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MITOCHONDRIAL OXIDANT STRESS INCREASES PDE5 ACTIVITY IN PERSISTENT PULMONARY HYPERTENSION OF THE NEWBORN

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Abstract

In the pulmonary vasculature, phosphodiesterase-5 (PDE5) degrades cGMP and inhibits nitric oxide-mediated, cGMP-dependent vasorelaxation. We previously reported that ventilation with 100% O₂ increased PDE5 activity in pulmonary arteries (PA) of pulmonary hypertension lambs (PPHN) more than in control lambs. In the present study, PA smooth muscle cells (PASMC) from PPHN lambs had increased basal PDE5 activity, decreased cGMP-responsiveness to NO, and increased mitochondrial matrix oxidant stress compared to control PASMC. Hyperoxia (24h) increased PDE5 activity and mitochondrial matrix oxidant stress above baseline to a similar degree in PPHN and control PASMC. Mitochondrially-targeted catalase decreased PDE5 activity at baseline and after hyperoxia in PPHN PASMC. Similarly, catalase treatment of PPHN lambs ventilated with 100% O₂ decreased PDE5 activity and increased cGMP in PA. We conclude that baseline PDE5 activity and oxidative stress is increased in PPHN PASMC, and scavenging H₂O₂ is sufficient to block oxidant-mediated increases in PDE5 activity in PPHN.

Keywords

pulmonary vasculature; reactive oxygen species; phosphodiesterase; cGMP; hyperoxia

1. Introduction

Persistent pulmonary hypertension of the newborn (PPHN) is a clinical syndrome occurring in 2–6 per 1000 live births with a significant risk of death, as well as short-term and long-term morbidity (Walsh-Sukys et al., 2000). It is multifactorial in origin, and defined by an abnormal transition at birth, resulting in elevated pulmonary vascular resistance, right to left-sided extrapulmonary shunting of deoxygenated blood, and severe hypoxemia. Pathological findings include pulmonary vascular remodeling and smooth muscle hyperplasia, often in the absence of significant lung parenchyma pathology (Haworth, 1988; Murphy et al., 1981). Current clinical management strategies to effect pulmonary vasodilation include mechanical ventilation with high levels of inspired oxygen and inhaled

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nitric oxide (iNO). While iNO decreases need for extracorporeal membrane oxygenation (ECMO), it has not been proven to improve survival or long-term outcomes, and 40% or more of infants have a limited or transient response (Clark et al., 2000; Group, 1997; Roberts et al., 1997). Thus, additional therapies are needed.

Increased oxygen tension after birth stimulates pulmonary vascular endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production. NO activates soluble guanylate cyclase (sGC) to produce cyclic GMP (cGMP), a critical second messenger for pulmonary vasodilation (Black et al., 1997; Lakshminrusimha and Steinhorn, 1999). Phosphodiesterase 5 (PDE5) is the major cGMP-hydrolytic phosphodiesterase in the lung, and it downregulates this pathway. In the normal postnatal transition period, PDE5 expression decreases shortly after birth, and then increases again 4 to 7 days later, suggesting developmental regulation (Farrow et al., 2010; Hanson et al., 1998; Okogbule-Wonodi et al., 1998).

While high concentrations of oxygen are widely used as a pulmonary vasodilator in the clinical setting of PPHN, emerging evidence in adult and neonatal disease raises concern about the potential for excess oxidative stress to lead to worsening tissue injury (Farrow et al., 2008a; Farrow et al., 2010; Farrow et al., 2008b; Lakshminrusimha et al., 2006a; Lakshminrusimha et al., 2009; Sinclair et al., 2004; Vento et al., 2001). During normal aerobic metabolism, eukaryotic cells constantly produce reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H_2O_2), which must be tightly regulated in order to prevent cellular injury. Previous data from our lab and others suggests that increased oxidative stress contributes to the vascular remodeling and dysfunction in fetal lambs with PPHN (Brennan et al., 2003; Konduri et al., 2007; Lakshminrusimha et al., 2006b; Wedgwood et al., 2005). Once mechanically ventilated with 100% O_2 , there is a further increase in ROS with accompanying dysregulation of multiple signaling pathways (Farrow et al., 2008b; Lakshminrusimha et al., 2006b). Recent data from our group and others suggests that PDE5 expression and activity may be upregulated by ROS in the pulmonary and systemic vasculature (Farrow et al., 2008a; Muzaffar et al., 2008). Further, we recently reported that the effects of hyperoxia may be amplified in PPHN, in that ventilated PPHN lambs have significantly more PDE5 activity and decreased cGMP in their resistance pulmonary arteries as compared to control lambs ventilated with 100% O_2 (Farrow et al., 2008a; Farrow et al., 2010). The present study sought to determine whether ROS play a role in amplifying PDE5 activity in PPHN lambs ventilated with 100% O_2 by testing the hypothesis that ROS-scavenging therapies such as catalase normalize PDE5 and steady-state cGMP levels in PPHN.

2. MATERIALS AND METHODS

2.1 Animal Surgery and Ventilation Protocols

This protocol was approved by the Laboratory Animal Care committees at University at Buffalo and Northwestern University. Time-dated pregnant ewes were obtained from Swartz family farm in Attica, NY. Thirteen lambs underwent antenatal ligation of the ductus arteriosus at 126 days gestation (term 143–145 days) to induce pulmonary hypertension as previously described. Lambs were delivered 9 days later. Lambs were intubated, fetal lung liquid was drained, and a dose of calfactant (3mL/kg, Infasurf, ONY laboratories, Amherst NY – a gift from Dr. Edmund A. Egan) was administered through the tracheal tube at birth. In five lambs, catalase combined with polyethylene glycol (PEG-catalase, 15,000 units/kg, Sigma) was mixed in calfactant prior to intratracheal administration. Lambs were placed under a servo-controlled radiant warmer and ventilated with 100% oxygen with a Servo300 ventilator (Siemens, Mississauga ON) as previously described (Lakshminrusimha et al., 2006b). Umbilical arterial and venous lines were placed. Fentanyl at 2–4 mcg/kg/h was given by continuous infusion, with additional boluses administered as needed to minimize

discomfort. Intravenous fluids were administered at 100 mL/kg/day. Arterial blood gases were drawn soon after birth and every two hours and as needed. At the end of 24 h ventilation, lambs were sacrificed with an overdose of thiopental sodium and exsanguination. Fifth generation pulmonary arteries were collected and snap-frozen for further analysis. Additional fetal control and ligated (PPHN) lambs were anesthetized and sacrificed as described above before their first breath for isolating primary pulmonary artery smooth muscle cells (PASMC).

2.2 Cell Culture

Primary cultures of pulmonary artery smooth muscle cells (PASMC) from both control lambs and fetal lambs with PPHN induced by in utero ductal ligation were prepared as previously described from intrapulmonary arteries isolated from 136-day gestation fetal lambs (Farrow et al., 2008a). Adventitia was gently removed under sterile conditions, and the vessel was washed in sterile phosphate-buffered saline (PBS; Mediatech, Herndon, VA), longitudinally cut, and placed endothelial side down in 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.

