PDE5 Expression and Activity and Restores cGMP Responsiveness in FPASMC

FPASMC were exposed to 21% O₂-5% CO₂ or 95% O₂-5% CO₂ for 24 hours ± NAC (500 μM). **A)** Cells were harvested for total protein and subjected to PDE5 Western Blot analysis, with β-actin normalization. Data are shown as mean±SEM (n=11, *p<0.01 vs. 21% O₂; #p<0.01 vs. 95% O₂). **B)** Representative Western blots are shown for PDE5 and β-actin in FPASMC. **C)** 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=11, read in duplicate; *p<0.05 vs. 21% O₂; #p<0.05 vs. 95% O₂). **D)** Cells were assayed for cGMP by EIA, and cGMP was normalized for mg total protein. Data are shown as mean±SEM (n=7, read in triplicate; *p<0.05 vs. untreated cells; #p<0.01 vs. 21% O₂+NO; + p<0.05 vs. 95% O₂+NO).

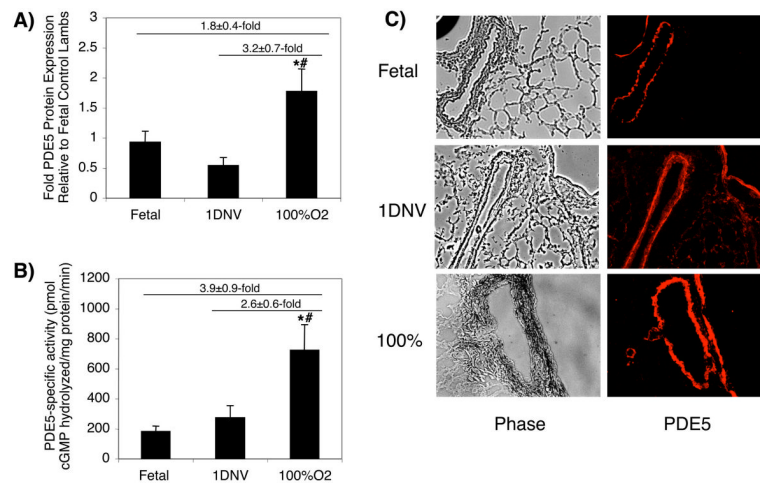


Figure 7. Ventilation of Healthy Neonatal Sheep with 100% O₂ Induces PDE5 Protein Expression and Activity

Ovine lung parenchyma and resistance pulmonary arteries (PA) from healthy lambs ventilated with 100% O₂ were harvested after 24 hours (n=5), and compared to both fetal lambs (Fetus, n=6) and healthy one-day non-ventilated lambs (1DNV, n=4). **A)** PA PDE5 protein expression was analyzed via Western blot, with β -actin normalization. Data are shown as mean \pm SEM (*p<0.05 vs. fetus, # p<0.05 vs. 1DNV). **B)** Lung 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 \pm SEM (*p<0.05 vs. fetus, #p<0.05 vs. 1DNV). **C)** PDE5 expression is localized within the PA smooth muscle and nearby airways. The left column shows phase contrast images (10X) of frozen lamb lung sections. The right column shows the corresponding sections stained for PDE5, with immunofluorescence shown as red.

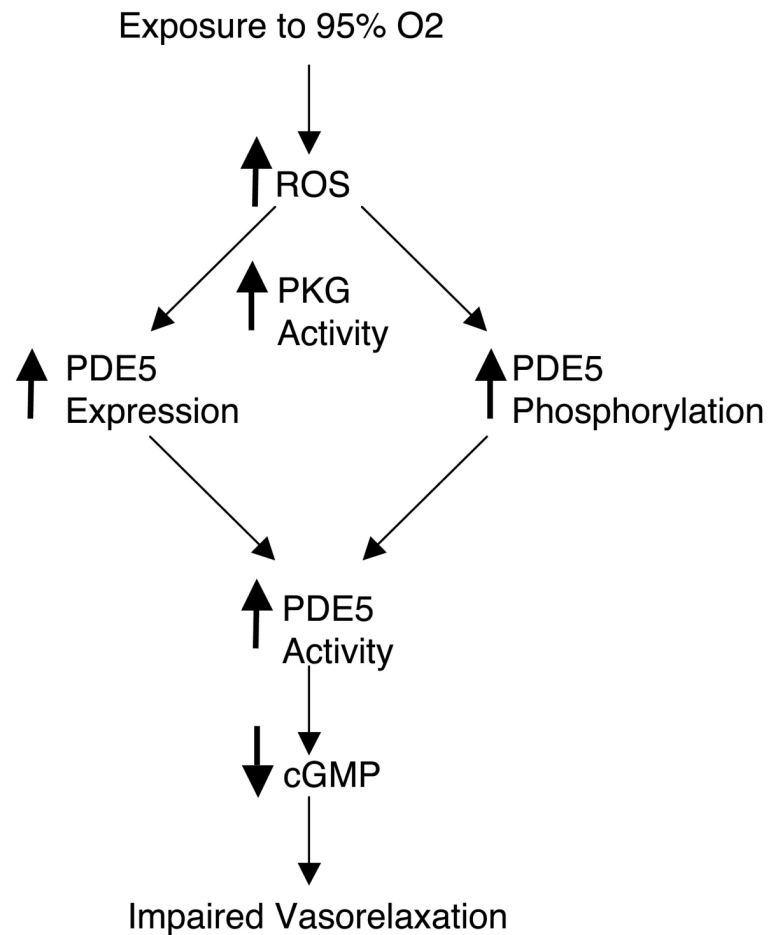


Figure 8. Model for the Effects of Hyperoxia on PDE5 in FPASMC

Exposure to hyperoxia leads to increased ROS production, which increases PDE5 expression and activity, likely in a PKG-mediated fashion. The increased PDE5 activity ultimately decreases the amount of available cGMP, leading to impaired vasorelaxation to exogenous NO.