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


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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 295: L979–L987, 2008. doi:10.1152/ajplung.90238.2008.—Endothelial nitric oxide (NO) synthase (eNOS) expression and activity are decreased in fetal lambs with persistent pulmonary hypertension (PPHN). We sought to determine the impact of mechanical ventilation with O2 with or without inhaled NO (iNO) or recombinant human SOD (rhSOD) on eNOS in the ductal ligation model of PPHN. PPHN lambs and age-matched controls were ventilated with 100% O2 for 24 h alone or combined with 20 ppm iNO continuously or a single dose of rhSOD (5 mg/kg) given intratracheally at delivery. In 1-day spontaneously breathing lambs, eNOS expression in resistance pulmonary arteries increased relative to fetal levels. eNOS expression increased in control lambs ventilated with 100% O2, but not in PPHN lambs. Addition of iNO or rhSOD increased eNOS expression and decreased generation of reactive oxygen species (ROS) in PPHN lambs relative to those ventilated with 100% O2 alone. However, only rhSOD restored eNOS function, increased tetrahydrobiopterin (BH4), a critical cofactor for eNOS function, and restored GTP cyclohydrolase I expression in isolated vessels and lungs from PPHN lambs. These data suggest that ventilation of PPHN lambs with 100% O2 increases ROS production, blunts postnatal increases in eNOS expression, and decreases available BH4 in PPHN lambs. Although the addition of iNO or rhSOD diminished ROS production and increased eNOS expression, only rhSOD improved eNOS function and levels of available BH4. Thus therapies designed to decrease oxidative stress and restore eNOS coupling, such as rhSOD, may prove useful in the treatment of PPHN in newborn infants.

reactive oxygen species; biopterin

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 dispersion of the lungs and increased P02, 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 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 Infasurf (3 ml/kg; Mediatech, Herndon, VA). Sections were blocked with 50 mM HEPES-150 mM NaCl-0.5% Triton X-100. Sections that were mounted onto charged slides for staining and stored at 80°C. Sections were subsequently fixed with acetone (Sigma) for 20 min at 4°C, allowed to air dry, and then washed with 1× PBS (Mediatech, Herndon, VA). Sections were blocked with 5% BSA (Sigma) and then incubated with polyclonal rabbit anti-GTP-CH1 (1:100 dilution in 5% BSA) 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 + 1× TBST. After sections were further incubated in Alexa Fluor 488 anti-mouse antibody (Molecular Probes/Invitrogen) at a 1:200 dilution in 1× TBST for 1 h, sections were mounted with DAPI (Sigma) and then mounted with DAPI for imaging. Magnification was obtained using a Leica microscope with a DMI 6500B camera.
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 \((10^{-5} \text{ M})\) to block endogenous prostaglandins and with propranolol \((10^{-6} \text{ M})\) to block \(\beta\)-adrenergic receptors. Two arterial rings from each lamb were pretreated with l-NA \((10^{-3} \text{ M})\) and then constricted with NE \((3 \times 10^{-7} \text{ M})\). Four arterial rings from each lamb were constricted with NE \((3 \times 10^{-7} \text{ M})\) 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 studied from each animal, and mean tension was used for analysis.

**Determination of total biopterin levels by HPLC.** Lung biopterin content was measured by HPLC analysis and a differential oxidation method, as previously described (24, 26). Briefly, frozen lung tissue was ground to a powder on liquid nitrogen. The powder was then extracted as 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.

**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% O2</th>
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<tbody>
<tr>
<td>Control lambs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fetus</td>
<td>5</td>
<td>1 ± 0.28</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>&lt;0.01</td>
<td></td>
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<tr>
<td>1DSB</td>
<td>7</td>
<td>17.1 ± 7.0</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>100% O2</td>
<td>6</td>
<td>8.6 ± 1.6</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>100% O2 + iNO</td>
<td>4</td>
<td>6.9 ± 2.7</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
<td></td>
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<tr>
<td>100% O2 + rhSOD</td>
<td>4</td>
<td>21.6 ± 11.9</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>&lt;0.05</td>
<td></td>
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<tr>
<td>PPHN lambs</td>
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<tr>
<td>Fetus</td>
<td>9</td>
<td>0.7 ± 0.2</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
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<tr>
<td>100% O2</td>
<td>4</td>
<td>1.9 ± 0.7</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>100% O2 + iNO</td>
<td>4</td>
<td>15.2 ± 5.4</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>100% O2 + rhSOD</td>
<td>4</td>
<td>18.7 ± 10.4</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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</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.

**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 (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, inasmuch 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,
Furthermore, Fig. 3 shows significant vessel remodeling 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 and 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 less dramatic increase in eNOS protein. Ventilation with 100% O₂ + iNO significantly increased eNOS protein expression in PPHN lambs relative to fetal PPHN lambs and PPHN lambs ventilated with 100% O₂ alone (P < 0.05; Fig. 2A). However, protein expression remained significantly less than that in comparably ventilated control lambs (2.0 ± 0.3 vs. 4.3 ± 0.8 fold, P < 0.05). eNOS expression in PPHN lambs ventilated with 100% O₂ + iNO remained confined to the endothelial layer of the vessels (Fig. 3).