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 2 and 4. PASMC exposed to hyperoxia were placed in a sealed humidified chamber and continuously maintained in 95%O₂-5%CO₂ (ProOx C-21, Biospherix, Lacona, NY). Some cells were treated with DETANONOate (100 µM; Cayman Chemical, Ann Arbor, MI). Other cells were infected with either an empty adenoviral vector, Y5 (750 pfu/cell; University of Iowa, Iowa City, IA), or an adenoviral construct expressing mitochondrially-targeted catalase (750 pfu/cell; ViraQuest, North Liberty, IA). Twenty-four hours after infection, cells were returned to normal culture medium, and forty-eight hours after infection, the cells were exposed to 21%O₂-5%CO₂ or 95%O₂-5%CO₂ for 24 hours as described above. PASMC were harvested for analysis after 24 hours.

2.3 Real-time PCR Analysis

PASMC were harvested for analysis of PDE5 mRNA as previously described (Farrow et al., 2008a). Briefly, RNA was harvested with 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 Beacon Designer software (Premier Biosoft International, Palo Alto, CA) and have previously been published (Farrow et al., 2008a). Primer sequences are as follows: PDE5 sense 5'-GCAAGGAAGGTATCAGAGG-3', PDE5 antisense 5'-ACAACAATGGGTCTAAGAGG-3', s-actin sense 5'-AACTACCTTCAACTCCATC-3', s-

actin antisense 5'-TGATCTTGATCTTCATTGTG-3'. Relative PDE5 amounts were normalized to β -actin expression using the $\Delta\Delta$ CT method (Livak and Schmittgen, 2001).

2.4 Western Blot Analysis

PASMC were harvested using 1X Mg-lysis buffer (Upstate, Charlottesville, VA) supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO) and a phosphatase inhibitor cocktail (EMD Biosciences, San Diego, CA) as previously described (Farrow et al., 2008a). Cell extracts were sonicated, and protein concentration was determined using the Bradford assay (Bradford, 1976). Isolated frozen PA tissue was homogenized, and total protein collected using the PARIS kit (Ambion, Austin, TX) as previously described (Farrow et al., 2008a; Farrow et al., 2010). Protein concentration was measured using the Bradford method (Bradford, 1976). Total protein for PASMC and PA tissue (40 μ g) was separated on a 4–20% SDS-polyacrylamide gel (Biorad) and then transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL). Western blot was then performed as previously described (Farrow et al., 2008a; Farrow et al., 2010). Briefly, membranes were blocked at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (1X TBST) and were then incubated overnight at 4°C with the primary antibody in 5% milk + 1X TBST at an appropriate dilution (1:333 for mouse anti-PDE5 [BD Transduction], 1:1000 for rabbit anti-MnSOD [Assay Designs, Ann Arbor, MI] and 1:2000 for mouse β -actin [Sigma, St. Louis, MO]). The membranes were washed and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) diluted 1:1000 in 5% milk + 1X TBST. Membranes were washed and exposed via chemiluminescence (Pierce). Bands were analyzed using a Digital Science Image Station (Kodak, Rochester, NY). PDE5 expression within each Western blot was normalized to β -actin. Data are shown as fold relative to untreated control PASMC or percent relative to PPHN lambs ventilated with 100% O₂.

2.5 PDE5 Activity Assay

PASMC were harvested for total protein in lysis buffer supplemented with protease and phosphatase inhibitors as described above. PASMC samples were immediately placed on ice and assayed the same day as previously described (Farrow et al., 2008a). Protein was prepared fresh from snap-frozen PA tissue as described above with the lysis buffer supplemented with protease and phosphatase inhibitors. The total PA protein was immediately placed on ice and assayed the same day as previously described (Farrow et al., 2008a; Farrow et al., 2010). The protein was purified over a Centri-Spin 10 column to remove any phosphate contamination (Princeton Separations, Adelphia, NJ). Protein concentration was determined as described above. Total protein (5 μ g) was assayed for cGMP hydrolytic activity using a commercially available colorimetric cyclic nucleotide phosphodiesterase assay kit (Biomol, Plymouth Meeting, PA). Each sample was read in four wells – two without sildenafil and two with sildenafil (100 nM), to determine PDE5 specific cGMP-hydrolytic activity. The samples were incubated at 30°C for 30 minutes, and the samples were then 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. 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.

2.6 Cyclic GMP Enzyme Immunoassay (EIA)

PASMC were lysed with 0.1N HCl, and extracts were dried and resuspended in EIA buffer (Cayman Chemical) as previously described (Farrow et al., 2008a). As previously described,

sheep PA tissue snap-frozen in liquid nitrogen was weighed and homogenized in 10 volumes of 5% trichloroacetic acid (TCA, Sigma). Precipitate was removed by centrifugation at 1,500×g for 10 min, and the TCA was extracted using water-saturated ether according to the manufacturer's protocol (Cayman Chemical) (Farrow et al., 2010). All samples were acetylated according to the manufacturer's protocol. PASMC cGMP content was measured by EIA in triplicate, and PA tissue cGMP content was measured by EIA in duplicate, using a commercially-available kit (Cayman Chemical). Results were measured using a Labsystems Multiskan EX automated plate reader (Thermo Electron) at 420 nm. Results are shown as pmol cGMP per mg protein for PASMC samples and as pmol cGMP per mg frozen tissue for PA tissue samples.

2.7 Detection of Reactive Oxygen Species (ROS)

PASMC in serum-free DMEM without phenol red and with antibiotics and antimycotics were infected in 60 mm culture dishes with 100 PFU/cell of a Mito-RoGFP adenoviral construct. Mito-RoGFP is a previously characterized ratiometric fluorescent probe sensitive to cellular oxidative stress (Farrow et al., 2008a; Hanson et al., 2004; Waypa et al., 2010). To create this probe, surface-exposed residues in green fluorescent protein (GFP) were replaced with cysteine residues capable of forming disulfide bonds. The mitochondrial matrix targeting sequence for cytochrome oxidase subunit IV was appended to the amino terminus, causing the expressed protein to be localized to the mitochondrial matrix compartment. Assessment of fluorescence ratios therefore provides a real-time measure of cysteine thiol redox status in that subcellular compartment (Farrow et al., 2008a; Hanson et al., 2004; Waypa et al., 2010). Briefly, forty-eight hours after infection, the PASMC were exposed to 21% O₂-5% CO₂ or 95% O₂-5% CO₂ for 24 hours. After exposure, PASMC 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 405 nm and 488 nm excitation wavelengths, while emission was assessed at 535 nm. 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.

