SOD and inhaled nitric oxide normalize phosphodiesterase 5 expression and activity in neonatal lambs with persistent pulmonary hypertension

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Persistent pulmonary hypertension of the newborn (PPHN) is a clinical syndrome occurring in 2–6 per 1,000 live births with a significant risk of death as well as short-term and long-term morbidity (47). It is caused by multiple disease processes that include pulmonary vascular remodeling and smooth muscle hyperplasia, often in the absence of significant lung parenchyma pathology (21, 38). Current clinical management strategies include mechanical ventilation with high levels of inspired oxygen and inhaled nitric oxide (iNO). Although iNO decreases need for extracorporeal membrane oxygenation (ECMO), it has not been proven to improve survival, and 40% or more of infants have a limited or transient response (8, 39a, 41).

Increased oxygen tension after birth stimulates pulmonary vascular endothelial nitric oxide synthase (eNOS) expression, activity, and NO production. NO activates soluble guanylate cyclase (sGC) to produce cGMP, a critical second messenger for pulmonary vasodilation (3, 29). Phosphodiesterase 5 (PDE5) is the major cGMP-hydrolytic PDE in the lung and downregulates this pathway. In lambs, PDE5 expression decreases shortly after birth and then increases again 4–7 days later, suggesting developmental regulation (19, 40).

Although oxygen is widely used as a pulmonary vasodilator in the clinical setting of PPHN, the effects of prolonged exposure to high O2 concentrations in combination with mechanical ventilation are not well-characterized. Emerging evidence in adult and neonatal disease states raises concern about the potential for oxidative stress inducing significant lung parenchymal and vascular injury (13, 14, 26, 27, 30, 42, 46). During normal aerobic metabolism, eukaryotic cells produce reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H2O2) that must be tightly regulated to prevent cellular injury. Multiple cell types present in the lung can produce ROS that may affect vascular tone and stimulate vascular smooth muscle cell growth (24).

We previously reported that NOS expression and activity are downregulated by excessive pulmonary ROS production in PPHN lambs. sGC and PDE5 are additional targets for ROS-mediated regulation in the pulmonary vasculature. sGC is a heterodimer consisting of α- and β-subunits, and earlier studies indicate its expression and activity are decreased in fetal lambs with PPHN (5, 35, 45). More recently, it has been suggested that the heme moiety at the sGC active site can be modified by oxidative stress, rendering the enzyme unresponsive to NO stimulation (43). Recent data from our group and others suggest that PDE5 expression and activity may be upregulated by ROS in the pulmonary and systemic vasculature (13, 39).

SOD catalyzes the dismutation of superoxide into H2O2 and oxygen, serving as an antioxidant and playing an important role in vascular tone, lung function, and the metabolism of NO (12, 15, 22). We recently reported that a single dose of recombinant human Cu/Zn SOD (rhSOD) at birth improved oxygenation in lambs with PPHN. Furthermore, rhSOD decreased the generation of ROS and normalized eNOS expression and function in the PPHN vasculature (14, 28). Additionally, iNO, the only Food and Drug Administration-approved therapy for PPHN, also decreased ROS and normalized eNOS expression in the PPHN vasculature (14). Our hypothesis is that ROS-scavenging therapies such as iNO and rhSOD may...
also normalize sGC, PDE5, and ultimately steady-state cGMP levels. A better understanding of these pathways will advance the effort to determine the potential harm of ROS and the therapeutic value of ROS scavengers such as rhSOD in the management of PPHN.

**MATERIALS AND METHODS**

**Fetal surgery and 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. Pregnant ewes and newborn lambs were obtained from the Swartz family farm (Attica, NY). Six total groups were studied and are described in detail below: fetal control lambs (n = 8), spontaneously breathing 1-day-old (1DSB) lambs (n = 4), fetal PPHN lambs (n = 7), PPHN lambs ventilated for 24 h with 100% O2 (100% O2, n = 4). 100% O2, 100% O2 + 100% O2 + rhSOD (100% O2 + rhSOD, n = 4). Fetal and ventilated lambs were studied at 135 days gestation (term = 143–145 days). 1DSB lambs were healthy newborn lambs that delivered spontaneously at comparable gestation with the experimental lambs, were fed normally, and breathed room air; three were born after time-dated pregnancies with gestational ages of 134–138 days, and one additional lamb exhibited hoof and physical maturity equivalent to 135-day-gestation lambs. At ~24 h of life, these 1DSB lambs were anesthetized with pentothal and killed by rapid exsanguination through a direct cardiac puncture. Pulmonary hypertension was established by antenatal ductus arteriosus ligation in lambs as previously described (28). Briefly, fetal surgery was performed on anesthetized pregnant ewes at 126 days of gestation, during which the fetal head and left upper extremity were exposed, a left thoracotomy was performed, and the ductus arteriosus was ligated. The fetal chest was closed, the fetus was returned to the uterus, and the ewe’s uterine and abdominal incisions were closed.

