Decreased Endothelial Nitric Oxide Synthase Expression and Function
Contribute to Impaired Mitochondrial Biogenesis and Oxidative Stress in
Fetal Lambs with Persistent Pulmonary Hypertension

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Running title: Mitochondrial biogenesis in PPHN.

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Abstract: Impaired vasodilation in persistent pulmonary hypertension of the newborn (PPHN) is characterized by mitochondrial dysfunction. We investigated the hypothesis that decreased endothelial nitric oxide synthase level leads to impaired mitochondrial biogenesis and function in a lamb model of PPHN induced by prenatal ductus arteriosus constriction. We ventilated PPHN lambs with 100% O\textsubscript{2} alone or with inhaled nitric oxide (iNO). We treated pulmonary artery endothelial cells (PAEC) from normal and PPHN lambs with detaNONOate, an NO donor. We observed decreased mitochondrial (mt) DNA copy number, electron transport chain (ETC) complex subunit levels and ATP levels in PAEC and lung tissue of PPHN fetal lambs at baseline compared to gestation matched controls. Phosphorylation of AMP-activated kinase (AMPK) and levels of PPAR gamma coactivator 1-alpha (PGC-1\alpha) and sirtuin1, which facilitate mitochondrial biogenesis, were decreased in PPHN. Ventilation with 100% O\textsubscript{2} was associated with larger decreases in ETC subunits in the lungs of PPHN lambs compared to unventilated PPHN lambs. INO administration, which facilitated weaning of FiO\textsubscript{2}, partly restored mtDNA copy number, ETC subunit levels and ATP levels. DetaNONOate increased eNOS phosphorylation and its interaction with HSP90, levels of superoxide dismutase 2 (SOD2) mRNA, protein and activity and decreased the mitochondrial superoxide levels in PPHN-PAEC. Knockdown of eNOS decreased ETC protein levels in control PAEC. We conclude that ventilation with 100% O\textsubscript{2} amplifies oxidative stress and mitochondrial dysfunction in PPHN, which are partly improved by iNO and weaning of oxygen.
Introduction

Endothelial nitric oxide synthase (eNOS) plays a central role in postnatal pulmonary vasodilation, a physiologic event required for successful transition at birth. Expression of eNOS increases towards term gestation in fetal lungs, to prime the neonatal lung for this adaptation. Exposure of the fetal lungs to higher oxygen tension at birth stimulates eNOS activity and increases nitric oxide (NO) release. The function of eNOS is regulated by interactions with the chaperone HSP90, phosphorylation at serine\textsuperscript{1179}, availability of arginine and tetrahydrobiopterin (BH4) and reactive oxygen species (ROS). NO has several downstream effects in the pulmonary circulation, including vasodilation, regulation of mitochondrial function and angiogenesis. Exposure of the fetal lung to higher oxygen levels at birth increases ATP production, which in-turn increases eNOS activity. Superoxide (O$_2^-$) anion, produced as a byproduct of mitochondrial oxidative phosphorylation, is scavenged by the mitochondrial superoxide dismutase (SOD2). Regulation of mitochondrial O$_2^-$ levels by SOD2 is necessary for normal endothelial function and ATP synthesis. Development of adequate number and mass of mitochondria through biogenesis is required as fetal lungs prepare for postnatal transition and increase in ATP synthesis. NO is an important upstream regulator of mitochondrial biogenesis, which involves both nuclear and mitochondrial encoded genes. Mitochondrial proteins produced by the nuclear genes induce mitochondrial DNA (mtDNA) transcription and replication, and synthesis of electron transport chain (ETC) protein subunits. Peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) is the master regulator of mitochondrial biogenesis and also integrates biogenesis with oxidative phosphorylation. AMP activated kinase (AMPK) and histone deacetylase, sirtulin-1 (SIRT1), together modulate PGC1α activity in a coordinated manner to maintain metabolic and redox homeostasis.
Studies in the ductal constriction model of persistent pulmonary hypertension of the newborn (PPHN) have shown that eNOS expression is down regulated and levels of reactive oxygen species (ROS) are increased in the pulmonary arteries. In addition, eNOS uncoupling from impaired HSP90 interactions with eNOS and decreased BH4 levels further decreases NO levels in PPHN. In experimental and clinical studies, inhaled nitric oxide (iNO) induces pulmonary vasodilation, leading to sustained improvement in oxygenation in infants with PPHN. However, mechanical ventilation and hyperoxia, even for a short period, increase ROS formation. ROS react with NO at a diffusion-limited rate, leading to rapid depletion of NO.

