Superoxide dismutase restores eNOS expression and function in resistance pulmonary arteries from neonatal lambs with persistent pulmonary hypertension

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AS PART OF THE NORMAL PHYSIOLOGICAL transition at birth, the pulmonary vascular resistance decreases through complex pathways allowing pulmonary blood flow to increase by 10-fold. Physical and biochemical processes that contribute to the normal newborn pulmonary transition include mechanical distension of the lungs and increased PO2, which stimulate endothelial nitric oxide (NO) synthase (eNOS) (28, 34, 40). eNOS converts L-arginine to L-citrulline and NO, which in turn activates soluble guanylate cyclase in vascular smooth muscle cells to generate cGMP, ultimately leading to vasodilation (1). Emerging evidence continues to increase the understanding of the complex regulation of eNOS expression and activity, including the potential effects of O2 (12, 33).

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- and long-term morbidity (19, 44). It is caused by multiple disease processes, which lead to an abnormal transition at birth, resulting in continued elevated pulmonary vascular resistance, right- to left-sided extrapulmonary shunting of deoxygenated blood, and hypoxemia. Pathological findings include pulmonary vascular remodeling and smooth muscle hyperplasia in the absence of significant lung parenchyma pathology (15, 29), changes that may be the result of prolonged fetal stress and hypoxia. Clinical management strategies include mechanical ventilation with high levels of inspired O2 and inhaled NO (iNO). Although iNO decreases the need for extracorporeal membrane oxygenation support, it has not been proven to improve survival, and ~50% of infants have a limited or transient response (7, 14, 32).

O2 stimulates NO production by fetal pulmonary artery (PA) endothelial cells (12), dilates the pulmonary vasculature, and increases pulmonary eNOS expression in the fetal lamb (2). Although O2 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 (10, 21, 22, 26, 38, 41, 43). Even during normal aerobic metabolism, eukaryotic cells produce reactive oxygen species (ROS), such as superoxide and H2O2, which must be tightly regulated to prevent undesired cellular injury. Multiple cell types present in the lung, particularly during inflammation, can produce ROS, which may affect vascular tone and stimulate vascular smooth muscle cell growth (20). ROS may also directly and indirectly interact with eNOS and NO, such as superoxide combining with NO to form the potent oxidant peroxynitrite (9, 12).

SOD catalyzes the dismutation of superoxide into H2O2 and O2, serving as an antioxidant and playing an important role in vascular tone, lung function, and metabolism of NO (9, 11, 17). Our hypothesis is that ROS play a critical role in the pathophysiology of PPHN and that ROS scavengers such as SOD may represent new therapeutic agents. We recently reported...
that a dose of recombinant human SOD (rhSOD) at or shortly after birth improved oxygenation in lambs with PPHN created by antenatal ligation of the ductus arteriosus (23). The goal of the present studies is to further investigate the pathways involved in PPHN, particularly those relating to the effects of SOD, on eNOS expression and function. 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 SOD, 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 Northwestern University approved this study. Pregnant ewes and newborn lambs were obtained from the Swartz family farm (Attica, NY). One-day spontaneously breathing (1DSB) lambs were healthy newborn lambs that delivered spontaneously at comparable gestation to the experimental lambs, fed normally, breathed room air, and then at ~24 h of life were anesthetized with thiopental sodium (Pentothal) and killed by rapid exsanguination through a direct cardiac puncture. Pulmonary hypertension was established by antenatal duct ligation in lambs, as previously described (23). Ewes with twin gestations were selected for fetal ductal ligation surgery, so that the additional fetus could serve as a control. Briefly, fetal surgery was performed on anesthetized pregnant ewes at 126 days gestation: 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 (full term = 145 days), the pregnant ewes were anesthetized with thiopental sodium and halothane, and the fetal lambs were delivered by cesarean section to avoid unattended spontaneous deliveries. Fetal control and ligated (PPHN) 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 lambs were delivered by cesarean section, placed under servo-controlled radiant warmers, intubated, given Infusurf (3 ml/kg; ONY, 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 U of activity; Savient Pharmaceuticals, Iselin, NJ). Ventilator settings (peak inspiratory pressure and rate) were adjusted with 20 ppm iNO, or 100% O₂ with 5 mg/kg intratracheal rhSOD (1 mg = 3.850 U of activity; Savient Pharmaceuticals, Iselin, NJ). Ventilator settings (peak inspiratory pressure and rate) were adjusted to maintain arterial Pco₂ between 35 and 50 mmHg. The subsequent care protocols are described in detail elsewhere (23). After 24 h of ventilation, lambs were anesthetized and killed as described above. The heart and lungs were removed en bloc, and fifth-generation PA (500 μm ID) were dissected and isolated. Tissue samples were frozen in liquid nitrogen and stored at −80°C until analysis.