At 135 days gestation, the pregnant ewes were anesthetized with pentothal and halothane, and the fetal lambs were delivered by cesarean section to avoid unattended spontaneous deliveries. Fetal control and ligated (PPHN) lambs were anesthetized and killed as described above before their first breath. Additional PPHN lambs were delivered by cesarean section, placed under servocontrolled radiant warmers, intubated, given 3 ml/kg calfactant (Onyx, Amherst, NY), and ventilated with 100% O2 alone, 100% O2 with 20 ppm iNO, or 100% O2 with 5 mg/kg intratracheal rhSOD (1 mg = 3,850 units of activity; Savient Pharmaceuticals, Iselin, NJ). The subsequent care protocols have been described in detail previously (14). After 24 h of ventilation, lambs were anesthetized and killed as above. The heart and lungs were removed en bloc, and 5th generation pulmonary arteries (PA; inner diameters of 500 μm) were dissected and isolated. Tissue samples were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

**Quantitative reverse transcription real-time PCR.** Frozen PA tissue was ground on liquid nitrogen, and RNA was isolated using the Aurum total RNA mini kit (Bio-Rad, Hercules, CA). RNA was quantified using the Quant-iT RiboGreen assay (Molecular Probes/Invitrogen, Carlsbad, CA). 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) and the iCycler iQ real-time PCR detection system (Bio-Rad) as previously described (13, 14). Briefly, real-time PCR for PDE5 with a β-actin internal control was performed with 35 cycles of real-time data collection using 95°C for 10 s and 49.6°C for 45 s. Real-time PCR for sGCβ with an 18S internal control was performed with 40 cycles of real-time data collection using 95°C for 10 s and 49°C for 45 s. PDE5, β-actin, sGCβ, and 18S primers were designed using Beacon Designer software (Premier BioSoft International, Palo Alto, CA) and were as follows: PDE5 sense 5′-GCAAGGAAGGTATCAGAGG-3′, PDE5 antisense 5′-ACAACAAATGGTCTAAGAGG-3′, β-actin sense 5′-AATCTCTCAACTACAT-3′, β-actin antisense 5′-TGATCCTTGTACGAGG-3′, sGCβ sense 5′-AACCTCTCAAGCCTCTATC-3′, sGCβ antisense 5′-CTACCCGTCATGACTGTCCTC-3′, 18S sense 5′-AGGGTCCGATTCGGAGAGGG-3′, 18S antisense 5′-CATCCAAATXGCGCTCG-3′. For all primers, amplicons between 75 and 150 bp in length were produced, and there was a single product on melt-curve analysis with good correlation for efficiency and standard curves (r² ≥ 0.98). All samples were analyzed in duplicate. Relative PDE5 amounts were normalized to β-actin, and relative sGCβ amounts were normalized to 18S using the ΔΔCT method (34). Data are shown as fold relative to fetal control lambs.

**Western blot analysis.** Frozen PA tissue was homogenized, and total protein was collected using the PARIS kit (Ambion, Austin, TX) supplemented with protease (Sigma, St. Louis, MO) and phosphatase inhibitors (EMD Biosciences, San Diego, CA) as previously described (13, 14). Protein concentration was measured using the Bradford method (6). PDE5 and sGC protein expression was assessed in all animals in all groups via Western blot. Total protein (40 μg) was separated on a 4–20% SDS-polyacrylamide gel (Bio-Rad) and then transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL). Western blot was then performed as previously described (13, 14). Briefly, membranes were blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (1 × TBST) and incubated overnight at 4°C with the primary antibody in 5% milk + 1 × TBST at an appropriate dilution: 1:500 for mouse anti-PDE5 (BD Transduction Laboratories, San Jose, CA), 1:500 for rabbit anti-sGCβ (BD Transduction Laboratories), and 1:2,000 for mouse β-actin (Sigma)). The membranes were washed and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) diluted 1:1,000 in 5% milk + 1 × TBST. Membranes were then washed and exposed via chemiluminescence (Pierce). Bands were analyzed using a Digital Science Image Station (Kodak, Rochester, NY). PDE5 or sGCβ expression within each Western blot was normalized to β-actin. Data are shown as fold relative to fetal control lambs.