Similar to the results we observed for lambs treated with iNO, treatment of control lambs with a single dose of rhSOD did not increase eNOS mRNA relative to ventilation of control lambs with 100% O₂ alone (Table 1). rhSOD significantly increased eNOS mRNA in PPHN lambs relative to 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 and 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).

**Fig. 1.** eNOS protein is increased in resistance pulmonary arteries (PA) after birth. Ovine resistance PA were harvested from fetal control lambs (n = 7) and 1-day spontaneously breathing (1DSB) lambs (n = 5). A: Western blot analysis of PA endothelial nitric oxide (NO) synthase (eNOS) protein expression, with β-actin normalization. Values are means ± SE relative to fetal control lambs. *P < 0.05 vs. 1DSB. B: representative Western blots for eNOS and β-actin in resistance PA. C: localization of eNOS expression within PA endothelium. Top: phase-contrast images of frozen lamb lung sections. Magnification ×20. Bottom: corresponding sections stained for eNOS, with immunofluorescence shown as green.

**Fig. 2.** eNOS protein expression is impaired in newborn lambs with persistent pulmonary hypertension (PPHN) ventilated with 100% O₂, but eNOS is rescued by inhaled NO (iNO) or recombinant human SOD (rhSOD). Ovine resistance PA were harvested from 1DSB lambs (n = 5), fetal nonventilated control and PPHN lambs (n = 9), control and PPHN lambs ventilated with 100% O₂ for 24 h (n = 4), control and PPHN lambs ventilated with 100% O₂ + iNO for 24 h (n = 4), and control and PPHN lambs ventilated with 100% O₂ + rhSOD for 24 h (n = 4). A: Western blot analysis of PA eNOS protein expression, with β-actin normalization. Values are means ± SE relative to control. *P < 0.05 vs. PPHN 100% O₂. +P < 0.05 vs. PPHN 100% O₂ + iNO. #P < 0.05 vs. 1DSB. B: representative Western blots for eNOS and β-actin in resistance PA. F: fetal nonventilated lambs; 100, lambs ventilated with 100% O₂; NO, lambs ventilated with 100% O₂ + iNO; S, lambs ventilated with 100% O₂ + rhSOD.
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. 6. Tetrahydrobiopterin (BH4) is decreased in PPHN lambs ventilated with 100% O2, but BH4 is rescued by treatment with rhSOD. Oxidized biopterins (BH2 + biopterin) were measured by HPLC with an alkaline 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.
Values are means ± SE relative to fetal control lambs. *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 was analyzed by Western blot, with eNOS in the resistance PA. In control lambs, eNOS protein expression 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 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 for (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 expression was analyzed by Western blot, with eNOS in the resistance PA. In control lambs, eNOS protein expression 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 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.
SOD RESTORES eNOS IN NEONATAL PULMONARY HYPERTENSION

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% O2 alone. This increase was comparable to IDSB lambs and control lambs ventilated with 100% O2 + 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 BH4 levels in the PPHN lambs relative to the lambs ventilated with 100% O2 alone and the lambs treated with iNO (Fig. 6) and increased GTP-CH1 expression relative to the lambs ventilated with 100% O2 alone and the lambs treated with iNO. Thus it is our hypothesis that rhSOD counteracts the effects of hyperoxia on eNOS and BH4/GTP-CH1 in the PA endothelial cell (Fig. 8).

The difference in BH4 levels in the rhSOD lambs likely plays a key role in the restoration of eNOS function in these lambs (Fig. 5). The oxidized biopterins (BH2 and BH3) 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 BH4 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 BH4 pools. It has been shown that significant deficits in BH4 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 BH4 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 BH4 levels and eNOS function in our ovine model of pulmonary hypertension is consistent with the larger body of data regarding the impact of BH4 on pulmonary hypertension. It is of particular interest that only rhSOD is sufficient to restore GTP-CH1 expression and BH4 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% O2 in the context of PPHN, may increase reactive nitrogen species and protein nitration, which impacts eNOS function directly as well as BH4 biosynthesis. Furthermore, recent studies in the literature have demonstrated that GTP-CH1 expression and BH4 production are upregulated by H2O2 (36, 37). Since rhSOD acts to convert superoxide to H2O2, it is reasonable to hypothesize that the H2O2 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 BH4 production, inasmuch as it likely clears ROS by reacting with them directly to produce reactive nitrogen species as opposed to the H2O2 produced by rhSOD. Thus the ability of rhSOD to restore eNOS expression and function as well as BH4 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 BH4 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 O2. 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 BH4 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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