Quantitative RT real-time PCR. Frozen isolated PA tissue was ground on liquid nitrogen, and RNA was isolated utilizing 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 utilizing the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) with the iCycler iQ detection system (Bio-Rad), with 40 cycles of real-time data collection at 95°C for 20 s and 59.6°C for 1 min, followed by melt-curve analysis to verify the presence of a single product. Primers were kindly provided by Dr. Girija G. Kunduri and have been previously described (6). The sequences of the primers are as follows: 5’ CCTCCACCCTCAACAATCT 3’ (sense) and 5’ GCACAGCAGGTTGTCCTC 3’ (antisense) for eNOS and 5’ CGGACACGAGCAGGATTGACAG 3’ (sense) and 5’ ATGCCAGGAGTCCTGTCGTCTTATCG 3’ (antisense) for 18S RNA. For eNOS and 18S primers, 75- to 150-bp-long amplicons were produced, and there was a single product on melt-curve analysis with good correlation for efficiency and standard curves (r² ≥ 0.98). PCR product size was verified by agarose gel electrophoresis, and all samples were analyzed in duplicate. For each reaction, negative controls containing reaction mix and primers without cDNA were performed to verify that primers and reaction mixtures were free of template contamination. Relative eNOS amounts were normalized to 18S expression using the cycle threshold (ΔΔCt) method (25). Data are fold values relative to fetal control lambs.

Western blot analysis. Isolated frozen lung and PA tissue was homogenized, and total protein was collected using the PARIS kit (Ambion, Austin, TX), as previously described (10). Protein concentration was measured using the Bradford method (3). Total protein (40 μg) was separated on a 4–20% SDS-polyacrylamide gel (Bio-Rad) and then transferred from the gel to a nitrocellulose membrane (Amersham, Arlington Heights, IL). Western blot was then performed as previously described (10). Briefly, membranes were blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (1X TBST) and then incubated overnight at 4°C with the primary antibody in 5% milk + 1X TBST at an appropriate dilution [1:1,000 for mouse anti-eNOS (BD Transduction, San Jose, CA); 1:200 for goat anti-GTP cyclohydrolase I (GTP-CH1, Santa Cruz Biotechnology, Santa Cruz, CA); and 1:2,000 for mouse β-actin (Sigma, St. Louis, MO)]. The membranes were then washed and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) diluted 1:1,000 in 5% milk + 1X TBST. Membranes were then washed and exposed via chemiluminescence (Pierce). Bands were analyzed using a Digital Science Image Station (Kodak, Rochester, NY). eNOS expression within each Western blot was normalized to β-actin. Data are fold values relative to fetal control lambs.

Immunohistochemistry. Lung sections were prepared and stained as described previously (10). Briefly, the right middle lobe of the lung was removed, and OCT compound (VWR Scientific, West Chester, PA) was pushed gently into the deflated lobe and allowed to solidify on ice for ~15–20 min. Blocks were prepared and cut into 8- to 10-μm sections that were mounted onto charged slides for staining and stored at −80°C. Sections were subsequently fixed with acetone (Sigma) for 10 min at 4°C, allowed to air dry, and then washed with 1X PBS (Mediatech, Herndon, VA). Sections were blocked with 5% BSA (Sigma) + 1X TBST at room temperature for 1 h and then stained overnight at 4°C with anti-eNOS antibody (BD Transduction) at a 1:100 dilution in 5% BSA + 1X TBST. After sections were further incubated in Alexa Fluor 488 anti-mouse antibody (Molecular Probes/Invitrogen) at a 1:200 dilution in 5% BSA, the tissue localization and expression of eNOS were visualized with a Nikon Eclipse TE-300 fluorescent microscope with excitation at 495 nm and emission at 519 nm. Fluorescent images were captured using a CoolSnap digital camera with Metamorph imaging software (Molecular Devices, Sunnyvale, CA).