**Caspase-3/7 activity assay.** Total lung protein was prepared as described above and was assayed the same day using the commercially available Caspase-Glo 3/7 Assay (Promega, Madison, WI). Briefly, 100 μg of total lung protein was incubated in duplicate with the caspase-3/7 substrate in a white-walled 96-well luminescence plate at room temperature for 1 h. Luminescence was measured using a SpectraMax Gemini XS dual-scanning microplate spectrophotometer (Molecular Devices). Data are shown as relative light units (RLU) per microgram total protein.

**PDE5 activity assay.** For the sheep PA tissue, protein was prepared fresh from snap-frozen tissue as described above. The total PA protein was immediately placed on ice and assayed the same day as previously described (13). The protein was purified over a CentriSpin column to remove any phosphate contamination (Princeton Separations, Adelphia, NJ). Protein concentration was determined as described above. Total protein (5 μg) was assayed for cGMP-hydrolytic activity using a commercially available colorimetric cyclic nucleotide PDE 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 min and then incubated with the Biomol Green reagent at room temperature for 20 min. Results were measured using a Labsystems Multiskan EX automated plate reader (Thermo Electron, Waltham, MA) at 620 nm. The difference between the picomoles cGMP hydrolyzed per milligram total protein per minute without sildenafil and the picomoles cGMP hydrolyzed per milligram total protein per minute with sildenafil represents the PDE5-specific cGMP-hydrolytic activity. Results are shown as the ratio of PDE5-specific picomoles cGMP hydrolyzed per milligram total protein per minute.

**sGC activity assay.** Total lung protein was prepared as described above, immediately placed on ice, and assayed the same day as...
previously described (5, 13, 31). Protein concentration was determined as described above. Total protein (50 μg) was incubated for 10 min at 37°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5; Fisher Scientific, Pittsburgh, PA), 4 mM MgCl2 (Fisher Scientific), 0.5 mM 3-isobutyl-1-methylxanthine (Biomol), 7.5 mM creatine phosphate (Sigma), 0.2 mg/ml creatine phosphokinase (Sigma), 1 mM sodium nitroprusside (Sigma), and 1 mM GTP (Sigma). The reaction was terminated by the addition of HCl (Sigma) to a final concentration of 0.1 N. Each sample was dried in a Speed-Vac and resuspended in 100 μl of cGMP enzyme immunoassay (EIA) buffer (Cayman Chemical, Ann Arbor, MI). cGMP in the reaction mixture was measured by EIA in duplicate using a commercially available kit (Cayman Chemical). Each sample was acetylated according to the manufacturer’s protocol, and then EIA was performed. Results were measured using a Labsystems Multiskan EX automated plate reader at 420 nm. sGC activity results are shown as picomoles cGMP per milligram total protein per minute for each sample.

**cGMP EIA.** To measure cGMP levels, sheep PA tissue snap-frozen in liquid nitrogen was weighed and homogenized in 10 volumes of 5% TCA (Sigma). Precipitate was removed by centrifugation at 1,500 g for 10 min. The TCA was then extracted using water-saturated ether according to the manufacturer’s protocol (Cayman Chemical). Samples were acetylated according to the manufacturer’s protocol. cGMP content of the PA samples was measured by EIA in duplicate in the absence of any phosphodiesterase inhibitor, using a commercially available kit (Cayman Chemical). Results were measured using a Labsystems Multiskan EX automated plate reader at 420 nm. Results are shown as picomoles cGMP per milligram frozen tissue.

**Statistical analysis.** All data are expressed as means ± SE with each n representing a single lamb studied. Results were analyzed by ANOVA with Bonferroni post hoc analysis using Prism software (GraphPad Software, San Diego, CA). Statistical significance was set at P < 0.05.

