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Hydrogen Peroxide Regulates Extracellular Superoxide Dismutase Activity and Expression in Neonatal Pulmonary Hypertension

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Abstract

We previously demonstrated that superoxide and H_2O_2 promote pulmonary arterial vasoconstriction in a lamb model of persistent pulmonary hypertension of the newborn (PPHN). Because extracellular superoxide dismutase (ecSOD) augments vasodilation, we hypothesized that H_2O_2-mediated ecSOD inactivation contributes to pulmonary arterial vasoconstriction in PPHN lambs. ecSOD activity was decreased in pulmonary arterial smooth muscle cells (PASMCs) isolated from PPHN lambs relative to controls. Exposure to 95% O_2 to mimic hyperoxic ventilation reduced ecSOD activity in control PASMCs. In both cases, these events were associated with increased protein thiol oxidation, as detected by the redox sensor roGFP. Accordingly, exogenous H_2O_2 decreased ecSOD activity in control PASMCs, and PEG-catalase restored ecSOD activity in PPHN PASMCs. In intact animal studies, ecSOD activity was decreased in fetal PPHN lambs, and in PPHN lambs ventilated with 100% O_2 relative to controls. In ventilated PPHN lambs, administration of a single dose of intratracheal PEG-catalase enhanced ecSOD activity, reduced superoxide levels, and improved oxygenation. We propose that H_2O_2 generated by PPHN and hyperoxia inactivates ecSOD, and intratracheal catalase enhances enzyme function. The associated decrease in extracellular superoxide augments vasodilation, suggesting that H_2O_2 scavengers may represent an effective therapy in the clinical management of PPHN. *Antioxid. Redox Signal.* 15, 1497–1506.

Introduction

After birth, with initiation of ventilation of the lungs, pulmonary vascular resistance normally decreases, and pulmonary blood flow increases by 10-fold (7, 37). This process is regulated by a complex and incompletely understood interaction between a series of mechanical and biochemical factors (11). Persistent pulmonary hypertension of the newborn (PPHN) is a serious disorder of newborn infants occurring in two to six per 1,000 live births, with a significant risk of morbidity and death (22). PPHN is characterized by a failure to decrease pulmonary vascular resistance normally at birth, resulting in pulmonary hypertension, right-to-left extrapulmonary shunting, and hypoxemia (40). In that regard, significant improvements in systemic arterial oxygenation can be achieved by vasodilation of the pulmonary circulation, which limits blood flow through extrapulmonary shunt pathways. Clinical management of PPHN includes mechanical ventilation with high levels of inspired oxygen and inhaled NO (39), although approximately 50% of infants have a limited or transient response (5, 31, 36).

In a newborn lamb model of PPHN generated by antenatal ligation of the ductus arteriosus, increasing evidence suggests that reactive oxygen species (ROS) play an important role in the vascular dysfunction and remodeling characteristic of PPHN. For example, superoxide levels were increased, and superoxide dismutase (SOD) activity was decreased in the pulmonary arteries of fetal PPHN lambs (3), which may lead to vasoconstriction. Hyperoxic ventilation may also promote the generation of ROS, which could further exacerbate the pulmonary vascular abnormalities of PPHN (25, 27). In agreement with this hypothesis, we previously demonstrated that pretreatment of isolated PPHN pulmonary arteries with superoxide scavengers enhanced relaxation responses (3). Furthermore, we recently reported that administration of intratracheal recombinant human SOD improved oxygenation and reduced oxidative stress in PPHN lambs ventilated with 100% O_2 for 24 h (26). Together,

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our data suggest that impaired endogenous SOD activity may contribute to PPHN.

Extracellular SOD (ecSOD) is the only known enzymatic scavenger of extracellular superoxide and is highly expressed within the lungs and vascular smooth muscle (28, 33, 34). Superoxide scavenging in the vascular extracellular space by ecSOD plays an important role in regulating vasodilation (19), and impaired ecSOD activity has been demonstrated in several models of systemic hypertension (13, 19, 20, 44). H_2O_2 has been shown to inactivate purified ecSOD (15, 29), and we previously demonstrated increased H_2O_2 levels in PPHN pulmonary arteries (43). From these data, we hypothesized that increased H_2O_2 reduces ecSOD activity, contributes to increased superoxide levels in PPHN pulmonary arteries, and promotes pulmonary vasoconstriction. The aim of this study was to investigate the role of H_2O_2 in the regulation of ecSOD activity in pulmonary arterial smooth muscle cells, and to determine whether H_2O_2 scavengers could augment ecSOD activity and improve oxygenation in PPHN lambs.

Materials and Methods

Cell culture

Primary cultures of fetal pulmonary arterial smooth muscle cells (FPASMCs) from control and PPHN fetal lambs were isolated by the explant technique, as described previously (9). In brief, a segment of the main pulmonary artery from 136-day-old fetal lambs was excised and placed in a sterile 10-cm dish containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 g/L glucose (Mediatech, Herndon, VA). The segment was stripped of adventitia with a sterile forceps. The main pulmonary artery segment was then cut longitudinally to open the vessel, and the endothelial layer was removed by gentle rubbing with a cell scraper. The vessel was then cut into 2-mm segments, inverted, and placed on a collagen-coated 35-mm tissue-culture dish. A drop of DMEM containing 10% fetal bovine serum (FBS; HyClone, Logan, UT), antibiotics (MediaTech), and antimycotics (Mediatech) was then added, and the cells were grown overnight at 37°C in a humidified atmosphere with 5% CO_2/95% air. The next day, an additional 2 ml of complete medium was added. The growth medium was subsequently changed every 2 days. When SMC islands could be observed under the microscope, the tissue segment was removed, and the individual cell islands were subcloned. Identity was confirmed as FPASMCs by immunostaining (>99% positive) with antibodies against α-smooth muscle actin, calponin, and caldesmon. This was taken as evidence that cultures were not contaminated with fibroblasts or with endothelial cells. All cultures for subsequent experiments were maintained in DMEM supplemented with 10% fetal calf serum (Hyclone), antibiotics (MediaTech), and antimycotics (MediaTech) at 37°C in a humidified atmosphere with 5% CO_2/95% air. All cultures were maintained in DMEM supplemented with 1 g/L glucose (Mediatech), 10% FBS (Hyclone), antibiotics (Mediatech), and antimycotics (Mediatech) at 37°C in a humidified atmosphere with 5% CO_2/95% air. Cells were synchronized before experiments by transfer to serum-free DMEM with antibiotics and antimycotics 12 h before the start of the experiment. All experiments were carried out by using 500,000 cells per condition between passages 2 and 5. FPASMCs were treated in incubators with 21% O_2/5% CO_2 or 95% O_2/5% CO_2 and harvested after 24 h. FPASMCs isolated from control lambs were treated with 0–50 μM H_2O_2 and harvested after 4 h. FPASMCs isolated from PPHN lambs were treated with 0–200 U/ml PEG-catalase (Sigma, St. Louis, MO) and harvested after 24 h.