We recently reported that mitochondrial oxidative stress is increased in pulmonary artery endothelial cells (PAEC) in PPHN. Exposure of PPHN infants with preexisting mitochondrial oxidative stress in their vessels to mechanical ventilation and hyperoxia could potentially exacerbate mitochondrial oxidative stress and vascular dysfunction. The decrease in NO and increase in ROS can lead to loss of mitochondria via decrease in biogenesis and removal of dysfunctional mitochondria by mitophagy. PAEC in PPHN have significantly reduced ATP levels, suggesting impaired oxidative phosphorylation.

We, therefore, hypothesized that increased mitochondrial oxidative stress and decreased NO availability impair mitochondrial biogenesis in PPHN. Our objectives for this study are to (1) determine the potential mechanistic link between reduced NO levels and alterations in mitochondrial biogenesis in PPHN, (2) the impact of ventilation with 100% O₂, typically used in PPHN infants, on mitochondrial biogenesis in PPHN and (3) determine the effects of iNO therapy and subsequent weaning of FiO₂, as typically used in the clinical management of PPHN, on mitochondrial biogenesis and redox balance in lambs with PPHN.
Study designs and methods.

Antibodies and chemicals: Rabbit anti-SOD-2 (1:10,000) was obtained from Assay Designs-Enzo Life Science (Ann Arbor, MI). Mouse anti-β-actin (1:10,000), rabbit anti-eNOS (1:5,000), rabbit anti-eNOS serine\(^{1179}\) (1:1,000), rabbit anti-HSP90 (1:1,000) and eNOS specific siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-mitochondrial ETC complex proteins were obtained from Mitoscience (Eugene, Oregon). SOD2 activity detection kit was from Cayman Laboratory. ATP extraction reagent kit was obtained from Sigma Aldrich (St Louise, MO). Primers for sheep NADH dehydrogenase 1 (ND1), NADH dehydrogenase 2 (ND2), SOD2 and eNOS and detaNONOate were obtained from Life-Science Technology-Invitrogen (Carlsbad, CA).

Creation of PPHN lamb model: This study was approved by the Institutional Animal Care and Use Committees of the Medical College of Wisconsin and University of Buffalo, NY and conformed to NIH Guidelines for Animal use in Research. Pulmonary hypertension was induced by prenatal ductus arteriosus constriction as previously described.\(^{18-19}\) Time-dated pregnant ewes at 128 d of gestation (term ~145 d) were anesthetized with inhaled isoflurane (2%). The fetal lamb was partially exteriorized by a midline laparotomy and hysterotomy, and an umbilical tape was tied around the ductus arteriosus. Fetal chest was then closed and the lamb was placed back in the uterus. Sham operated and gestation-matched twin fetal lambs were used as controls. Ewe was then allowed to recover for 8 d. Previous studies have shown that these lambs consistently develop severe PPHN with this intervention.\(^{20,23,28}\).

Ventilation studies were done at the University of Buffalo. Lambs were ventilated for 24 hours and sacrificed. Right carotid and jugular venous lines were placed at the time of C-section at birth. Blood gases were measured every 10-15 mins in the first hour and every hour for 24 h. Ventilator rates and pressures were adjusted to keep arterial partial
pressure of CO2 (PaCO2) in the target range of 40-55 mmHg and PH>7.20 (Table 2).

PPHN lambs received one dose of 3ml/kg of Calfactant (Infasurf, ONY Laboratories, Amherst, NY, a gift from Dr. Edmund A. Egan), administered via endotracheal tube at birth to avoid the contribution of surfactant deficiency to hypoxemia, followed by mechanical ventilation and were assigned to 2 groups: 1) ventilation with 100% O2 for 24 h without weaning and without iNO (n=7), or 2) ventilation with 20 parts per million (ppm) iNO administered via endotracheal tube, which was started between 6 and 12h when the oxygenation index (OI= Mean airway pressure x FiO2 x 100 / PaO2 in mmHg) was >25 (n=7). Inhaled NO was started at a mean of 7.6±1.1 h and before 10h age in all 7 lambs.

The inspired O2 concentration was titrated to maintain PaO2 between 60-80mmHg, to simulate the clinical management of infants with PPHN.

In the PPHN fetal lambs that responded well to iNO treatment, FiO2 was weaned by 10-15% based on PaO2 and oxygen saturation. When FiO2 was <60% and PaO2 remained >60, iNO was weaned by 5ppm every 4 h until the dose was at 5 ppm and then by 1 ppm every 4 h. If the FiO2 requirement increased by more than 15% following a wean, the previous dose of iNO was resumed. All lambs were ventilated for 24 h and sacrificed. Lung tissue samples were collected from the left apical lobes, washed in cold buffer and snap frozen in liquid nitrogen.

Gestation-matched unventilated PPHN fetal lambs and normal fetal lambs were used as controls. Fetal lungs were harvested for the isolation of lung tissue which was snap frozen in liquid N2 for protein studies.