2.8 MnSOD Activity Assay

PASMC were scraped from tissue culture plates, washed in PBS and centrifuged. Cell pellets were resuspended and sonicated in ice-cold 50mM potassium phosphate buffer (pH 7.6) containing 0.3M KBr and protease inhibitors. Protein content was measured by the Bradford method as above. Samples were treated with 1mM KCN to inactivate CuZnSOD and ecSOD and analyzed for enzyme activity using a commercially available SOD assay kit (Stressgen, Ann Arbor, MI). Briefly, superoxide generated by xanthine oxidase converts WST-1 to produce a formazan dye that absorbs light at 450nm. SOD reduces the rate of color formation and sample activity is calculated from a standard curve using purified SOD of known activity. Activity was normalized to protein content and expressed relative to normoxic control samples.

2.9 Immunohistochemistry

Lung sections were prepared and stained as described previously (Farrow et al., 2008a; Farrow et al., 2008b). Briefly, the right middle lobe of the lung was removed, and OCT compound (VWR Scientific, West Chester, PA) was injected into the trachea to inflate the lobe; it was then allowed to solidify on ice for 15–20 minutes. Blocks were prepared and cut into 8–10 µm sections that were mounted onto charged slides and stored at –80°C. Sections were subsequently fixed with acetone (Sigma) for 10 minutes at 4°C, allowed to air dry, and

then washed with PBS (Mediatech). Sections were blocked with 5% BSA (Sigma) + 1X TBST at room temperature for one hour and then stained overnight at 4°C anti-PDE5 (1:50 dilution) antibody in 5% BSA + 1X TBST. 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 PDE5 was visualized with a Nikon Eclipse TE-300 fluorescent microscope with excitation at 495 nm and emission at 519 nm for Alexa Fluor 488. Fluorescent images were captured using a CoolSnap digital camera with Metamorph imaging software (Molecular Devices, Sunnyvale, CA).

2.10 Statistical Analysis

All data are expressed as the mean $\pm$ SEM. Results were analyzed by two-sided unpaired t-test or ANOVA with Bonferroni's post-hoc analysis when appropriate using Prism software (GraphPad Software Inc., San Diego, CA). Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1 PDE5 Expression is Decreased and PDE5 Activity is Increased in PPHN PASM

We recently published that PDE5 expression is decreased in the resistance PA of fetal PPHN lambs relative to fetal control lambs but also noted a trend toward increased PDE5 activity, suggesting an increase in PDE5 specific activity (Farrow et al., 2010). In the present study, we studied primary PASM from fetal PPHN lambs in parallel with passage-matched PASM from fetal control lambs. Like the intact lambs, PPHN PASM have decreased PDE5 RNA and protein expression as compared to control PASM (Figure 1A and 1B; $21 \pm 6\%$ and $40 \pm 8\%$, respectively, relative to control PASM). A representative Western blot is shown in Figure 1B. Like the intact lambs, PPHN PASM also had increased basal PDE5 activity compared to control PASM (Figure 1C; $171 \pm 23\%$ relative to control PASM).

3.2 Hyperoxia Induces PDE5 Activity in Both Control and PPHN PASM

Exposure to hyperoxia for 24 hours increases PDE5 expression and activity in both isolated PASM and resistance PA from mechanically ventilated control lambs (Farrow et al., 2008a). Also, mechanical ventilation with 100% O₂ for 24 hours increased PDE5 activity to an even greater extent in PPHN lambs (Farrow et al., 2008a; Farrow et al., 2010). In the present study, exposure to hyperoxia for 24h led to a 1.6 ± 0.2 -fold increase in PDE5 protein expression in control PASM (Figure 2A), similar to our previously published results. However, in the PPHN PASM, there was no significant increase in PDE5 protein expression in response to 24h of hyperoxia (Figure 2A). A representative blot is shown in Figure 2B.

As shown in Figure 3A, hyperoxia increased PDE5 activity in control PASM from 529 ± 112 to 985 ± 109 pmol cGMP hydrolyzed/mg protein/min. Similar to the results shown in Figure 1C, the baseline PDE5 activity in PPHN PASM is significantly higher than in control PASM and is equivalent to that seen in the control PASM exposed to hyperoxia (Figure 3A; 951 ± 132 pmol cGMP hydrolyzed/mg protein/min). Exposure of the PPHN PASM to 24h hyperoxia further increased PDE5 activity relative to baseline, but the absolute amount of the increase is comparable to that seen in the control PASM (Figure 3A; 1394 ± 127 pmol cGMP hydrolyzed/mg protein/min).

PDE5 primarily acts to decrease NO-stimulated cGMP within the cell (Dukarm et al., 1999; Farrow et al., 2008a). Consistent with that mechanism, there was no difference between unstimulated, room air cGMP levels in control and PPHN PASM (Figure 3B). However, the PPHN PASM generated significantly less cGMP in response to exogenous NO in room

air compared to control PASMC (Figure 3B; 2.0 ± 0.4 vs. 3.2 ± 0.8 pmol cGMP/mg protein, respectively). In cells exposed to hyperoxia for 24h, both control and PPHN PASMC produced significantly less cGMP in response to exogenous NO as compared to matched cells in normoxia (Figure 3B).

3.3 Mitochondrial Matrix Oxidant Stress is Increased in PPHN PASMC

Previous reports have established that fetal PPHN lambs have evidence of increased ROS (Brennan et al., 2003; Konduri et al., 2007; Lakshminrusimha et al., 2006b; Wedgwood et al., 2005). In the present study, we utilized the ratiometric fluorescent probe, Mito-RoGFP, to measure the cysteine thiol redox status in the mitochondrial matrix of PPHN and control PASMC. Consistent with the data in the intact lamb, PPHN PASMC had increased oxidative stress in the mitochondrial matrix at baseline relative to control PASMC (Figure 4; $43.4 \pm 2.7\%$ vs. $19.8 \pm 2.7\%$ Mito-RoGFP oxidation respectively). Exposure to hyperoxia for 24h increased mitochondrial matrix oxidative stress to a similar degree over baseline in both control and PPHN PASMC (Figure 4). The sources of mitochondrial matrix oxidative stress in hyperoxia are unknown, but likely arise from the capture of electrons from mitochondrial electron transport chain proteins by the increased levels of O_2 . MnSOD is expressed in the mitochondrial matrix of cells and converts the resulting superoxide to hydrogen peroxide (H_2O_2), which has previously been shown to increase PDE5 activity in control PASMC (Farrow et al., 2008a). At baseline, PPHN PASMC had significantly increased MnSOD activity and expression vs. control PASMC (Figure 5A and Figure 5C; 2.5 ± 0.3 -fold and 2.8 ± 0.5 -fold, respectively, relative to control cells). A representative blot is shown in Figure 5B. Exposure to hyperoxia (24h) did not increase MnSOD activity control PASMC, but did increase MnSOD activity in PPHN cells compared to normoxic PPHN cells (Figure 5C; 4.5 ± 1 -fold relative to normoxic control cells).