Detection of reactive oxygen species

FPASMCs in serum-free DMEM without phenol red and with antibiotics and antimycotics were infected in 60-mm culture dishes with 100 PFU/cell of a RoGFP adenoviral construct. RoGFP is a previously characterized ratiometric fluorescent probe sensitive to cellular oxidative stress (8, 14). Twelve hours after infection, cells were returned to normal culture medium, and 48 h after infection, the FPASMCs were exposed to 21% O_2/5% CO_2 or 95% O_2/5% CO_2 for 24 h. After exposure, FPASMCs were lifted from the plates and divided into three aliquots. The first aliquot was used to assess redox status, whereas the other two were used to calibrate the probe. One aliquot was fully reduced with dithiothreitol (1 m*M*, Sigma), and the second was fully oxidized by using *t*-butyl hydroperoxide (1 m*M*; Sigma). All aliquots were analyzed with a DakoCytomation CyAn multilaser flow cytometer by using 400-nm and 485-nm excitation wavelengths; emission was assessed at 535 nm. An uninfected control was also assessed to provide a signal for background subtraction. For each condition, the cysteine thiol redox status was calculated as percentage oxidized, by comparison with the values obtained for the fully reduced and fully oxidized conditions.

Western blot analysis

Lung tissue was homogenized, and total protein collected by using the PARIS kit (Ambion, Austin, TX), as previously described (9). Protein lysates from FPASMCs were prepared by using 1×Mg-lysis buffer (Upstate, Charlottesville, VA) supplemented with a protease inhibitor cocktail (Sigma). Protein concentration was measured by using the Bradford method (2). Total protein (40 μg) was separated on a 4–20% SDS-polyacrylamide gel (Biorad, Hercules, CA) and then transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL). Western blot was then performed as previously described (9, 10). In brief, membranes were blocked at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (1×TBST) and were then incubated overnight at 4°C with the primary antibody in 5% milk + 1×TBST at a 1:500 dilution for rabbit-anti-ecSOD (Santa Cruz Biotechnology, Santa Cruz, CA), and a 1:2,000 dilution for mouse anti-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 washed and exposed via chemiluminescence (Pierce). Bands were analyzed by using a Digital Science Image Station (Kodak, Rochester, NY). ecSOD expression within each Western blot was normalized to β-actin.

ecSOD purification and activity assay

Frozen lung tissue was ground in liquid nitrogen and sonicated in ice-cold 50 m*M* potassium phosphate buffer (pH 7.6) containing 0.3 *M* KBr and protease inhibitors. Cells were scraped from tissue-culture plates, washed in PBS, and centrifuged. Cell pellets were resuspended and sonicated in

ice-cold 50 m*M* potassium phosphate buffer (pH 7.6) containing 0.3 *M* KBr and protease inhibitors. These protein fractions contain intracellular ecSOD and extracellular ecSOD sequestered within the extracellular matrix. Protein concentration was determined by the Bradford method. ecSOD was separated from the other SOD isoforms with affinity chromatography by using a concavalin A sepharose column (Pierce), as previously described (12). Protein content in the eluted fraction was measured by the Bradford method and samples analyzed immediately for enzyme activity by using a commercially available SOD assay kit (Stressgen, Ann Arbor, MI). In brief, superoxide generated by xanthine oxidase converts WST-1 to produce a formazan dye that absorbs light at 450 nm. SOD reduces the rate of color formation, and sample activity is calculated from a standard curve by using purified SOD of known activity. Activity was normalized to protein content and expressed relative to control samples.

Animal surgery and ventilation protocols

The Laboratory Animal Care Committees at the State University of New York (SUNY) Buffalo and Northwestern University approved this study. Fetal ductal ligation was performed at 125 days of gestation (term, 145 days) in time-dated pregnant sheep (Swartz family farm, Attica, NY), as previously described (45, 46). After a 9-day recovery, six fetal lambs with PPHN and twin controls were delivered by cesarean section and were killed before their first breath. Additional PPHN lambs were intubated and administered calfactant, 3 ml/kg (Infasurf; ONY, Inc., Amherst, NY). The umbilical cord was clamped and cut, and the lamb was placed under a radiant warmer and ventilated with a Servo 300 ventilator (Siemens, Mississauga, ON, Canada) with the following initial settings: positive end-expiratory pressure, 4 cm H_2O; positive inspiratory pressure (PIP), 20 to 25 cm H_2O (targeted to provide a tidal volume of 10 ml/kg); and rate, 40 breaths/min. Umbilical arterial and venous lines were placed, and ventilator settings were adjusted to maintain $Paco_2$ between 35 and 50 mm Hg, as described previously (24). In brief, abnormal $Paco_2$ values were managed by adjusting the PIP to maintain a tidal volume of 10 ml/kg and/or change in the ventilator rate by five breaths/min. Intravenous fluids (dextrose 10% solution with 25 mEq of NaCl, 20 mEq of KCl, 10 mEq of $NaHCO_3$/L) were administered continuously at 100 ml/kg/d. Fluid composition and rate were adjusted based on serum electrolyte values. Lambs were sedated with a continuous infusion of fentanyl (2–4 μg/kg/h) and paralyzed with pancuronium bromide (0.1 mg/kg/dose every 4 h as needed). Additional boluses of fentanyl were administered for signs of pain or discomfort. Lambs with hypotension (mean blood pressure, <40 mm Hg) and tachycardia (heart rate >200 beats/min) received 10 ml/kg of whole blood (collected from either the placenta or the mother). A second transfusion was repeated if these signs persisted. If hypotension persisted despite two blood transfusions; dopamine was started at 5 μg/kg/min and titrated based on the therapeutic response. Thoracotomy and instrumentation of the PAs were not performed. Oxygenation was measured as arterial-to-alveolar oxygen ratios (a/A ratio = umbilical arterial $Pao_2 \div [745 - 47] \times Fio_2 - Paco_2/0.8$, where 745 mm Hg is the barometric pressure at SUNY Buffalo, and 47 mm Hg is the partial pressure of water).