**RESULTS**

**Treatment with iNO or rhSOD does not impact sGCβ expression in ovine PA.** Others have previously described that sGC-α- and β-subunit expression normally increases after birth in PA from newborn rats and piglets (5, 35). In contrast, we (45) previously reported that the sGC-α subunit protein expression is decreased in PA from fetal PPHN lambs compared with healthy controls. Here, we demonstrate that sGCβ mRNA and protein expression increase in 1DSB compared with fetal lambs (mRNA: 9.3 ± 2.7-fold vs. fetal control; protein: 1.6 ± 0.2-fold vs. fetal control; Fig. 1A and B). However, in contrast to our previous findings for sGCα, we observed no change in sGCβ mRNA and protein expression in the PA of fetal PPHN lambs vs. fetal control lambs (Fig. 1, A and B). In PPHN lambs ventilated with 100% O2 for 24 h, sGCβ protein expression increased in the small PA to levels comparable with 1DSB lambs (1.6 ± 0.1-fold vs. fetal control; Fig. 1B), but sGCβ mRNA expression was unchanged (Fig. 1A). Concurrent administration of iNO or rhSOD produced no significant change in sGCβ mRNA or protein expression in the small PA compared with PPHN lambs ventilated with 100% oxygen alone (Fig. 1, A and B). A representative Western blot demonstrating these changes in sGCβ protein expression is shown in Fig. 1C.

**Ventilation with 100% O2 in PPHN lambs increases sGC activity in ovine lungs.** We (45) previously reported decreased sGC activity in endothelium-denuded small PA from PPHN fetuses vs. fetal controls. In the current study, we demonstrate no significant difference in sGC activity in ovine lung between fetal control, fetal PPHN, and 1DSB lambs (Fig. 2). Interestingly, ventilation with 100% O2 significantly increased sGC activity vs. fetal control, fetal PPHN, and 1DSB lambs (2 ± 0.1-fold vs. fetal controls; Fig. 2). Finally, treatment with iNO or rhSOD reduced sGC activity to levels comparable with the fetal control, 1DSB, and fetal PPHN lambs (Fig. 2).

**Treatment with iNO or rhSOD decreases PDE5 expression in ovine PA.** Others have previously demonstrated that PDE5 in the lungs is developmentally regulated, decreasing shortly after birth and then rising again 4–7 days later (13, 19, 40). More recently, we (13) reported that mechanical ventilation with 100% O2 for 24 h increases PDE5 expression in small PA in control, healthy lambs. Here, we demonstrate that there is no
significant change in PDE5 mRNA levels in small PA between fetal controls, healthy 1DSB lambs, and PPHN fetuses (Fig. 3A). However, PDE5 protein expression significantly decreases in small PA of the 1DSB compared with fetal control lambs (1DSB: 0.55 ± 0.1-fold vs. fetal controls; Fig. 3B). We also observed decreased PDE5 protein in PPHN fetuses (0.32 ± 0.04-fold vs. fetal controls; Fig. 3B).

In the PPHN lambs ventilated with 100% O₂ for 24 h, there was a trend toward increased PDE5 mRNA expression in small PA relative to fetal control, 1DSB, and fetal PPHN lambs (1.5 ± 0.2-fold vs. fetal controls; Fig. 3A). PDE5 protein expression was significantly increased in small PA of the lambs ventilated with 100% O₂ for 24 h relative to both 1DSB and fetal PPHN lambs, reaching levels comparable with fetal control lambs (Fig. 3B). Ventilation of PPHN lambs with iNO or rhSOD significantly decreased PDE5 mRNA and protein expression in the small PA compared with PPHN lambs ventilated with 100% oxygen alone (Fig. 3, A and B). A representative Western blot demonstrating these changes in PDE5 protein expression is shown in Fig. 3C.

Since PDE5 mRNA was relatively unchanged but PDE5 protein was different across our three nonventilated experimental groups, we sought to determine PDE5 protein stability in the 1DSB and PPHN fetal lambs. The only enzyme currently known to regulate PDE5 protein stability is caspase-3. There are five consensus caspase-3 sites in the PDE5 enzyme including one at the cGMP-hydrolytic site. Others have previously demonstrated that caspase-3 can cause decreased PDE5 activity by proteolytic degradation of PDE5 (16, 17). Consistent with this hypothesis, we found a significant increase in caspase-3 activity in lung tissue from 1DSB lambs relative to fetal controls and a nonsignificant trend toward increased caspase-3 activity in PPHN fetal lambs relative to fetal controls (Fig. 4). Finally, in the lambs ventilated with 100% O₂ with or without iNO or rhSOD, caspase-3 activity is decreased relative to the 1DSB lambs, suggesting that the PDE5 downregulation seen in these lambs is not dependent on proteolytic degradation (Fig. 4).