3.4 Mitochondrially Targeted Catalase Decreases PDE5 Activity in PPHN Cells

Since both PDE5 activity and mitochondrial matrix oxidative stress were increased in PPHN cells at baseline and with exposure to hyperoxia, we sought to determine if abrogating mitochondrial matrix oxidative stress was sufficient to block PDE5 activity. We have previously demonstrated that overexpression of mitochondrially-targeted catalase can block hyperoxia-induced mitochondrial matrix oxidant stress in control PASMC (Farrow et al., 2009b). Treatment with mitochondria-targeted catalase had no significant effect on baseline PDE5 activity in control PASMC, but it significantly decreased the baseline PDE5 activity in PPHN cells (Figure 6, $40 \pm 12\%$ decrease compared to PPHN cells infected with Y5 control virus). Similarly, mitochondrially-targeted catalase decreased PDE5 activity in both control and PPHN cells exposed to 24h of hyperoxia (Figure 6, $36 \pm 15\%$ decrease and $37 \pm 8\%$ decrease compared to hyperoxia-exposed control and PPHN cells, respectively, infected with Y5 control virus).

3.5 Treatment with PEG-Catalase Decreases PDE5 Expression in Resistance PA from PPHN Lambs

We previously reported that while PDE5 expression normally decreases after birth, ventilation with 100% oxygen significantly increases PDE5 expression (Farrow et al., 2010). Intratracheal PEG-catalase delivered to PPHN lambs ventilated with 100% oxygen for 24 hours significantly decreased PDE5 expression in resistance pulmonary arteries compared to PPHN lambs ventilated with 100% oxygen alone (Figure 7A; $48 \pm 12\%$ of control). A representative blot is shown in Figure 7B. As shown in Figure 7C, the PDE5 expression in the PPHN lambs treated with catalase is confined to the smooth muscle layer of the resistance PA and airways.

3.6 Treatment with PEG-Catalase Decreases PDE5 Activity and Increases Steady-State cGMP in Resistance PA from PPHN Lambs

Similar to the results for expression, intratracheal PEG-catalase significantly decreased PDE5 activity in the resistance PA versus ventilation with 100% oxygen alone (Figure 8A; 810 ± 203 vs. 1346 ± 284 pmol cGMP hydrolyzed/mg protein/min respectively). PEG-catalase also significantly increased steady-state cGMP in the resistance PA compared to ventilation with 100% oxygen alone (Figure 8B; 0.127 ± 0.028 pmol cGMP/mg PA tissue vs. 0.028 ± 0.007 pmol cGMP/mg PA tissue, respectively).

4. DISCUSSION

PPHN is a clinical syndrome with multiple etiologies that involve complex alterations in signaling pathways. Following ductal ligation of the fetal lamb, fetal pulmonary artery pressure increases, and there is rapid pulmonary vascular remodeling, consistent with that seen in idiopathic PPHN in human infants (Morin, 1989; Wild et al., 1989). Previous studies utilizing the ductal ligation model suggest that ROS are involved in the *in utero* pathogenesis of PPHN (Brennan et al., 2003; Konduri et al., 2007; Lakshminrusimha et al., 2006b; Wedgwood et al., 2005). Additionally, mechanical ventilation with high concentrations of O₂, as typically utilized in clinically significant PPHN, may lead to production of ROS from multiple cellular compartments (Farrow 2008, Lakshminrusimha 2009). In the present study, we sought to determine the downstream effects of the elevated antenatal and postnatal ROS on key intracellular mediators, such as PDE5 and cGMP, using PASMC derived from PPHN lambs. We also sought to determine whether postnatally delivered antioxidants, such as PEG-catalase, could rescue PPHN lambs from the downstream effects of elevated ROS.

We recently reported that fetal PPHN lambs sacrificed before their first breath have evidence for increased ROS in the vascular wall, in part due to increased expression of NADPH oxidase (Brennan et al., 2003). We also noted decreased PDE5 protein expression, but increased PDE5 specific activity (Farrow et al., 2010). Consistent with that finding, we show here that PASMC derived from intrapulmonary arteries of these PPHN lambs maintain this phenotype in culture through 4 passages with decreased PDE5 protein expression and increased PDE5 activity (Figures 1A, 1B, and 1C). These findings suggest that early-passage PASMC from PPHN lambs represent a valuable tool to better understand the complex alterations in intracellular signaling that occur in the ductal ligation model of PPHN.

Interestingly, we did observe some differences between intact PPHN lambs and isolated PPHN PASMC. We previously reported that mechanical ventilation with 100% O₂ for 24h leads to increased PDE5 expression and activity in both control and PPHN lambs, but found a proportionally greater increase in PDE5 activity in response to hyperoxic ventilation in the PPHN lambs (Farrow et al., 2008a; Farrow et al., 2010). In contrast, in isolated PASMC, exposure to 100% O₂ for 24h led to a significant increase in PDE5 expression in the control PASMC, but not in PPHN cells (Figures 2A and 2B). Further, exposure to 100% O₂ for 24 hours increased PDE5 activity to a similar degree in both control and PPHN PASMC (Figure 3A). Since the PPHN PASMC start from a higher basal level of PDE5 activity, hyperoxia induced higher absolute PDE5 activity than the control PASMC, but we did not observe the amplified response seen in the intact lamb. We acknowledge that the control and PPHN PASMC were cultured at baseline in 21% oxygen, which does not completely duplicate fetal conditions and may contribute to some of the differences seen between the *in vitro* cell data and the intact lamb data. However, our findings suggest that the amplified response to hyperoxia seen in the intact PPHN lambs may also require additional co-factors extrinsic to the smooth muscle cell. Possible explanations are that increases in PDE5 activity are partly dependent on crosstalk with an intact endothelium, or that altered shear stress and

stretch forces associated with *in vivo* pulmonary hypertension are required. Future studies involving endothelial and smooth muscle cell co-cultures may be able to partly address this key mechanistic question.

PDE5 primarily acts to decrease NO-stimulated cGMP within the cell (Dukarm et al., 1999; Farrow et al., 2008a). As such, we did not see any significant difference in basal, unstimulated cGMP levels between control and PPHN PASMC (Figure 3B). However, the PPHN PASMC generated significantly less cGMP in response to exogenous NO compared to control PASMC in room air, likely due to their baseline elevation of PDE5 activity. Furthermore, both control and PPHN PASMC exhibited blunted cGMP accumulation in response to exogenous NO after exposure to hyperoxia for 24h (Figure 3B), consistent with the increase in PDE5 activity seen in both control and PPHN PASMC after hyperoxia exposure. Thus, the data in the isolated control and PPHN PASMC suggests that the primary difference between these two cell types is their baseline PDE5 activity, rather than changes induced by hyperoxia. This in part may be due to the observed difference that PDE5 protein expression does not increase in response to hyperoxia in the PPHN cells (Figure 2A), which is unlike what we have reported in the intact animal. This suggests that part of the amplification of PDE5 activity in response to hyperoxia that we previously reported in the PPHN lambs is also due to increased PDE5 expression in response to hyperoxia (Farrow et al., 2008a; Farrow et al., 2010).