Lambs were randomized before delivery to receive 100% oxygen and mechanical ventilation alone ($n = 12$) or in combination with intratracheal PEG-catalase, 20,000 U/kg (Sigma) at birth ($n = 5$). The Fio_2 in the inspiratory limb of the ventilator circuit was continuously measured and kept constant at close to 1.0. After 24 h of ventilation, the lambs were anesthetized with 10 mg/kg of pentothal and killed by rapid exsanguination. The heart and lungs were removed *en bloc*, and fifth-generation PA (500 μm I.D.) were dissected and isolated. Lung-tissue samples were frozen in liquid nitrogen and stored at −80°C until analysis.

In situ *analysis of superoxide generation*

Lung sections were prepared and stained as described previously (26). In brief, the right middle lobe of the lung was removed, and OCT compound (VWR Scientific, West Chester, PA) was pushed 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 and stored at −80°C. The sections were exposed to 5μM dihydroethidium (DHE; Molecular Probes, Eugene, OR) in PBS. Slides were incubated in a light-protected humidified chamber at 37°C for 15 min. Slides were washed 3 times in PBS and observed by using a Nikon Eclipse TE-300 fluorescent microscope with excitation at 518 nm and emission at 605 nm. Fluorescent images were captured by using a CoolSnap digital camera, and the average fluorescent intensities (to correct for differences in pixel number) of pulmonary arteries quantified by using Metamorph imaging software (Fryer Corp., Huntley, IL). Tissue sections were processed and imaged in parallel.

Statistical analysis

Cellular roGFP oxidation and ecSOD expression and activity data were normalized to normoxic untreated cells from control lambs. Lung DHE fluorescence intensities and ecSOD expression and activity data were normalized to fetal control lambs. All data are expressed as the mean ± SEM. Each n represents a single lamb studied. Results were analyzed with ANOVA with Newman-Keuls *post hoc* test by using Prism software (GraphPad Software, Inc., San Diego, CA). Statistical significance was set at $p < 0.05$.

Results

PPHN and hyperoxia increase ecSOD expression but decrease activity in PASMCs

We isolated fifth-generation pulmonary artery SMCs from control and PPHN lambs and confirmed that the PPHN cells maintained their phenotype in culture by demonstrating a decrease in expression of PKG-1α (data not shown), in agreement with the previous study of Resnik *et al.* (35). We then exposed the cells to 95% O_2 for 24 h to mimic the effects of hyperoxia on lambs ventilated with 100% O_2. Combined intracellular and extracellular ecSOD was purified from protein lysates with affinity chromatography. Figure 1A shows a representative Western blot for ecSOD, with band intensities quantified and normalized to β-actin. We found a 3.6 ± 0.7-fold increase in ecSOD protein in normoxic PPHN cells relative to normoxic controls (Fig. 1B). Hyperoxia increased ecSOD protein to 2.0 ± 0.4-fold in control cells but did not increase expression further in PPHN cells (Fig. 1B). ecSOD

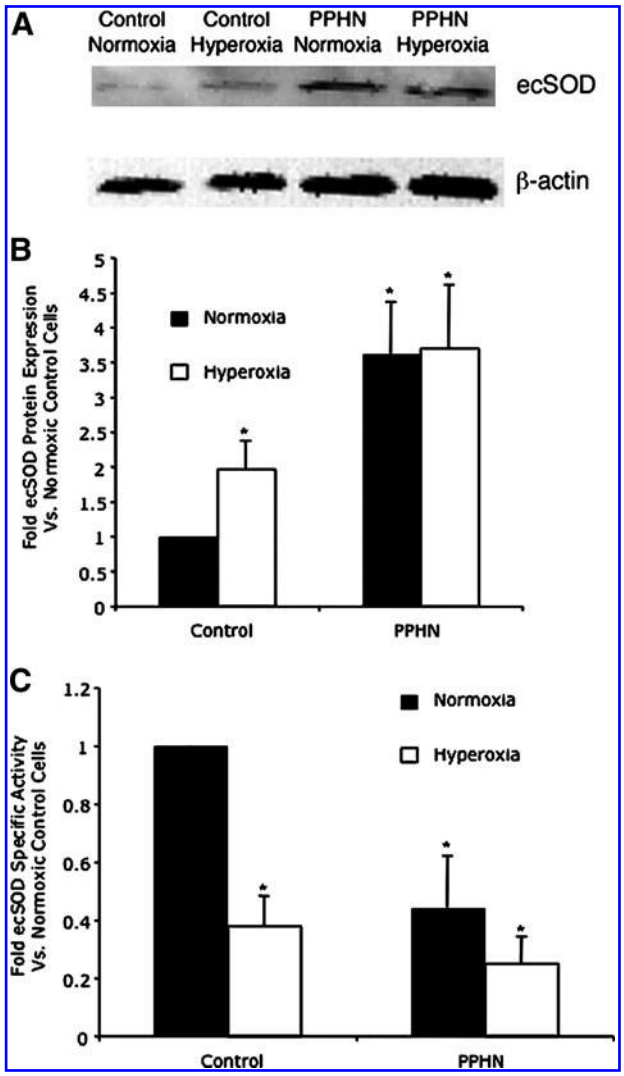

FIG. 1. PPHN and hyperoxia increase ecSOD expression but decrease activity in PASMCs. PASMCs isolated from control and PPHN lambs were maintained under normoxic or hyperoxic conditions for 24 h, and protein was analyzed with Western blotting. **(A)** Representative blots for ecSOD and β-actin. **(B)** ecSOD band intensities were quantified and normalized to β-actin. Data are shown as mean ± SEM relative to normoxic control cells; $n = 4$. $^*p < 0.05$ versus normoxic control cells. **(C)** ecSOD protein was purified from cell extracts with affinity chromatography, and SOD activity was determined and normalized to ecSOD expression. Data are shown as mean ± SEM relative to normoxic control cells; $n = 4$. $^*p < 0.05$ versus normoxic control cells.

protein was purified from cell extracts with affinity chromatography, and ecSOD-specific activity was 0.38 ± 0.18-fold in normoxic PPHN cells relative to normoxic control cells (Fig. 1C). Hyperoxia decreased ecSOD specific activity to 0.38 ± 0.10-fold in control cells, and to 0.25 ± 0.09-fold in PPHN cells, relative to normoxic control cells (Fig. 1C).