In situ analysis of superoxide generation. Frozen lung sections were exposed to 5 μM dihydroethidium (DHE; Molecular Probes/Invitrogen) in PBS. Slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Ethidium-stained slices were observed by fluorescence microscopy with excitation at 518 nm and emission at 605 nm. Fluorescent images were captured as described above. Tissue sections were processed and imaged in parallel. Isolated vessel studies. Fifth-generation intralobar PA (500 μm ID) were isolated, dissected with care to preserve the integrity of the endothelium, cut into ~1- to 2-mm-long, 1- to 2-mm rings, and studied using standard tissue bath techniques, as described previously (39). Rings were suspended in water-jacketed chambers filled with aerated (94% O₂-6% CO₂) Krebs-Ringer solution. To obtain a continuous recording of isometric force generation, we tied each vessel ring to a force displacement transducer (model UC2, Statham Instruments, Hato Rey, PR) that was connected to a recorder (Gould Instrument Systems, Valley View, OH). After the arterial rings were mounted, they were allowed to equilibrate in 20 min in the bathing solution. A micrometer was used to stretch the tissues repeatedly in small incre-
ments over the following 45 min until resting tone remained stable at a passive tension of 0.8 g for PA isolated from control lambs and 1.0 g for PA isolated from PPHN lambs. Preliminary experiments determined that this procedure provided an optimal length for generation of active tone to exogenous norepinephrine (NE). Wet tissue weights were obtained at the end of each experiment, and contraction responses were normalized to tissue weight.

The following pharmacological agents were used: indomethacin, dl-propranolol, norepinephrine hydrochloride (NE), and N-nitro-L-arginine (L-NA). Indomethacin was dissolved in ethyl alcohol. Sonication was used to dissolve L-NA in warmed Krebs solution. All other drugs were purchased from Sigma Aldrich (St. Louis, MO). Isolated PA were pretreated with indomethacin (10^{-3} M) to block endogenous prostaglandins and with propranolol (10^{-6} M) to block β-adrenergic receptors. Two arterial rings from each lamb were pretreated with L-NA (10^{-5} M) and then constricted with NE (3 × 10^{-7} M). Four arterial rings from each lamb were constricted with NE (3 × 10^{-7} M) without L-NA. This concentration of NE provided 40–50% of contraction force (expressed as grams of force per grams of tissue weight) generated by 118 mM KCl. The starting tensions generated by NE in the protocols with and without L-NA were recorded and corrected for wet tissue weight. The mean starting tension (expressed in grams of force per gram of wet tissue weight) from two to four PA rings per protocol was studied from each animal, and mean tension was used for analysis.

**Results**

**eNOS expression is increased in resistance PA after birth.** Significant literature implies that eNOS plays a critical role in normal transition after birth, and multiple studies have demonstrated that eNOS activity increases after birth, both as a consequence of increased O_{2} and mechanical forces (2, 8, 39). Consistent with these studies, eNOS RNA was increased in resistance PA from 1DSB lambs compared with fetal controls (Table 1). Similarly, eNOS protein expression in resistance PA from 1DSB lambs was increased compared with fetal controls (3.1 ± 1.1-fold, P < 0.05; Fig. 1, A and B). In contrast, there was no change in neuronal (nNOS) or inducible NO synthase (iNOS) protein expression in 1DSB lambs compared with fetal controls (see supplemental Fig. 1 in the online version of this article). Furthermore, as shown in Fig. 1C, eNOS expression was confined to the endothelial layer of the vessels in control and 1DSB lambs (Fig. 1C). In contrast, expression of nNOS and iNOS is largely confined to the smooth muscle layer of the vessels in control and 1DSB lambs (see supplemental Figs. 2 and 3). Ventilation with 100% O_{2} also significantly increased eNOS mRNA in control lambs compared with fetal lambs (Table 1). As shown in Fig. 2, ventilation with 100% O_{2} also significantly increased eNOS protein in control lambs compared with fetal lambs (2.5 ± 0.4-fold, P < 0.05), and this increase was similar in magnitude to that in the 1DSB lambs. The increase in eNOS protein expression in the control lambs ventilated with 100% O_{2} is an effect specific to eNOS, as much as nNOS protein expression was decreased and there was no change in iNOS protein expression in these lambs (see supplemental Fig. 1). Inasmuch as eNOS appears to be the primary NOS isoform that is upregulated in the pulmonary endothelium after birth and the primary NOS isoform that is impacted by mechanical ventilation with O_{2}, the remainder of the studies presented here will focus on regulation of eNOS in the neonatal pulmonary vasculature.