Treatment with iNO or rhSOD decreases PDE5 activity in ovine PA. Others have previously suggested that PDE5 activity may be increased in fetal lambs with PPHN (10, 11, 20, 51). More recently, we demonstrated that ventilation with 100% O₂ for 24 h increases pulmonary PDE5 activity in healthy, control lambs (13). Although we observed decreased PDE5 protein expression in small PA of 1DSB (Fig. 3B), this was not associated with a significant change in PDE5 activity compared with fetal control lambs (Fig. 5). We observed a trend toward increased PDE5 activity in the small PA of fetal PPHN vs. fetal control lambs, (2.3 ± 0.7-fold vs. fetal controls; Fig. 5). However, in the PPHN lambs ventilated with 100% O₂ for 24 h, there was a dramatic increase in the PDE5 activity relative to fetal control, 1DSB, and fetal PPHN lambs (11.7 ± 2-fold vs. fetal controls; Fig. 5). Treatment with iNO or rhSOD significantly decreased PDE5 activity compared with PPHN
PA to levels comparable with the 1DSB lambs (Fig. 6). Concurrent iNO or rhSOD restored cGMP levels in the small PA of neonatal sheep ventilated with 100% O2 for 24 h compared with healthy 1DSB lambs (Fig. 6). However, cGMP concentrations were significantly lower in PPHN lambs ventilated with 100% O2 for 24 h compared with healthy 1DSB lambs (Fig. 6). Concurrent iNO or rhSOD restored cGMP levels in the small PA to levels comparable with the 1DSB lambs (Fig. 6).

**DISCUSSION**

PPHN is a clinical syndrome with multiple etiologies that involve complex alterations in signaling pathways. Following ductal ligation of the fetal lamb, fetal PA pressure increases, leading to rapid pulmonary vascular remodeling and a clinical and histological disease process consistent with that seen in idiopathic PPHN (36, 49). Previous studies using the ductal ligation model suggest that ROS may play a significant role in the pathogenesis of PPHN (7, 23, 28, 48). In addition to the implications for antenatal remodeling, these findings raise concerns about the impact of traditional therapies on disease progression. Mechanical ventilation with high concentrations of O2 is typically used in clinically significant PPHN to minimize hypoxemia and to promote pulmonary vasodilation. Our findings represent the first comprehensive study of the sGC-cGMP-PDE5 signaling axis in small PA from PPHN lambs treated with therapies commonly used in human infants with PPHN, i.e., mechanical ventilation, high concentrations of O2, and iNO.

Although total lung tissue includes multiple different components such as vessels, airways, and parenchymal tissue, the studies presented here focused on endogenous sGC, PDE5, and cGMP in the small PA of neonatal sheep. In healthy control lambs, we demonstrate that following birth, sGCβ mRNA and protein expression is increased (Fig. 1, A and B), PDE5 protein expression is decreased (Fig. 3B), and steady-state cGMP is increased (Fig. 6) in the small PA. These results are consistent with previous studies showing an increase in sGC expression and a decrease in PDE5 expression after birth in a variety of lung and PA animal tissues (5, 13, 19, 35, 40). These changes in sGC, PDE5, and cGMP represent key events for a normal pulmonary vascular transition to extrauterine life, and disruption of this normal pattern could cause or exacerbate PPHN.

Interestingly, we did observe some differences in PPHN fetuses in the current study compared with those previously reported in the literature. Previous studies showed that ductal ligation of ovine fetuses decreased sGCα protein expression, sGC activity, and cGMP levels in small PA (45) as well as decreased sGCα and sGCβ expression in whole lung tissue (4). In the current study, we examined the sGCβ subunit expression as opposed to sGCα subunit expression since the sGCβ subunit is the heme-containing and active subunit. We found no difference in sGCβ protein expression between fetal control and
fetal PPHN lambs (Fig. 1B), indicating probable subtle regulatory differences between the two sGC subunits in the pulmonary vasculature. We also found no difference in sGC activity between fetal control and fetal PPHN lambs (Fig. 2).