PPHN and hyperoxia increase cytosolic protein thiol oxidation in PASMCs

We determined the cytosolic redox status of control and PPHN PASMCs under normoxic and hyperoxic conditions by using the ratiometric protein probe roGFP. The probe was 13.7 ± 3.5% oxidized in normoxic control cells and 50.4 ± 5.1% oxidized in normoxic PPHN cells (Fig. 2). In agreement with our previous study (9), 24-h hyperoxia increased cytosolic roGFP oxidation to 27.3 ± 2.4% in control cells, whereas hyperoxia oxidized the probe further to 67.6 ± 14.0% in PPHN cells (Fig. 2).

H_2O_2 increases ecSOD expression but decreases activity in control PASMCs

Because endogenous H_2O_2 oxidizes roGFP (42), we next examined the effects of exogenous H_2O_2 on ecSOD expression and activity in control PASMCs. A 4-h exposure to H_2O_2 dose-dependently increased levels of ecSOD mRNA (Fig. 3A) and protein (Fig. 3B) to 2.29 ± 0.38-fold and 2.05 ± 0.18-fold of untreated cells, respectively, at 50 μM. This increase in expression was accompanied by a decrease in ecSOD-specific activity, to levels of 0.50 ± 0.07 in cells treated with 50 μM H_2O_2 for 4 h relative to untreated cells (Fig. 3D).

PEG-catalase restores ecSOD activity in PPHN PASMCs

Because overexpression of catalase attenuates hypoxia-induced roGFP oxidation in PASMCs (42), we looked at the effects of catalase on ecSOD activity in PPHN PASMCs. After 24 h of treatment with PEG-catalase, ecSOD-specific activity was increased in normoxic and hyperoxic PPHN PASMCs to 1.51 ± 0.09-fold and 1.55 ± 0.31-fold, respectively, relative to untreated normoxic PPHN cells (Fig. 4).

Intratracheal PEG-catalase decreases ecSOD expression but increases enzyme activity in PPHN lungs

We quantified ecSOD expression and activity in lung-protein extracts from fetal control and fetal PPHN lambs, and from PPHN lambs ventilated with 100% O_2 for 24 h with or without a single dose of intratracheal PEG-catalase. Figure 5A

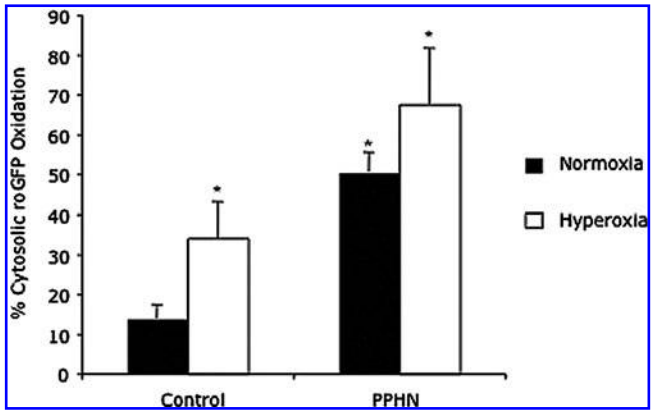

FIG. 2. PPHN and hyperoxia increase roGFP oxidation in PASMCs. PASMCs isolated from control and PPHN lambs were infected with an adenovirus expressing the redox sensor roGFP and maintained under normoxic or hyperoxic conditions for 24 h. The percentage of roGFP oxidation was determined with flow cytometry after probe calibration. Data shown are mean ± SEM; $n = 4$. $^*p < 0.05$ versus normoxic control cells.