**eNOS expression does not increase in PPHN lambs ventilated with 100% O_{2}**. In contrast to our observations in control lambs, ventilation of PPHN lambs with 100% O_{2} did not increase eNOS mRNA or protein relative to fetal nonventilated PPHN lambs. eNOS mRNA and protein expression was also significantly less in ventilated PPHN lambs than healthy 1DSB lambs and control lambs ventilated with 100% O_{2} (Table 1).

**Table 1. eNOS mRNA expression in ovine resistance pulmonary arteries**

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>eNOS mRNA Expression</th>
<th>vs. Control Fetus</th>
<th>vs. PPHN Fetus</th>
<th>vs. 1DSB</th>
<th>vs. Control 100% O_{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control lambs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetus</td>
<td>5</td>
<td>1±0.28</td>
<td>NS</td>
<td>&lt;0.01</td>
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<tr>
<td>1DSB</td>
<td>7</td>
<td>17.1±7.0</td>
<td>&lt;0.01</td>
<td></td>
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<tr>
<td>100% O_{2}</td>
<td>6</td>
<td>8.6±1.6</td>
<td>&lt;0.05</td>
<td></td>
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<tr>
<td>100% O_{2} + iNO</td>
<td>4</td>
<td>6.9±2.7</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
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<tr>
<td>100% O_{2} + rhSOD</td>
<td>4</td>
<td>21.6±11.8</td>
<td>&lt;0.01</td>
<td></td>
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<td></td>
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<tr>
<td>PPHN lambs</td>
<td></td>
<td></td>
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<tr>
<td>Fetus</td>
<td>9</td>
<td>0.7±0.2</td>
<td>NS</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
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<tr>
<td>100% O_{2}</td>
<td>4</td>
<td>1.9±0.7</td>
<td>NS</td>
<td>&lt;0.05</td>
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<td>&lt;0.05</td>
</tr>
<tr>
<td>100% O_{2} + iNO</td>
<td>4</td>
<td>15.2±5.4</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% O_{2} + rhSOD</td>
<td>4</td>
<td>18.7±10.4</td>
<td>&lt;0.01</td>
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<td></td>
<td></td>
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</tbody>
</table>

Endothelial nitric oxide (NO) synthase (eNOS) values are means ± SE, expressed as fold relative to fetal control. 1DSB, 1-day spontaneously breathing lambs; PPHN, persistent pulmonary hypertension of the newborn; iNO, inhaled NO; rhSOD, recombinant human SOD; NS, not significant.

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Fig. 2). Furthermore, Fig. 3 shows significant vessel remodelling with marked smooth muscle hypertrophy in fetal and ventilated PPHN. Similar to control lambs, eNOS expression was confined to the endothelial layer of the vessels. Treatment with iNO or rhSOD rescues eNOS expression in PPHN lambs.

Treatment of control lambs with iNO did not cause any further increase in eNOS mRNA relative to ventilation of control lambs with 100% O₂ alone (Table 1). In contrast, addition of iNO significantly increased eNOS mRNA in PPHN lambs ventilated with 100% O₂ alone (Table 1; 18.6 ± 10.4 vs. 1.9 ± 0.7 fold, P < 0.05). In contrast to iNO treatment, ventilation with 100% O₂ + rhSOD increased eNOS protein equally in control and PPHN lambs (Fig. 2A; 4.1 ± 1.0 and 4.1 ± 1.1 fold in control and PPHN lambs, respectively). The
increases were significantly greater in the PPHN lambs than in fetal PPHN lambs and PPHN lambs ventilated with 100% O₂ alone (Fig. 2, A and B; P < 0.05). Furthermore, this eNOS expression in the PPHN lambs treated with 100% O₂ + rhSOD was appropriately localized in the endothelial layer of the vessels (Fig. 3).