We previously reported that ROS such as H_2O_2 activate PDE5 (Farrow et al., 2008a). Previous studies have provided evidence for increased ROS in the pulmonary vasculature of intact fetal PPHN lambs at birth, before exposure to any postnatal therapies (Brennan et al., 2003; Konduri et al., 2007; Lakshminrusimha et al., 2006b; Wedgwood et al., 2005). We sought to determine if ROS were similarly increased in early-passage PPHN PASMC, and then to determine potential sources of elevated ROS at baseline and in response to hyperoxia. Previous studies have suggested that mitochondria are a key source of ROS production in human lung fibroblasts exposed to hyperoxia (Klimova et al., 2009). Our group has previously found an increase in cytosolic oxidant stress after 24h of hyperoxia in PASMC isolated from normal neonatal lambs, but the effect of similar hyperoxic exposures on mitochondrial matrix oxidant stress in the cell is not known (Farrow et al., 2008a). Here we use a redox sensor targeted to the mitochondria to demonstrate that PPHN PASMC have a higher basal level of oxidant stress in the mitochondrial matrix relative to control PASMC (Figure 4). This indicates that the global increase in oxidant stress in the PPHN PASMC is due to increases in more than one subcellular compartment, as we have noted increased oxidant stress in the cytosol as well (Wedgwood et al., 2010). Further, both control and PPHN PASMC experience increased mitochondrial matrix oxidant stress after exposure to hyperoxia for 24 hours (Figure 4). The magnitude of the increase over basal levels is similar between the two cell types, and parallels the hyperoxia-induced increases in PDE5 activity. Thus, while the PPHN PASMC reach a significantly higher absolute level of oxidant stress after hyperoxia exposure, we speculate this is primarily due to a higher level of baseline oxidant stress in the mitochondrial matrix.

One potential cause of the mitochondrial matrix oxidant stress is H_2O_2 derived from the dismutation of superoxide by MnSOD. The resulting H_2O_2 can then diffuse from the mitochondria into the cytosol (Pani et al., 2009). We and others have previously shown that H_2O_2 can inhibit eNOS (Kumar et al., 2008), downregulate sGC (Wedgwood et al., 2005), and stimulate PDE5 (Farrow et al., 2008a). Furthermore, catalase normalized vasodilator responses to NO in pulmonary arteries isolated from fetal PPHN lambs (Wedgwood et al., 2005), indicating the effects of H_2O_2 are dynamic and reversible. In the present study, we show for the first time that the PPHN PASMC have significantly higher MnSOD activity at baseline than control PASMC (Figure 5C). Elevated MnSOD activity could increase

mitochondrial matrix H_2O_2 , which in turn could impact basal PDE5 activity in the cytosol. However, despite the increase in MnSOD activity and mitochondrial matrix oxidant stress seen in the PPHN PASMCMC at baseline, PDE5 protein expression is decreased relative to control lambs. We have previously reported that exogenous H_2O_2 is sufficient to increase PDE5 protein expression in control PASMCMC, so we conclude that factors other than H_2O_2 must depress PDE5 protein in PPHN lambs. One potential mechanism could be increased PDE5 protein degradation in the PPHN cells. We recently found a trend towards increased caspase 3 activity in isolated PA from PPHN fetuses, and note that caspase 3 is the only enzyme that has been described to play a role in PDE5 protein degradation (Farrow et al., 2010). Future studies into caspase 3 activity in the PPHN cells, as well as careful studies examining the PDE5 promoter regulation in the PPHN should provide additional information about the mechanism for decreased PDE5 protein expression in the PPHN PASMCMC.

Interestingly, exposure to hyperoxia did not increase MnSOD activity in control PASMCMC, but it did in the PPHN cells. This suggests that distinct sources of increased oxidant stress exist in PPHN vs control PASMCMC, both at baseline and in response to hyperoxia. Other reports also indicate that changes in MnSOD activity may vary among different cell types or conditions resulting in increased oxidative stress (Clerch and Massaro, 1992, 1993; Sam et al., 2005). Further investigation will be needed to determine the intrinsic differences between the control and PPHN PASMCMC that facilitate this different response of MnSOD following hyperoxia exposure.

We then sought to determine if decreasing mitochondrial matrix H_2O_2 by overexpressing a mitochondrially-targeted catalase would be sufficient to decrease basal PDE5 activity in the PPHN PASMCMC. We speculated that the increased mitochondrial matrix oxidant stress was responsible for the increased PDE5 activity in the PPHN PASMCMC since we have previously demonstrated that hyperoxia-induced mitochondrial matrix oxidative stress in control PASMCMC is sufficient to increase PDE5 activity (Farrow et al., 2009c). Scavenging mitochondrial matrix H_2O_2 indeed decreased baseline PDE5 activity in the PPHN PASMCMC, consistent with our hypothesis (Figure 6). Further, mitochondrially-targeted catalase also blunted the hyperoxia-induced increase in PDE5 activity in both control and PPHN cells (Figure 6). Thus, we conclude that the increased mitochondrial matrix oxidative stress is sufficient to generate an intracellular signal that increases PDE5 activity.

While these studies in isolated cells allow us to gain important mechanistic insights, our ultimate goal is to derive new therapies for those infants with PPHN who do not respond to conventional therapies. Currently there is no feasible mechanism to deliver mitochondrially-targeted catalase into the lungs of intact animals. However, it is likely that the increased H_2O_2 in the mitochondrial matrix in PPHN PASMCMC leaks out into the cytosol to mediate at least some of its downstream effects, since the PPHN PASMCMC have evidence of increased baseline cytosolic oxidative stress as well (Wedgwood et al., 2010). We also recently reported that PEG-catalase treatment of PPHN lambs being mechanically ventilated with 100% O_2 led to improved oxygenation (Farrow et al., 2009a), and therefore sought to determine if this clinical improvement was associated with normalization of PDE5 and cGMP. As shown in Figure 7, treatment with PEG-catalase led to a significant decrease in PDE5 protein expression in the resistance PA of PPHN lambs ventilated with 100% O_2 . Similarly, treatment with PEG-catalase significantly decreased PA PDE5 activity (Figure 8A) and greatly increased PA cGMP levels in PPHN lambs ventilated with 100% O_2 (Figure 8B). These studies in intact lambs suggest that scavenging H_2O_2 may restore activity of key enzymes in NO-cGMP signaling, and represent a useful future therapeutic strategy for infants with PPHN. Additionally, consistent with our animal data showing that increased PDE5 activity contributes to the vascular dysfunction of PPHN, early clinical studies of

sildenafil, a selective inhibitor of PDE5, in human infants have shown a potential benefit of this therapeutic approach (Atz and Wessel, 1999; Baquero et al., 2006; Steinhorn et al., 2009).