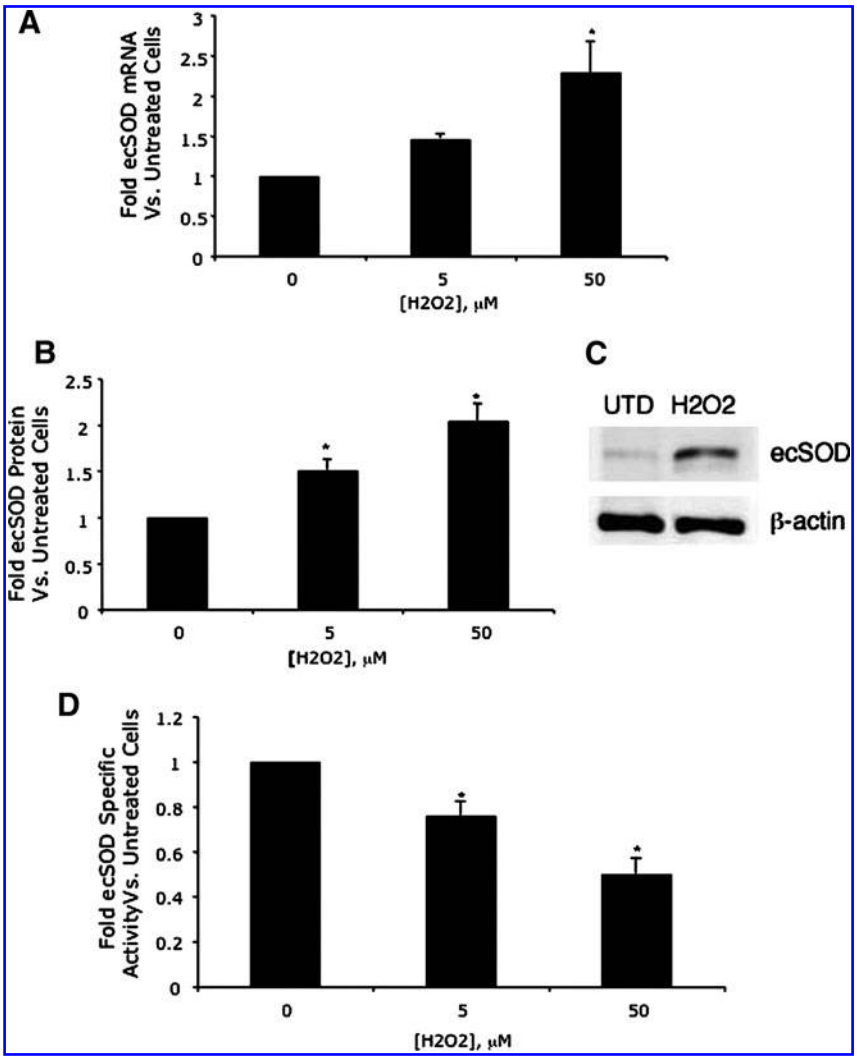

FIG. 3. H_2O_2 Increases ecSOD expression but decreases activity in PASMCs. PASMCs isolated from control lambs were treated with 0–50 μM H_2O_2 for 4 h in serum-free medium. **(A)** RNA was isolated and ecSOD mRNA levels quantified with qRT-PCR. Data are shown as mean ± SEM relative to untreated cells; $n = 4$. *$p < 0.05$ versus untreated cells. **(B)** ecSOD protein was quantified with Western blotting and normalized to β-actin. Data are shown as mean ± SEM relative to untreated cells; $n = 6$. *$p < 0.05$ vs. untreated cells. **(C)** Representative Western blots for ecSOD and β-actin. **(D)** ecSOD protein was purified from cell extracts with affinity chromatography, and SOD activity was determined and normalized to ecSOD expression. Data are shown as mean ± SEM relative to untreated cells; $n = 4$. *$p < 0.05$ versus untreated cells.

shows a representative Western blot for ecSOD and β-actin. ecSOD band intensities were normalized to β-actin and are presented in Fig. 5B. Relative to fetal control lambs, ecSOD protein was increased to 1.8 ± 0.3-fold and 2.2 ± 0.2-fold in fetal PPHN lambs and PPHN lambs ventilated with 100% O_2, respectively, whereas catalase treatment reduced ecSOD protein levels in ventilated PPHN lambs to 0.9 ± 0.2-fold (Fig. 5B). Relative to fetal control lambs, ecSOD-specific activity was decreased to 0.48 ± 0.08-fold and 0.40 ± 0.04-fold in fetal PPHN lambs and PPHN lambs ventilated with 100% O_2, respectively, whereas catalase treatment increased activity in ventilated PPHN lambs to 2.05 ± 0.33-fold (Fig. 5C). Previously we found no significant differences in expression of CuZnSOD and MnSOD between fetal control and fetal PPHN lambs (3), and in this study, we found no differences in the specific activities of these enzymes (Fig. 5D).

Intratracheal PEG-catalase decreases superoxide levels in PPHN pulmonary arteries

We next looked at the correlation between ecSOD activity and superoxide generation in lung sections from fetal control and fetal PPHN lambs, and from PPHN lambs ventilated with 100% O_2 with or without PEG-catalase. Lung sections were stained with the superoxide-sensitive fluorescent probe dihydroethidium (DHE) (Fig. 6A). Fluorescence intensities within the pulmonary arteries were normalized to pixel area to correct for differences in vessel thickness and are presented in Fig. 6B. In agreement with our previous studies (10), DHE fluorescence was increased in the pulmonary arteries of fetal PPHN lambs to 1.76 ± 0.21-fold and was increased further in ventilated PPHN lambs to 4.35 ± 0.87-fold relative to fetal controls (Fig. 6B). Treatment with intratracheal PEG-catalase significantly decreased pulmonary artery DHE fluorescence in ventilated PPHN lambs to 1.14 ± 0.18-fold relative to fetal controls (Fig. 6B).

Intratracheal PEG-catalase improves oxygenation in PPHN lambs

At birth, oxygenation was significantly impaired in both groups of PPHN lambs (a/A ratio = 0.04 ± 0.01) compared with their control twins without PPHN (0.62 ± 0.08). Four lambs in the oxygen group died at 9, 10, 12, and 16 h of age, and oxygenation remained low at 24 h among survivors (Fig. 7). In the catalase group, two lambs died early at 4 and 8 h of age. Oxygenation improved at 6 h of age in this group and remained significantly higher than that in the oxygen group for the duration of the 24-h ventilation period.

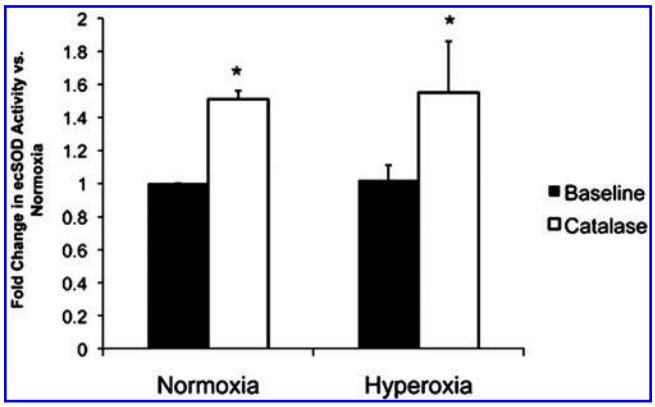

FIG. 4. PEG-catalase restores ecSOD activity in PPHN PASMCs. PASMCs isolated from PPHN lambs were treated with 0–200 U/ml PEG-catalase for 24 h in serum-free medium. ecSOD protein was purified from cell extracts with affinity chromatography, and SOD activity was determined and normalized to ecSOD expression. Data are shown as mean ± SEM relative to normoxic untreated cells; $n = 3$. *$p < 0.05$ versus untreated cells.

Discussion

The ductal-ligation lamb model of persistent pulmonary hypertension exhibits increased pulmonary arterial pressures and vascular remodeling and produces clinical and histologic disease processes characteristic of severe idiopathic PPHN (30, 45). Reactive oxygen species such as H_2O_2 contribute to abnormal vascular tone through a variety of mechanisms (38, 47) and appear to play an important role in the pathogenesis of PPHN (43). We, and others, previously showed that H_2O_2 can inhibit eNOS (23), downregulate sGC (43), and stimulate PDE5 (9). Furthermore, catalase normalized vasodilator responses to NO in fetal PPHN pulmonary arteries (43), indicating that the effects of H_2O_2 are dynamic and reversible. We therefore wished to learn whether H_2O_2 played a role in the vascular abnormalities of PPHN, and whether scavenging H_2O_2 would inhibit ROS-mediated vasoconstriction and improve the efficacy of hyperoxic ventilation in lambs with PPHN.