**Ventilation with 100% O₂ increases ROS in PPHN lambs, but treatment with iNO or rhSOD decreases ROS.** Ventilation with high levels of O₂ was associated with accumulation of ROS, as measured by DHE fluorescence, in PPHN lambs that was greater than ROS accumulation in 1DSB control and fetal nonventilated PPHN lambs (Fig. 4; P < 0.05). Various strategies were employed, including treatment with iNO or rhSOD, in an attempt to reduce oxidative stress in these lambs. These strategies were equally effective at decreasing oxidative stress compared with ventilation with 100% O₂ alone in PPHN lambs, as measured by DHE fluorescence (1.8 ± 0.8 and 2.2 ± 0.9 fold in 100% O₂ + iNO and 100% O₂ + rhSOD lambs, respectively; P < 0.05 vs. 100% O₂ PPHN lambs; Fig. 4).

**Treatment with rhSOD, but not iNO, restores endogenous eNOS function in PPHN lambs ventilated with 100% O₂.** Recent studies demonstrated that ventilation with 100% O₂ increases NE-induced contractility of resistance PA in control and PPHN lambs relative to fetal and 1DSB lambs (21, 23). As shown in Fig. 5, ventilation of PPHN lambs with 100% O₂ with iNO or rhSOD significantly reduced NE-induced contractility, restoring it to levels similar to those observed in fetal and 1DSB control lambs. The difference in the contraction response of the resistance PA to NE alone vs. NE + L-NA, an eNOS inhibitor, was subsequently used as a measure of endogenous eNOS function. In Fig. 5, we demonstrated that addition of L-NA, an eNOS inhibitor, had no effect on vessel contractility in fetal PPHN lambs or those ventilated with 100% O₂ or 100% O₂ + iNO. However, treatment with a single dose of rhSOD restored the contraction response to L-NA, indicating restored endogenous eNOS function.

**Treatment with a single dose of rhSOD, but not iNO, increases BH₄ in PPHN lambs ventilated with 100% O₂.** Decreased levels of BH₄, a key cofactor for eNOS function, can lead to uncoupling of eNOS in PA endothelial cells (26). In addition, recent reports indicate that biopterin pools may be altered in other models of pulmonary hypertension (13, 16, 31). We hypothesized that rhSOD may improve eNOS function through an increase in available BH₄. Using HPLC, we determined that the total biopterin pool (BH₄ + BH₂ + biopterin), but not the BH₂ + biopterin pool, was increased in 1DSB lamb
lungs relative to fetal controls. This suggests that BH₄ specifically increases as part of normal postnatal transition (Fig. 6; \( P < 0.05 \) vs. control fetuses). However, BH₄ levels in the lungs of ventilated PPHN lambs were significantly decreased compared with 1DSB lambs (Fig. 6; \( P < 0.05 \)). Addition of iNO did not significantly increase BH₄ levels. In contrast, treatment of PPHN lambs with a single dose of rhSOD increased BH₄ to levels comparable to those observed in 1DSB lambs (Fig. 6).

Treatment with a single dose of rhSOD, but not iNO, increases GTP-CH₁ in PPHN lambs ventilated with 100% O₂. Since treatment with rhSOD normalized BH₄ levels in the PPHN lambs ventilated with 100% O₂, we hypothesized that rhSOD might impact GTP-CH₁ expression or activity in these animals. GTP-CH₁, the rate-limiting enzyme in the synthesis of BH₄, has previously been demonstrated to be developmentally regulated in the perinatal vasculature (30). In Fig. 7, we demonstrate that GTP-CH₁ increased dramatically in the lung tissue within 24 h of birth, as shown by the large increase in the 1DSB lambs compared with fetal control lambs. GTP-CH₁ expression was decreased in the PPHN lambs ventilated with 100% O₂ relative to the healthy 1DSB lambs (Fig. 7). Similar to the BH₄ results, treatment with rhSOD, but not iNO, was sufficient to restore GTP-CH₁ expression to levels comparable to those in the healthy 1DSB lambs (Fig. 7).