Previous reports also showed increased PDE5 activity in lung tissue of PPHN fetuses, which contains many diverse cell types including airway smooth muscle cells that express PDE5 (20). In the current study, we measured PDE5 expression and activity specifically in the small PA, an approach that may be more effective at determining effects relevant to pulmonary vascular tone. We found a decrease in PDE5 protein expression (Fig. 3B) and a modest trend toward increased PDE5 activity in the small PA of PPHN vs. control fetuses (Fig. 5). Consistent with the minimal changes in sGC and PDE5 activity, there was no significant difference in cGMP levels in the small PA of PPHN vs. control fetuses (Fig. 6).

A key methodological difference in the present study may explain these differences. We delivered the PPHN fetuses 9 days after ductal ligation to minimize an unacceptably high rate of stillbirth and to allow for lambs with pulmonary hypertension that was survivable for the 24-h ventilation period. In contrast, the previous studies were performed 10–16 days after ductal ligation. Despite the earlier delivery, the lambs in the current study had severe hypoxemia and maintained all the histopathological hallmarks of PPHN (28). Thus the previously published effects of ductal ligation on sGCα protein expression, sGC activity, cGMP levels, and PDE5 protein expression may be due to progression of vascular dysfunction and remodeling in response to a more prolonged period of exposure to antenatal pulmonary hypertension (4, 45).

Interestingly, lambs undergoing a normal postnatal transition (1DSB) and fetal lambs with PPHN both demonstrated decreased PDE5 protein, but not mRNA, expression (Fig. 3, A and B). Since PDE5 mRNA is unchanged in both groups, it seems likely that the decrease in PDE5 protein is a result of increased proteolytic degradation. Caspase-3 is the only enzyme that has previously been described to regulate PDE5 degradation (16, 17). Consistent with this hypothesis, we observed increased caspase-3 activity in the 1DSB lambs and a trend toward increased caspase-3 activity in PPHN fetuses relative to the control fetuses (Fig. 4). The mechanism by which caspase activity is induced in these two groups is unknown. As pulmonary blood flow increased in both groups relative to the control fetuses, we speculate that mechanical forces such as cyclic stretch may be involved in regulating caspase activity in neonatal lambs.

In the current study, we also extend our previous findings to include an evaluation of the changes that occur following birth and onset of mechanical ventilation. One of the key findings in our study is that ventilation of PPHN lambs with 100% O2 was associated with a significant decrease in steady-state cGMP levels in the small PA relative to normal 1-day-old lambs (Fig. 6). Decreased cGMP is likely to lead to increased pulmonary vascular tone, as evidenced in the increased contractile responses to norepinephrine previously reported in PPHN lambs ventilated with 100% O2 for 24 h (28). We recently demonstrated that postnatal ventilated PPHN lambs have decreased eNOS expression and function in their small PA, which could decrease NO production and subsequent activation of the sGC enzyme (14). Interestingly, ventilation with 100% O2 did not negatively impact sGCβ protein expression (Fig. 1B) or sGC activity (Fig. 2), suggesting that sGC would still be responsive to NO. In contrast, we observed an increase in PDE5 protein expression (Fig. 3B) and activity (Fig. 5) in the PPHN lambs ventilated with 100% O2, suggesting that dysregulation of PDE5 is an important cause of the depressed cGMP levels we observed in these lambs. Taken together, these data suggest that ventilating PPHN lambs with 100% O2 downregulates eNOS and upregulates PDE5, which together significantly decrease steady-state cGMP levels in these animals (14).

Mechanical ventilation with 100% O2 may produce multiple stresses on the vascular smooth muscle cells, including mechanical forces from the ventilation and increased ROS from the high fraction of inspired oxygen. We (14, 28) previously demonstrated that ventilation of PPHN lambs with 100% O2 leads to increased oxidative stress or ROS as measured by increased dihydrothioglycolic staining and increased 8-isoprostane levels. Although we noted decreased caspase-3 activity as a possible cause of the increased PDE5 protein levels, we also observed a modest trend toward increased PDE5 mRNA expression (Fig. 3A), suggesting a potential role for ROS-mediated transcriptional regulation in these lambs as well. Consistent with a model that incorporates ROS-mediated transcriptional regulation, we (13) previously demonstrated that exposure to 95% O2 for 24 h leads to increased ROS and increased PDE5 mRNA and protein expression in isolated fetal PA smooth muscle cells and in ventilated control lambs. In the current study, we now report that a 24-h period of ventilation with 100% O2 increases PDE5 expression and activity in PPHN lambs (Figs. 3 and 5) and note that the increase in PDE5 activity in the PPHN lambs is much more dramatic than that seen in the control lambs (13). The mechanism by which ROS impacts PDE5 transcription regulation, and thus expression, remains unknown, but the PDE5 promoter has been described to contain binding sites for Sp1, a redox-sensitive transcription factor (32, 33).