We previously showed that superoxide levels were increased in fetal PPHN pulmonary arteries relative to fetal controls, and that superoxide scavenging enhanced the relaxation of isolated fetal PPHN pulmonary arteries to the NO donor SNAP (3). Recent studies also showed that administration of intratracheal recombinant human SOD improved oxygenation (26) and reduced superoxide levels (10) in PPHN lambs ventilated with 100% O_2. These findings led us to hypothesize that impaired endogenous SOD activity may contribute to increased superoxide levels in PPHN. Our previous studies also indicated that total SOD activity was decreased in the pulmonary arteries of fetal PPHN lambs relative to fetal controls, although expression of the CuZnSOD and MnSOD isoforms remained unchanged (3).

In the current study, we now report that, similar to our previous expression studies, no significant differences in CuZnSOD- and MnSOD-specific activities were found in the lungs of fetal control and fetal PPHN lambs. This led us to investigate the potential role of impaired ecSOD activity in the pathogenesis of PPHN.

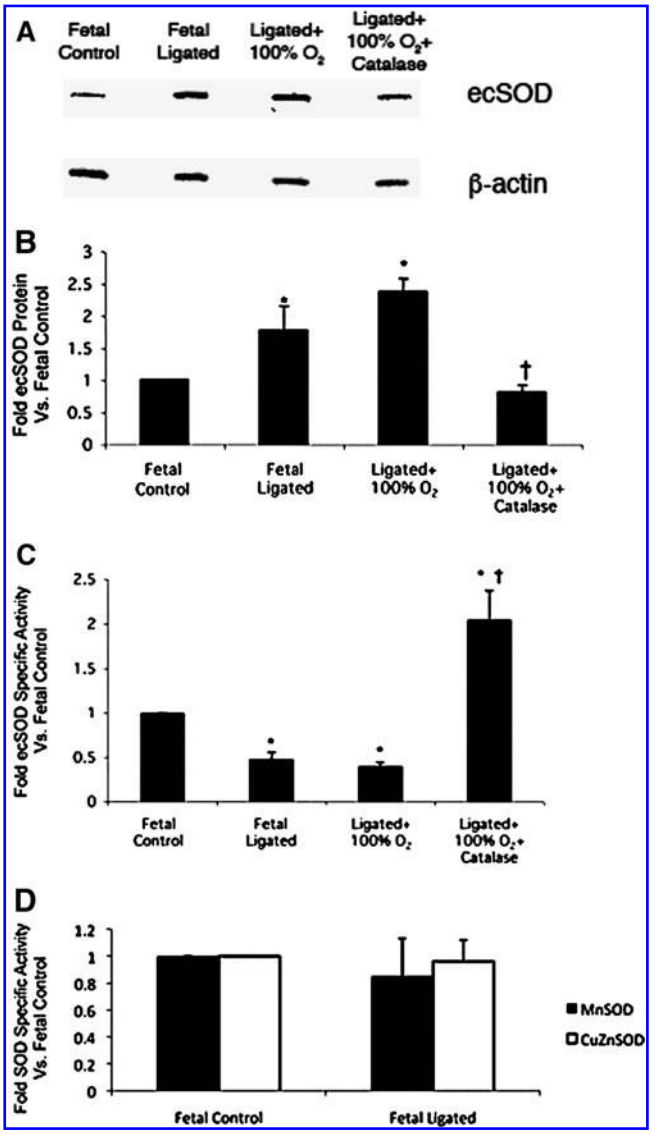

FIG. 5. PEG-catalase decreases ecSOD expression but increases activity in PPHN lungs. Lung tissue was harvested from fetal control and fetal PPHN lambs ($n = 6$ each), and from PPHN lambs ventilated for 24 h with 100% O_2 alone (L100; $n = 7$) or in combination with PEG-catalase ($n = 5$). **(A)** Representative Western blots are shown for ecSOD and β-actin in total lung protein. **(B)** Protein expression was analyzed with Western blot, with β-actin normalization. Data are shown as mean ± SEM relative to fetal control lambs. *$p < 0.05$ versus fetal control lambs. †$p < 0.05$ versus L100. **(C)** ecSOD protein was purified from lung tissue with affinity chromatography, and SOD activity was determined and normalized to ecSOD expression. Data are shown as mean ± SEM relative to fetal control lambs. *$p < 0.05$ versus fetal control lambs. †$p < 0.05$ versus L100. **(D)** CuZnSOD and MnSOD activities were determined from fetal control and fetal PPHN lung protein extracts and normalized to expression as detected with Western blotting. Data are shown as mean ± SEM relative to fetal control lambs.

Vascular ecSOD is the only known enzymatic scavenger of extracellular superoxide and is highly expressed within the lungs and vascular tissue of many species, including humans and lambs (28, 34). Overexpression of ecSOD ameliorated pulmonary hypertension in monocrotaline-treated rats (21)