5. CONCLUSIONS

PDE5 and cGMP are key mediators in the regulation of pulmonary vascular tone in the immediate postnatal period. In the present study, PASMC isolated from fetal PPHN lambs at baseline had increased PDE5 activity, decreased cGMP-responsiveness to NO, and increased mitochondrial matrix oxidant stress compared to control PASMC. Hyperoxia (24h) induced increased PDE5 activity and mitochondrial matrix oxidant stress above baseline to a similar degree in PPHN vs. control PASMC, but produced an additive effect that resulted in higher PDE5 activity and mitochondrial matrix oxidant stress in the PPHN cells. Mitochondrially-targeted catalase decreased PDE5 activity at baseline and after hyperoxia in PPHN PASMC, indicating a role for H₂O₂ in the regulation of PDE5 activity. Similarly, PEG-catalase treatment of PPHN lambs ventilated with 100% O₂ decreased PDE5 activity and increased cGMP in PA. Thus, H₂O₂ derived from the mitochondria appears to be a key regulator of basal PDE5 activity in the neonatal pulmonary vasculature. In disease states that have elevated ROS such as PPHN, antioxidants such as catalase may represent important future therapeutic options for those PPHN infants that do not respond to conventional therapies.

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References

- Atz AM, Wessel DL. Sildenafil ameliorates effects of inhaled nitric oxide withdrawal. *Anesthesiology*. 1999; 91:307–310. [PubMed: 10422958]
- Baquero H, Soliz A, Neira F, Venegas ME, Sola A. Oral sildenafil in infants with persistent pulmonary hypertension of the newborn: a pilot randomized blinded study. *Pediatrics*. 2006; 117:1077–1083. [PubMed: 16585301]
- Black SM, Johengen MJ, Ma ZD, Bristow J, Soifer SJ. Ventilation and oxygenation induce endothelial nitric oxide synthase gene expression in the lungs of fetal lambs. *J Clin Invest*. 1997; 100:1448–1458. [PubMed: 9294110]
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72:248–254. [PubMed: 942051]
- Brennan LA, Steinhorn RH, Wedgwood S, Mata-Greenwood E, Roark EA, Russell JA, Black SM. Increased superoxide generation is associated with pulmonary hypertension in fetal lambs: a role for NADPH oxidase. *Circ Res*. 2003; 92:683–691. [PubMed: 12609968]
- Clark RH, Kueser TJ, Walker MW, Southgate WM, Huckaby JL, Perez JA, Roy BJ, Keszler M, Kinsella JP. Low-dose nitric oxide therapy for persistent pulmonary hypertension of the newborn. Clinical Inhaled Nitric Oxide Research Group. *The New England journal of medicine*. 2000; 342:469–474. [PubMed: 10675427]
- Clerch LB, Massaro D. Rat lung antioxidant enzymes: differences in perinatal gene expression and regulation. *Am J Physiol*. 1992; 263:L466–470. [PubMed: 1415724]

- Clerch LB, Massaro D. Tolerance of rats to hyperoxia. Lung antioxidant enzyme gene expression. *J Clin Invest.* 1993; 91:499–508. [PubMed: 8432858]
- Dukarm RC, Russell JA, Morin FC 3rd, Perry BJ, Steinhorn RH. The cGMP-specific phosphodiesterase inhibitor E4021 dilates the pulmonary circulation. *Am J Respir Crit Care Med.* 1999; 160:858–865. [PubMed: 10471609]
- Farrow KN, Groh BS, Schumacker PT, Lakshminrusimha S, Czech L, Gugino SF, Russell JA, Steinhorn RH. Hyperoxia increases phosphodiesterase 5 expression and activity in ovine fetal pulmonary artery smooth muscle cells. *Circ Res.* 2008a; 102:226–233. [PubMed: 17991881]
- Farrow KN, Lakshminrusimha S, Wedgwood S, Czech L, Smith CL, Gugino SF, Russell JA, Steinhorn R. PEG-Catalase decreases PDE5 activity and improves oxygenation in an ovine model of persistent pulmonary hypertension (PPHN). *E-PAS* 2009. 2009a:2540.5.
- Farrow KN, Lakshminrusimha S, Czech L, Groh BS, Gugino SF, Davis JM, Russell JA, Steinhorn RH. Superoxide Dismutase and Inhaled Nitric Oxide Normalize Phosphodiesterase 5 Expression and Activity in Neonatal Lambs with Persistent Pulmonary Hypertension. *Am J Physiol Lung Cell Mol Physiol.* 2010; 299:L109–116. [PubMed: 20400523]
- Farrow KN, Lakshminrusimha S, Reda WJ, Wedgwood S, Czech L, Gugino SF, Davis JM, Russell JA, Steinhorn RH. Superoxide Dismutase Restores eNOS Expression and Function in Resistance Pulmonary Arteries from Neonatal Lambs with Persistent Pulmonary Hypertension. *Am J Physiol Lung Cell Mol Physiol.* 2008b; 295:L979–987. [PubMed: 18790993]
- Farrow KN, Smith CL, Czech L, Gugino SF, Russell JA, Schumacker PT, Steinhorn R. Abstract P20: Catalase Blocks Hyperoxia-Induced Mitochondrial Oxidative Stress and Decreases Phosphodiesterase 5 (PDE5) Activity in Ovine Fetal Pulmonary Artery Smooth Muscle Cells (FPASMC). *Circulation.* 2009b; 120:S1445.
- Farrow KN, Smith CL, Czech L, Lakshminrusimha S, Gugino SF, Russell JA, Schumacker PT, Steinhorn R. Mitochondrial oxidative stress increases PDE5 expression and activity in ovine fetal pulmonary artery smooth muscle cells (FPASMC). *E-PAS* 2009. 2009c:5755.1.
- Group NINOS. Inhaled nitric oxide in full-term and nearly full-term infants with hypoxic respiratory failure. *New Eng J Med.* 1997; 336:597–604. [PubMed: 9036320]
- Hanson GT, Aggeler R, Oglesbee D, Cannon M, Capaldi RA, Tsien RY, Remington SJ. Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. *J Biol Chem.* 2004; 279:13044–13053. [PubMed: 14722062]
- Hanson KA, Burns F, Rybalkin SD, Miller JW, Beavo J, Clarke WR. Developmental changes in lung cGMP phosphodiesterase-5 activity, protein, and message. *Am J Respir Crit Care Med.* 1998; 158:279–288. [PubMed: 9655741]
- Haworth SG. Pulmonary vascular remodeling in neonatal pulmonary hypertension. *Chest.* 1988; 93:133S–138S. [PubMed: 3277815]
- Klimova TA, Bell EL, Shroff EH, Weinberg FD, Snyder CM, Dimri GP, Schumacker PT, Budinger GR, Chandel NS. Hyperoxia-induced premature senescence requires p53 and pRb, but not mitochondrial matrix ROS. *Faseb J.* 2009; 23:783–794. [PubMed: 18948382]
- Konduri GG, Bakhutashvili I, Eis A, Pritchard K Jr. Oxidant stress from uncoupled nitric oxide synthase impairs vasodilation in fetal lambs with persistent pulmonary hypertension. *Am J Physiol Heart Circ Physiol.* 2007; 292:H1812–1820. [PubMed: 17142346]
- Kumar S, Sun X, Wedgwood S, Black SM. Hydrogen peroxide decreases endothelial nitric oxide synthase promoter activity through the inhibition of AP-1 activity. *Am J Physiol Lung Cell Mol Physiol.* 2008; 295:L370–377. [PubMed: 18556800]
- Lakshminrusimha S, Russell JA, Steinhorn RH, Ryan RM, Gugino SF, Morin FC 3rd, Swartz DD, Kumar VH. Pulmonary arterial contractility in neonatal lambs increases with 100% oxygen resuscitation. *Pediatr Res.* 2006a; 59:137–141. [PubMed: 16326983]
- Lakshminrusimha S, Russell JA, Wedgwood S, Gugino SF, Kazzaz JA, Davis JM, Steinhorn RH. Superoxide dismutase improves oxygenation and reduces oxidation in neonatal pulmonary hypertension. *Am J Respir Crit Care Med.* 2006b; 174:1370–1377. [PubMed: 17008638]
- Lakshminrusimha S, Steinhorn RH. Pulmonary vascular biology during neonatal transition. *Clin Perinatol.* 1999; 26:601–619. [PubMed: 10494467]