**DISCUSSION**

PPHN is a clinical syndrome with multiple etiologies that involve complex interrelated pathways that we are just beginning to elucidate. The ductal ligation lamb model of PPHN increases fetal PA pressures, leads to pulmonary vascular remodeling, and produces a clinical and histological disease process consistent with that seen in idiopathic PPHN (27, 46).

**Fig. 4.** Oxidative stress in ventilated PPHN lambs is increased by ventilation with 100% O₂ and reduced by iNO or rhSOD. Unfixed frozen lung sections from 1DSB lambs \( (n = 4) \), fetal nonventilated PPHN lambs \( (n = 3) \), PPHN lambs ventilated with 100% O₂ for 24 h \( (n = 3) \), PPHN lambs ventilated with 100% O₂ + iNO \( (n = 3) \), and PPHN lambs ventilated with 100% O₂ + rhSOD \( (n = 3) \) were incubated with dihydroethidium (DHE) and imaged using fluorescence microscopy. Conversion of DHE by superoxide to ethidium results in red nuclear fluorescence. A: fluorescent intensity of each image was quantified using Metamorph imaging software. Values are means ± SE (5–7 vessels quantified per animal imaged) relative to 1DSB lambs. *\( P < 0.05 \) vs. PPHN 100% O₂. #\( P < 0.05 \) vs. 1DSB. B: representative fluorescent images, with DHE fluorescence in red. Magnification \( ×20 \).

**Fig. 5.** Endogenous eNOS function is restored in PPHN lambs ventilated with 100% O₂ and treated with a single dose of rhSOD. Contraction response of 5th-generation PA was measured to 300 nM norepinephrine (NE) with and without pretreatment with the NOS inhibitor N-nitro-l-arginine (L-NA, 1 mM) and expressed as grams of tension per gram of wet tissue weight. *\( P < 0.05 \) vs. PPHN 100% O₂. †\( P < 0.05 \) vs. corresponding vessel without L-NA.

**Fig. 6.** Tetrahydrobiopterin (BH₄) is decreased in PPHN lambs ventilated with 100% O₂, but BH₄ is rescued by treatment with rhSOD. Ovine lungs were harvested from fetal control lambs \( (n = 9) \), 1DSB lambs \( (n = 5) \), fetal PPHN lambs \( (n = 9) \), PPHN lambs ventilated with 100% O₂ for 24 h \( (n = 4) \), PPHN lambs ventilated with 100% O₂ + iNO for 24 h \( (n = 4) \), and PPHN lambs ventilated with 100% O₂ + rhSOD for 24 h \( (n = 4) \). Total biopterins [BH₄ + dihydrobiopterin (BH₂) + biopterin] were measured by HPLC with an acid extraction. Oxidized biopterins (BH₂ + biopterin) were measured by HPLC with an alkaline extraction. BH₄ was determined by the difference between total and oxidized biopterins. Values are means ± SE. *\( P < 0.05 \) vs. PPHN 100% O₂. †\( P < 0.05 \) vs. 1DSB.
Previous studies utilizing the ductal ligation model suggest that ROS may play a significant role in the pathogenesis of PPHN (4, 18, 23, 45). These findings raise concerns about the impact of traditional therapies on disease progression. Mechanical ventilation with high concentrations of O₂ is typically utilized in clinically significant PPHN to minimize hypoxemia. The purpose of the present study was to examine the effects of mechanical ventilation, O₂, iNO, and rhSOD on endogenous eNOS expression and function in the lamb model of PPHN.

Since total lung tissue includes multiple different components, such as vessels, airways, and parenchymal tissue, the studies presented here focused on the effects on endogenous eNOS in the resistance PA. In control lambs, eNOS protein expression increased after birth (Fig. 1, A and B), consistent with results reported previously (2, 8, 35). When control lambs were delivered and ventilated with 100% O₂ for 24 h, the increase in eNOS protein was similar to that in spontaneously breathing lambs. Our findings suggest that the ROS generated by ventilating control lambs with high O₂ for 24 h are not sufficient to impair endogenous eNOS expression. However, we recently reported in control lambs that hyperoxic ventilation increased expression and activity of the cGMP-specific phosphodiesterase PDE5, indicating that other components of the NO-cGMP pathway may be affected under these conditions (10).