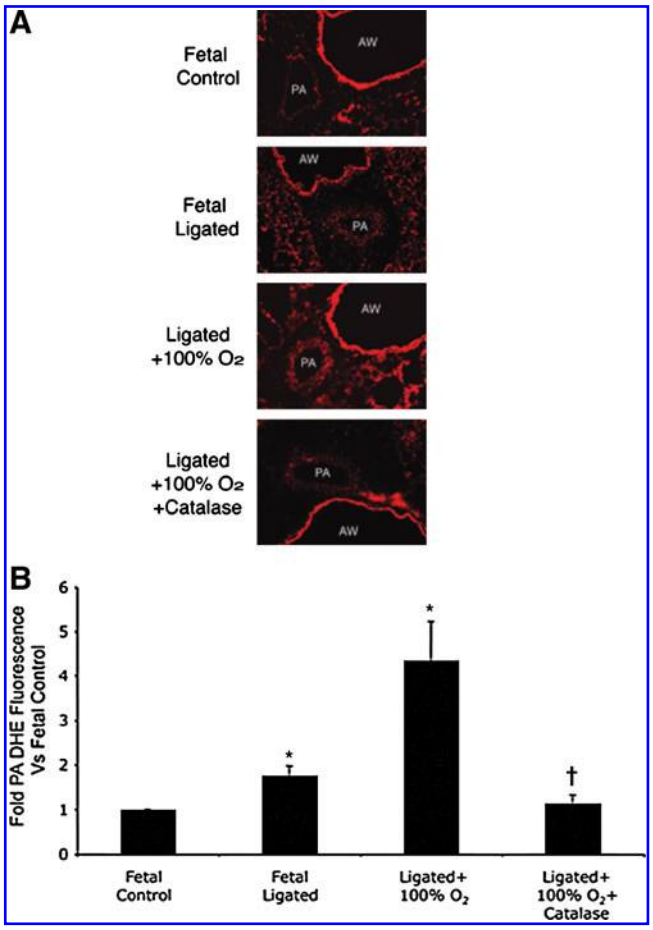

FIG. 6. PEG-catalase decreases superoxide levels in resistance PAs. (A) Frozen lung sections from PPHN lambs ventilated for 24 h with 100% oxygen alone (L100; $n = 7$) or in combination with 20,000 U/kg PEG-catalase administered at birth ($n = 5$) were stained with the fluorescent probe dihydroethidium (DHE) to detect superoxide. Sections were viewed at 20×with fluorescence microscopy, and images were captured by using a digital camera. Structures detected include pulmonary arteries (PAs) and airways (AWs). **(B)** Average fluorescence intensities within the pulmonary arteries were quantified by using MetaMorph Imaging software. Values are expressed as fold change relative to L100 lambs and shown as mean ± SEM. $*p < 0.05$ versus L100 lambs. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

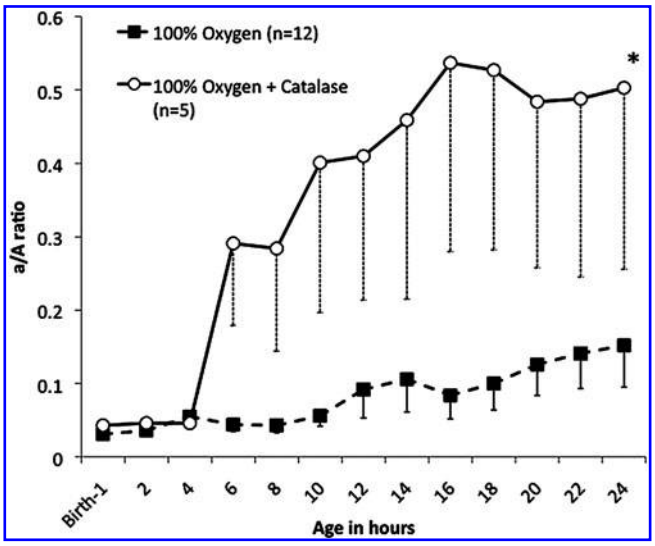

FIG. 7. PEG-catalase improves the arterial-to-alveolar oxygenation (a/A) ratio in PPHN lambs. The a/A ratio was calculated over a 24-h period for lambs with PPHN ventilated with 100% oxygen alone ($n = 12$) or with 100% oxygen in combination with 20,000 U/kg of PEG-catalase administered at birth ($n = 5$). a/A ratios are shown as mean ± SEM. $*p < 0.05$ versus L100 lambs. $*p < 0.05$ versus L100 lambs.

and attenuated pulmonary vascular remodeling in mice exposed to chronic hypoxia (32). We now report that ecSOD-specific activity is decreased in PASMCs and lung extracts of fetal PPHN lambs relative to fetal controls, a finding that further highlights the potential importance of this antioxidant enzyme in protecting against superoxide-induced pulmonary hypertension.

By using a cell-based approach, we sought to investigate the mechanisms that regulate ecSOD expression and activity in the fetal pulmonary circulation. We found that basal ecSOD expression was higher, but specific activity was lower in PASMCs isolated from fetal PPHN lambs relative to cells isolated from fetal control lambs. Control cells exposed to 95% O_2 to mimic the conditions in lambs ventilated with 100% O_2 similarly displayed a pattern of increased ecSOD expression but reduced activity. RoGFP is a ratiometric protein sensor created by substitution of surface-exposed cysteines of a green fluorescent protein that allows disulfide bond formation and thus confers redox sensitivity (8, 14). RoGFP exhibits two emission maxima, the ratio of which is independent of protein concentration and photobleaching, and has been used to demonstrate increased cytosolic ROS levels in PASMCs exposed both to hyperoxia (9) and to hypoxia (42). Our new studies with this redox sensor in fetal PASMCs indicate that increased ecSOD expression correlates with increased cytosolic protein thiol oxidation. Although our previous reports indicate that enzymatic sources such as NADPH oxidase may produce ROS, the precise sources of PPHN- and hyperoxia-induced ROS in PASMCs are currently unknown. Our previous studies indicated that endogenous H_2O_2 increases cytosolic roGFP oxidation in PASMCs exposed to hypoxia (42), and we believe the present study to be the first showing that exogenous H_2O_2 increases ecSOD expression in vascular cells.

Furthermore, our real-time PCR data indicate that H_2O_2 increases ecSOD mRNA, similar to the way angiotensin II was found to increase ecSOD transcription and RNA stability in human aortic SMCs (12). However, the mechanisms that regulate ecSOD expression are poorly understood. Antioxidant-1 (Atox-1) increased ecSOD expression in mouse fibroblasts by binding to the ecSOD promoter in a copper-dependent fashion (17). Induction of heme oxygenase-1 (HO-1) was accompanied by an increase in ecSOD expression in bovine pulmonary arteries (1), whereas exogenous H_2O_2 increased the expression of HO-1 in human aortic SMCs (4). Conversely, angiotensin II was shown to increase ecSOD expression in mouse aortas in organoid culture independent of superoxide or NADPH oxidase (12). Additional studies are warranted to investigate the mechanisms of H_2O_2-induced