- Lakshminrusimha S, Swartz DD, Gugino SF, Ma CX, Wynn KA, Ryan RM, Russell JA, Steinhorn RH. Oxygen Concentration and Pulmonary Hemodynamics in Newborn Lambs with Pulmonary Hypertension. *Pediatric Research*. 2009; 66:539–544. [PubMed: 19680165]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- Morin FC 3rd. Ligating the ductus arteriosus before birth causes persistent pulmonary hypertension in the newborn lamb. *Pediatric research*. 1989; 25:245–250. [PubMed: 2704590]
- Murphy JD, Rabinovitch M, Goldstein JD, Reid LM. The structural basis of persistent pulmonary hypertension of the newborn infant. *The Journal of pediatrics*. 1981; 98:962–967. [PubMed: 7229803]
- Muzaffar S, Shukla N, Bond M, Sala-Newby GB, Newby AC, Angelini GD, Jeremy JY. Superoxide from NADPH oxidase upregulates type 5 phosphodiesterase in human vascular smooth muscle cells: inhibition with iloprost and NONOate. *British journal of pharmacology*. 2008; 155:847–856. [PubMed: 18660830]
- Okogbule-Wonodi AC, Ibe BO, Yue BW, Hsu S, Raj JU. Phosphodiesterase activity in intrapulmonary arteries and veins of perinatal lambs. *Mol Genet Metab*. 1998; 65:229–237. [PubMed: 9851888]
- Pani G, Koch OR, Galeotti T. The p53-p66shc-Manganese Superoxide Dismutase (MnSOD) network: a mitochondrial intrigue to generate reactive oxygen species. *The international journal of biochemistry & cell biology*. 2009; 41:1002–1005.
- Roberts JD, Fineman JR, Morin FC 3rd, Shaul PW, Rimar S, Schreiber MD, Polin RA, Zwass MS, Zayek MM, Gross I, Heymann MA, Zapol WM, Thusu KG, Zellers TM, Wylam ME, Zaslavsky A. Inhaled nitric oxide and persistent pulmonary hypertension of the newborn. *New Eng J Med*. 1997; 336:605–610. [PubMed: 9032045]
- Sam F, Kerstetter DL, Pimental DR, Mulukutla S, Tabaei A, Bristow MR, Colucci WS, Sawyer DB. Increased reactive oxygen species production and functional alterations in antioxidant enzymes in human failing myocardium. *Journal of cardiac failure*. 2005; 11:473–480. [PubMed: 16105639]
- Sinclair SE, Altemeier WA, Matute-Bello G, Chi EY. Augmented lung injury due to interaction between hyperoxia and mechanical ventilation. *Crit Care Med*. 2004; 32:2496–2501. [PubMed: 15599157]
- Steinhorn RH, Kinsella JP, Pierce C, Butrous G, Dilleen M, Oakes M, Wessel DL. Intravenous sildenafil in the treatment of neonates with persistent pulmonary hypertension. *J Pediatr*. 2009; 155:841–847. [PubMed: 19836028]
- Vento M, Asensi M, Sastre J, Garcia-Sala F, Pallardo FV, Vina J. Resuscitation with room air instead of 100% oxygen prevents oxidative stress in moderately asphyxiated term neonates. *Pediatrics*. 2001; 107:642–647. [PubMed: 11335737]
- Walsh-Sukys MC, Tyson JE, Wright LL, Bauer CR, Korones SB, Stevenson DK, Verter J, Stoll BJ, Lemons JA, Papile L, Shankaran S, Donovan EF, Oh W, Ehrenkranz RA, Fanaroff AA. Persistent pulmonary hypertension of the newborn in the era before nitric oxide: practice variation and outcomes. *Pediatrics*. 2000; 105:14–20. [PubMed: 10617698]
- Waypa GB, Marks JD, Guzy R, Mungai PT, Schriewer J, Dokic D, Schumacker PT. Hypoxia triggers subcellular compartmental redox signaling in vascular smooth muscle cells. *Circ Res*. 2010; 106:526–535. [PubMed: 20019331]
- Wedgwood S, Lakshminrusimha S, Gugino SF, Schumacker PT, Steinhorn R. Increased peroxynitrite is associated with decreased ecSOD activity in PPHN. *E-PAS 2010*. 2010:4335.5.
- Wedgwood S, Steinhorn RH, Bunderson M, Wilham J, Lakshminrusimha S, Brennan LA, Black SM. Increased hydrogen peroxide downregulates soluble guanylate cyclase in the lungs of lambs with persistent pulmonary hypertension of the newborn. *American journal of physiology*. 2005; 289:L660–666. [PubMed: 15937064]
- Wild LM, Nickerson PA, Morin FC 3rd. Ligating the ductus arteriosus before birth remodels the pulmonary vasculature of the lamb. *Pediatr Res*. 1989; 25:251–257. [PubMed: 2704591]

Figure 1.