In contrast, we found the impact of mechanical ventilation with O₂ to be significantly different in the PPHN lambs. In fetal PPHN lambs, we found trends toward decreased eNOS mRNA and protein expression compared with fetal controls. In contrast to previous reports, these differences did not reach statistical significance, likely because the lambs in the present study were delivered 9 days after in utero ligation, compared with 10 days in previous studies (35, 42). Despite this difference, the PPHN lambs in the present study demonstrate all the histopathological and clinical features of PPHN (Figs. 3 and 5) (21, 23). The earlier time point of delivery was chosen because of an unacceptably high rate of fetal loss due to preterm labor and stillbirth.

After delivery and ventilation of PPHN lambs, eNOS protein was suppressed relative to healthy 1DSB lambs and control lambs ventilated with 100% O₂ (Fig. 2). This finding correlated with significantly greater ROS production (Fig. 4) and increased contractile responses to NE (Fig. 5). It should be noted that the resting tension used for the arteries isolated from the PPHN lambs was higher than that used for the controls. This is not surprising, given the higher intravascular pressure in vivo and known remodeling of these vessels. We also noted that contractile responses in PPHN arteries were unaffected by the addition of the eNOS inhibitor L-NA, suggesting that the endogenous eNOS was not producing NO (Fig. 8). We previously demonstrated increased ROS production, associated with decreased endogenous SOD activity and increased protein expression of the p67phox subunit of NADPH oxidase, in fetal nonventilated PPHN lambs (4). The present study suggests that, when delivered and ventilated with high levels of O₂, PPHN lambs are particularly susceptible to further increases in ROS production and ROS-mediated damage, which lead to continued suppression of eNOS expression and function.

The only pulmonary vasodilator approved by the US Food and Drug Administration for infants with PPHN is iNO. As such, we sought to determine whether addition of iNO would restore endogenous eNOS expression and function in PPHN lambs. When PPHN lambs were ventilated with iNO, there was a significant increase in eNOS mRNA and protein relative to PPHN lambs ventilated with 100% O₂ alone, which was comparable to the increase in 1DSB lambs (Table 1, Fig. 2). However, the lack of L-NA enhancement of vascular contractility suggests that this endogenous eNOS remains relatively inactive in these lambs. It is particularly interesting that iNO successfully upregulates eNOS expression, but not function, in the PPHN lambs. Previous studies demonstrated that exogenous NO upregulates eNOS mRNA levels in fetal intrapulmonary artery endothelial cells (47) but inhibits eNOS activity by protein nitration (5). Our results are consistent with these findings in isolated PA endothelial cells. Furthermore, we present new data that ventilation of PPHN lambs with 100% O₂ leads to decreased BH₄ levels and treatment with exogenous NO is unable to rescue these BH₄ levels (Fig. 6) and GTP-CH₁ expression (Fig. 7). Since BH₄ represents a critical cofactor
eNOS function, the decreased BH$_4$ levels in the PPHN lambs treated with iNO likely also contribute to the decreased eNOS function in the isolated vessel studies (Fig. 5). The relative inability of iNO to induce endogenous eNOS function in PPHN lambs may explain in part why ~50% of infants with PPHN either fail to respond or do not sustain their response to iNO (7, 14, 32).

We previously demonstrated decreased SOD activity and increased NADPH oxidase expression in PPHN lambs (4). If this decreased SOD activity affects endogenous eNOS expression and/or function, then restoration of SOD activity should restore endogenous eNOS expression and function. We found that administration of a single dose of rhSOD at birth increased eNOS mRNA and protein expression levels relative to PPHN lambs ventilated with 100% O$_2$ alone. This increase was comparable to IDSB lambs and control lambs ventilated with 100% O$_2$ + rhSOD (Table 1, Fig. 2). We also found that contractile responses to L-NA were restored, suggesting that rhSOD restored endogenous eNOS function (Fig. 5). Furthermore, treatment with rhSOD increased BH$_4$ levels in the PPHN lambs relative to the lambs ventilated with 100% O$_2$ alone and the lambs treated with iNO (Fig. 6) and increased GTP-CH1 expression relative to the lambs ventilated with 100% O$_2$ alone and the lambs treated with iNO. Thus it is our hypothesis that rhSOD counteracts the effects of hyperoxia on eNOS and BH$_4$/GTP-CH1 in the PA endothelial cell (Fig. 8).