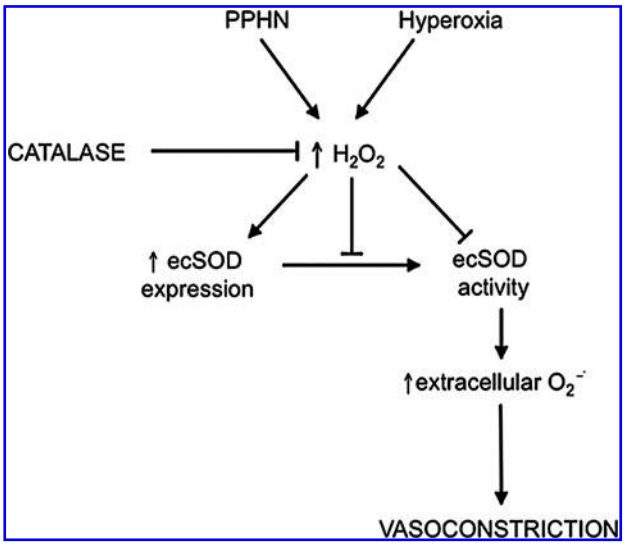

FIG. 8. Model for the effects of catalase on abnormal signaling pathways in PPHN. PPHN and hyperoxia increase H_2O_2 levels, which inactivates ecSOD, resulting in extracellular superoxide-induced vasoconstriction. Catalase treatment restores normal ecSOD activity, resulting in a reduction in extracellular superoxide, leading to vasorelaxation.

ecSOD expression in the fetal pulmonary circulation and to examine the potential involvement of ROS-sensitive transcription factors, including Atox-1 and HO-1.

Our data suggest that induction of ecSOD protein may result from a compensatory mechanism to counteract an increase in oxidative stress, although ROS-mediated ecSOD inactivation overrides the increase in expression. ecSOD activation requires Atox-1, which acts as a chaperone to deliver copper to the trans-Golgi network (TGN) before protein secretion (18). H_2O_2 has been shown to inactivate ecSOD through oxidation of copper at the enzyme active site (15, 29) and can potentially inhibit ecSOD activity in several subcellular locations by diffusing across biologic membranes. These locations include the cytosol, where H_2O_2 can oxidize copper bound to Atox-1; within the TGN, where it can oxidize copper bound to ecSOD before secretion; and within the extracellular matrix, where the enzyme resides after export. PEG-catalase would be expected to decrease H_2O_2 levels in these locations, which could explain the increase in ecSOD activity observed in PPHN cells treated with PEG-catalase. It also is possible that ROS inactivate ecSOD by oxidizing cysteine thiols and inducing a conformational change in the protein, although such an inhibitory mechanism has not been reported previously. ecSOD possesses peroxidase activity speculated to reduce extracellular H_2O_2 (15), and ecSOD inactivation may result in further increases in H_2O_2 levels, leading to sustained enzyme inhibition. H_2O_2 also has been shown to inactivate CuZnSOD (16), although our current data indicate that CuZnSOD activity is unchanged in PPHN lung homogenates relative to control samples. Subcellular H_2O_2 generation in close proximity to SOD isoform location may account for these differences in enzyme inactivation. Future experiments targeting roGFP and catalase to specific subcellular locations may reveal the precise nature of H_2O_2-mediated ecSOD inhibition in PPHN cells and in control cells exposed to hyperoxia.

In agreement with our *in vitro* data, we found an increase in ecSOD expression associated with a decrease in specific activity in lung-protein extracts from fetal PPHN lambs relative to controls. A similar pattern has been reported in models of systemic hypertension caused by renal clipping or atherosclerosis (12, 20), indicating that increased ecSOD protein levels are not necessarily accompanied by equivalent increases in enzyme activity. We propose that this effect may be mediated by excess H_2O_2 levels. In addition, we found that a single dose of intratracheal PEG-catalase at birth restores ecSOD activity in PPHN lambs ventilated with 100% O_2 for 24 h, which further supports our hypothesis that H_2O_2 increases ecSOD expression but decreases specific activity in the fetal and newborn pulmonary circulation. We previously demonstrated elevated H_2O_2 levels in pulmonary arteries isolated from fetal PPHN lambs relative to fetal controls, although endogenous catalase and glutathione peroxidase activities were unchanged (43), suggesting that increased H_2O_2 generation is primarily involved. The sources of increased H_2O_2 levels are currently unknown, although future studies using our isolated PPHN pulmonary vascular cells may help elucidate underlying mechanisms. Our current data also demonstrate that intratracheal catalase improves oxygenation in PPHN lambs ventilated with 100% O_2 for 24 h. This lamb model of PPHN is not associated with parenchymal lung disease, suprasystemic pulmonary pressures, or profound hypoxemia created by extrapulmonary shunts. Therefore, improved oxygenation after catalase treatment is most likely due to significant reductions in pulmonary vascular resistance. From these data, we speculate that increased pulmonary arterial H_2O_2 generation decreases ecSOD activity in PPHN, whereas removal of H_2O_2 by catalase restores ecSOD function and facilitates vasodilation (Fig. 8).

In this study, PEG-catalase decreased pulmonary arterial DHE fluorescence in ventilated PPHN lambs, suggesting that enhanced ecSOD activity resulted in increased superoxide scavenging. The antioxidant uric acid has been shown to restore ecSOD activity after H_2O_2–induced inactivation by rereducing copper at the active site, and infusion with oxonic acid to increase serum levels of uric acid increased aortic ecSOD activity in the $ApoE^{-/-}$ mouse model of atherosclerosis (15). Furthermore, reduced serum levels of uric acid may be associated with a higher risk of cardiovascular disease and hypertension (6, 41). Serum levels of uric acid in PPHN lambs relative to controls are currently unknown, but treatments such as oxonic acid infusion to increase uric acid levels may restore ecSOD activity and improve ventilation in these lambs.

Many factors potentially mediate abnormal ecSOD expression and activity in PPHN, and further characterization of the mechanisms involved may lead to better treatment strategies, in particular for those newborns who respond poorly to hyperoxia or inhaled NO or both. The development of antioxidant therapies that restore ecSOD activity while maintaining high levels of expression may be advantageous in the clinical management of PPHN.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ANOVA = analysis of variance
DHE = dihydroethidium
ecSOD = extracellular superoxide dismutase
FPASMC = fetal pulmonary artery smooth muscle cell
ID = internal diameter
OCT = optimal cutting temperature
PA = pulmonary artery
PBS = phosphate-buffered saline
PCR = polymerase chain reaction
PEG = polyethylene glycol
PFU = plaque-forming unit
PPHN = persistent pulmonary hypertension of the newborn
roGFP = redox-sensitive green fluorescent protein
ROS = reactive oxygen species
SMC = smooth muscle cell
SOD = superoxide dismutase
TBST = Tris-buffered saline-Tween 20
TGN = trans-Golgi network

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