PDE5 Expression is Decreased, but PDE5 Specific Activity is Increased in PPHN PASMC. Protein was harvested from control and PPHN PASMC. A) PDE5 mRNA expression is analyzed by real-time RT-PCR, normalized to β -actin (n=4). B) PDE5 protein expression is analyzed by Western blot, normalized for β -actin (n=6). Data are shown as mean $\pm$ SEM relative to control PASMC. A representative Western blot is shown for PDE5 and β -actin in control and PPHN PASMC. C) PDE5-specific cGMP hydrolytic activity is measured using a commercially-available colorimetric assay (n=13, read in duplicate). Data are shown as mean $\pm$ SEM pmol cGMP hydrolyzed/mg protein/min. * p<0.05 vs. control PASMC.

Figure 2.

Twenty-four Hours of Hyperoxia Increases PDE5 Protein Expression in Control PASMC but not PPHN PASMC. Protein was harvested from control and PPHN PASMC exposed to either 21% O₂-5% CO₂ or 95% O₂-5% CO₂ for 24 hours. A) PDE5 protein expression is analyzed by Western blot, normalized for β-actin (n=8). Data are shown as mean ± SEM relative to control PASMC in 21% O₂-5% CO₂. B) A representative Western blot is shown for PDE5 and β-actin in control and PPHN PASMC. C21 - Control PASMC in 21% O₂-5% CO₂, C95 - Control PASMC in 95% O₂-5% CO₂, P21 - PPHN PASMC in 21% O₂-5% CO₂, P95 - PPHN PASMC in 95% O₂-5% CO₂. * p<0.05 vs. control PASMC in 21% O₂-5% CO₂; # p<0.05 vs. control PASMC in 95% O₂-5% CO₂.

Figure 3.

Twenty-four Hours of Hyperoxia Increases PDE5 Activity and Decreases cGMP

Responsiveness to Exogenous NO in Control and PPHN PASM. A) Protein was harvested from control and PPHN PASM exposed to either 21%O₂-5%CO₂ or 95%O₂-5%CO₂ for 24 hours. PDE5-specific cGMP hydrolytic activity is measured using a commercially-available colorimetric assay (n=15, read in duplicate). Data are shown as mean ± SEM pmol cGMP hydrolyzed/mg protein/min. B) Control and PPHN PASM were exposed to 21%O₂-5%CO₂ or 95%O₂-5%CO₂ ± DETANONOate (100μM) for 24 hours. Cells were harvested and assayed for cGMP by EIA. cGMP levels were normalized for mg total protein. Data are shown as mean±SEM (n=6, read in triplicate). * p<0.05 vs. control

PASMC in 21% O₂-5% CO₂; [#] p<0.05 vs. control PASMC in 95% O₂-5% CO₂; ⁺ p<0.05 vs. PPHN PASMC in 21% O₂-5% CO₂.

Figure 4.

Mitochondrial Matrix Oxidative Stress is Increased in PPHN PASMC. Mito-RoGFP contains surface-exposed cysteine residues that are sensitive to the local redox environment. After measuring the cellular environment using multilaser flow cytometry, the probe is calibrated by maximally reducing it with dithiothreitol (DTT, 1 mM) and maximally oxidizing it with t-butyl hydroperoxide (TBH, 1mM). Control and PPHN FPASMC were exposed to 21% O₂-5% CO₂ or 95% O₂-5% CO₂ for 24h, and oxidation of the MitoRoGFP probe was measured (n=4). Data are shown as mean ± SEM for the % maximal oxidation of the Mito-RoGFP probe. * p<0.05 vs. control PASMC in 21% O₂-5% CO₂; # p<0.05 vs. control PASMC in 95% O₂-5% CO₂; + p<0.05 vs. PPHN PASMC in 21% O₂-5% CO₂.

Figure 5.

MnSOD Expression and Activity are Increased in PPHN PASMC. A) MnSOD protein expression is analyzed by Western blot, normalized for β -actin ($n=6$). Data are shown as mean $\pm$ SEM relative to control PASMC in 21% O₂-5% CO₂. B) A representative Western blot is shown for MnSOD and β -actin in control and PPHN PASMC. C) Protein was harvested from control and PPHN PASMC exposed to either 21% O₂-5% CO₂ or 95% O₂-5% CO₂ for 24 hours. MnSOD activity was measured with a commercially-available colorimetric assay as previously described ($n=5$). * $p<0.05$ vs. control PASMC in 21% O₂-5% CO₂; # $p<0.05$ vs. control PASMC in 95% O₂-5% CO₂; + $p<0.05$ vs. PPHN PASMC in 21% O₂-5% CO₂.

Figure 6.

Mitochondrially-Targeted Catalase Decreases PDE5 Activity in PPHN PASM. Protein was harvested from control and PPHN PASM exposed to either 21% O₂-5% CO₂ or 95% O₂-5% CO₂ for 24 hours. Cells were infected with either an empty adenoviral vector, Y5, or with an adenoviral construct expressing mitochondrially-targeted catalase (Mcat). PDE5-specific cGMP hydrolytic activity is measured using a commercially-available colorimetric assay (n=8 for control PASM and n=15 for PPHN PASM, read in duplicate). Data are shown as mean ± SEM pmol cGMP hydrolyzed/mg protein/min. * p<0.05 vs. control PASM in 21% O₂-5% CO₂; # p<0.05 vs. control PASM in 95% O₂-5% CO₂; + p<0.05 vs. PPHN PASM in 21% O₂-5% CO₂; & p<0.05 vs. PPHN PASM in 95% O₂-5% CO₂.

Figure 7.

Treatment with PEG-Catalase Decreases PDE5 Expression in PPHN Resistance PA. Ovine resistance PA were harvested from PPHN lambs ventilated with 100% O₂ for 24 hours (PPHN100, n=8) and PPHN lambs ventilated with 100% O₂ for 24 hours and treated with PEG-Catalase (PPHN100+Cat, n=5). A) PA PDE5 protein expression was analyzed via Western blot, with β-actin normalization. Data are shown as mean±SEM relative to PPHN100 lambs. * p<0.05 vs. PPHN100 lambs. B) Representative Western blots are shown for PDE5 and β-actin in resistance PA. C) The left column shows phase contrast images (20X) of frozen lamb lung sections. The right column shows the corresponding sections stained for PDE5 with immunofluorescence shown as green.

Figure 8.

Treatment with PEG-Catalase Decreases PDE5 Activity and Increases cGMP in PPHN Resistance PA. Ovine resistance PA were harvested from PPHN lambs ventilated with 100% O₂ for 24 hours (PPHN100, n=8) and PPHN lambs ventilated with 100% O₂ for 24 hours and treated with PEG-Catalase (PPHN100+Cat, n=5). A) PA PDE5 specific activity was measured as the sildenafil-inhibitable fraction of total cGMP hydrolysis, normalized for total mg of protein (read in duplicate). Data are shown as mean±SEM. B) PA were assayed for cGMP by EIA, and cGMP was normalized for mg PA tissue (read in duplicate). Data are shown as mean±SEM. *p<0.05 vs. PPHN100 lambs.