We previously reported that treatment of PA smooth muscle cells with an antioxidant, N-acetylcysteine, was sufficient to decrease PDE5 expression and activity. These data suggested to us that ROS are a primary regulator of both PDE5 expression and activity in the vascular smooth muscle (13). If ROS,
rather than mechanical forces from ventilation, are the primary regulators of PDE5 in the intact PPHN lambs, then antioxidant therapy should be sufficient to decrease PDE5 expression and activity. We (14, 28) have previously shown that treatment with iNO or rhSOD in PPHN lambs ventilated with 100% O_2 decreases oxidative stress as measured by decreased dihydroethidium staining and decreased 8-isoprostane levels. We now extend those observations and demonstrate that iNO or rhSOD treatment also significantly decreases PDE5 mRNA expression (Fig. 3A), protein expression (Fig. 3B), and activity (Fig. 5) relative to PPHN lambs ventilated with 100% O_2 alone, suggesting that increased ROS are the primary mediators of increased PDE5 expression and activity in the remodeled pulmonary vasculature associated with PPHN. Similarly, iNO or rhSOD normalize cGMP levels in small PA to levels comparable with the 1DSB lambs (Fig. 6), suggesting that these treatments are effective in restoring signaling patterns more consistent with those associated with the normal postnatal pulmonary vascular transition. Interestingly, iNO or rhSOD also decreased sGC activity relative to PPHN lambs ventilated with 100% O_2 alone, although the levels did not differ significantly from healthy 1DSB lambs (Fig. 2). This finding lends further support to the hypothesis that PDE5 is a primary regulator of cGMP concentrations in the pulmonary vasculature. Taken together, these data are most consistent with a hypothesis where mechanical ventilation with 100% O_2 leads to increased ROS, which in turn increase PDE5 expression and activity, leading to decreased cGMP (Fig. 7). Treatment with iNO or rhSOD has an antioxidant effect leading to decreased ROS, decreased PDE5 expression and activity, and increased cGMP (Fig. 7). This finding is in contrast to our (14) previously published data where rhSOD, but not iNO, fully restored eNOS function in the PPHN lambs. We speculate that the combination of iNO with 100% O_2 results in nitration of some, but not all, proteins, leading to differential effects on eNOS and PDE5 expression and activity. Based on recently published findings by Zhao et al. (50), we would further speculate that mechanical ventilation with 100% O_2 + iNO may nitrate and inactivate PKG, which phosphorylates and activates PDE5 to downregulate NO signaling. We (13) previously published that 24 h of hyperoxia leads to increased PKG-mediated phosphorylation of PDE5 in PA smooth muscle cells. Thus we propose that iNO treatment in the lambs may result in nitration and inactivation of PKG with a subsequent decrease in PDE5 phosphorylation and activity. However, rhSOD treatment appears to normalize both eNOS and PDE5 in the PPHN vasculature, thus explaining why those lambs have steady-state cGMP levels most like the 1DSB lambs (Fig. 6). In intact PPHN lambs, delayed rhSOD administration at 4 h after delivery still led to improved oxygenation, likely due to normalization of both eNOS and PDE5 expression and function (28). An interesting and important extension of our studies in the future will be to examine the effects of iNO and rhSOD in PPHN lambs ventilated with lower O_2 concentrations. Although technically challenging due to the severity of hypoxemia in PPHN lambs (28), these studies will reveal whether the degree of hypoxemia affects the mechanisms under investigation.

Previous studies have examined the utility of both sGC activators, such as BAY 41-2272, and PDE5 inhibitors, such as dipyridamole, in the ovine PPHN model (9, 10, 51). Although both classes of drugs are effective in decreasing pulmonary vascular resistance, our data suggest that the effectiveness of sGC activators could be lessened by PDE5 upregulation. Consistent with our animal data, early preliminary studies of sildenafil, a selective inhibitor of PDE5, in human infants have shown the potential of this therapeutic approach (1, 2, 37, 44). We speculate that either antioxidant therapies, such as rhSOD, or direct inhibitors of PDE5 may represent important future therapies for infants with PPHN.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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