The difference in BH$_4$ levels in the rhSOD lambs likely plays a key role in the restoration of eNOS function in these lambs (Fig. 5). The oxidized biopterins (BH$_2$ + biopeterin) do not change across any of the ventilation groups (Fig. 6). This is in contrast to previously published data in another ovine model of chronic pulmonary hypertension (13). However, other groups have demonstrated that GTP-CH1, the rate-limiting enzyme for BH$_4$ production, is developmentally regulated and increases at 12–24 h after birth in the pulmonary vasculature. However, after 24 h, GTP-CH1 levels fall, reaching a nadir at 3 days of life (30). This decrease places the neonate at risk for having limited BH$_4$ pools. It has been shown that significant deficits in BH$_4$ biosynthesis, such as in GTP-CH1 knockout mice, lead to pulmonary hypertension and pulmonary vascular remodeling, even in normoxia (16, 31). Finally, postnatal cotreatment with BH$_4$ and a superoxide mimetic, MnTMPyP, has been reported to improve endothelial function and increase vessel relaxation in a porcine model of pulmonary hypertension (30). Thus the ability of rhSOD in the present study to restore GTP-CH1 expression and subsequently restore BH$_4$ levels and eNOS function in our ovine model of pulmonary hypertension is consistent with the larger body of data regarding the impact of BH$_4$ on pulmonary hypertension. It is of particular interest that only rhSOD is sufficient to restore GTP-CH1 expression and BH$_4$ levels in the PPHN lambs (Figs. 6 and 7) when both rhSOD and iNO reduce oxidative stress in the PPHN lambs, as evidenced by DHE staining (Fig. 5). However, we would hypothesize that iNO, when delivered with 100% O$_2$ in the context of PPHN, may increase reactive nitrogen species and protein nitration, which impacts eNOS function directly as well as BH$_4$ biosynthesis. Furthermore, recent studies in the literature have demonstrated that GTP-CH1 expression and BH$_4$ production are upregulated by H$_2$O$_2$ (36, 37). Since rhSOD acts to convert superoxide to H$_2$O$_2$, it is reasonable to hypothesize that the H$_2$O$_2$ produced by rhSOD is in part responsible for the increase in GTP-CH1 expression we observed (Fig. 7). This hypothesis would also further explain why iNO is less effective at normalizing GTP-CH1 expression and BH$_4$ production, inasmuch as it likely clears ROS by reacting with them directly to produce reactive nitrogen species as opposed to the H$_2$O$_2$ produced by rhSOD. Thus the ability of rhSOD to restore eNOS expression and function as well as BH$_4$ levels may explain in part the significant increase in oxygenation we recently reported when PPHN lambs are treated with rhSOD (23).

Thus our studies indicate that rhSOD is at least as effective as iNO on eNOS expression and function in resistance PA from PPHN lambs, suggesting that superoxide conversion and clearance have a significant role in eNOS expression and function and a significant effect on BH$_4$ levels. Although iNO has been a successful therapy in PPHN, a significant portion of infants do not respond or sustain their response to iNO, nor does iNO significantly impact mortality or morbidity due to PPHN (14, 32). The data presented here suggest that the complex pathophysiology of PPHN is likely due, in part, to inhibition of eNOS expression and function by ROS, such as superoxide. This pathophysiology is further negatively impacted by the most common PPHN treatment, i.e., mechanical ventilation with high levels of O$_2$. The data presented here suggest that treatment with rhSOD is able to enhance eNOS expression and function through enhancement of ROS clearance and restoration of BH$_4$ levels (Fig. 8). Therefore, rhSOD may represent a future adjunctive or alternative therapy to iNO in the clinical management of severe PPHN.

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