**Cell culture:** Control and PPHN fetal lambs delivered at term gestation without ventilation at Medical College of Wisconsin were used for isolation of pulmonary arteries and PAECs, using techniques we described previously.1, 20 PAEC isolated from control (normal) and unventilated PPHN fetal lambs were cultured in DMEM with 20% fetal calf serum (FCS) at 37°C in room air and 5% CO2. We used PAEC between passages 2 and
4 for our experiments. Endothelial cell identity was verified by staining for factor VIII antigen and acetylated LDL uptake.\(^2\)

**NO donor studies:** PAEC isolated from normal fetal lambs and fetal lambs with PPHN were grown to 60% confluence in 60 mm-well plates. Cells in selected plates were treated with 200nM detaNONOate, which was added to the culture medium followed by incubation for 18 h at 37°C. We assessed the effects of detaNONOate on mitochondrial ETC subunit levels, levels of mitochondrial superoxide, SOD2 expression and activity, and cellular oxygen consumption as described below.

**NO measurements:** The concentration of NO released by detaNONOate was assessed by measuring NO level in cultured medium of rapidly dividing PAECs following detaNONOate treatment using an NO analyzer (inNO-T-II, Innovative Instruments, Inc.), connected to a computer. PAECs were grown in 100 mm tissue culture plates until 90% confluent. Cells were then transferred to the measuring platform in 21% O\(_2\) at room temperature (25°C) and allowed to equilibrate for 1 h prior to measurement. After sensor calibration, 200nM detaNONOate was added to the medium, and NO level was measured for the next 18 h.

**Western blot analysis of proteins:** PAEC were grown to 90% confluence and washed twice in ice-cold Hank’s Balanced Salt Solution (HBSS). The cells were lysed in modified RIPA buffer. Protein content of the lysates was determined by bicinchoninic acid (BCA) assay. Separated proteins were transferred to nitrocellulose membranes and were blotted with specific polyclonal antibodies for SOD2, eNOS, ETC I-V proteins and β-actin, overnight at 4°C. The membranes were blotted with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:10,000; Bio-Rad) or HRP-conjugated anti-rabbit IgG antibody (1:10,000; Bio-Rad) and exposed to CL-XPosure film (Pierce) after treatment with SuperSignal West Pico (Pierce). The signals were analyzed with Image J.
and normalized to the expression of loading control, β-actin for whole cell and tissue lysates.

Peripheral lung tissue samples were flash-frozen in liquid nitrogen, pulverized, and placed in modified RIPA buffer to obtain homogenates. An aliquot of protein (50 μg) was used for immunoblots for electron transport chain complex I-V proteins, SOD2, eNOS and β-actin as described above.

**Quantification of mtDNA copy number:** We quantified mtDNA and nuclear DNA copy numbers using the iQ5 multicolor real-time PCR detection system (Bio-Rad). Genomic DNA was extracted from 100μg tissue samples using Qiagen mini DNA extraction kit. 5μg of the purified genomic DNA was used for the PCR as described previously. NADH dehydrogenases 1 and 2 (ND1 and ND2) genes were selected from the D-loop of mtDNA and PCR primers were designed using Primer3 with sequence as follows; ND1 forward 5’-GGAGGACTGAACCAAACCCA-3’, reverse 5’-GCCTGAGGATAGGGGAAT-3’ and ND2 forward 5’-TACTTCAACCCATCGCCGAC-3’ and reverse 5’-ACGGCTAGGCTTGA-TATGGC-3’. The specificity of each primer was confirmed by performing a blast search to ensure that the primer sequences are unique for the template sequence. The PCR cycle was started at 95°C for 3 min followed by 40 cycles of 95°C for 10 s and then 58°C for 1 min. Melting temperatures were monitored for each pair of primers, and single-peak melting temperature was observed for all of the primer pairs. MtDNA copy number was normalized to 18S and β-actin copy numbers. The number of the threshold cycle (Ct) for each target DNA was corrected against the corresponding Ct of 18S and β-actin to obtain the Ct, and then \(2^{-\Delta\Delta Ct}\) was calculated against the corresponding control for estimation of DNA abundance.

**Quantification of PGC1α, SOD2 and eNOS mRNA:** RNA was extracted via the TRizol (Sigma) method from lung tissue and the contaminated DNA was removed by the TURBO DNA-free Kit (Ambion) in a 37°C water bath for 60 min. cDNA was synthesized
from the extracted RNA using the iScript cDNA synthesis kit (Bio-Rad). The PCR
primers were designed using Primer3 as previously described and sequences are as
follows: PGC1α (forward 5’-CAGACCTGACACAACGC-3’, reverse 5’CTTGAAAAATT
GCTTGCGT-3’), SOD2 (forward 5’-ATTGCTGGAAGCCCATCAAAC-3’) and reverse 5’-
AGCAGGGGGATAAGACCT-3’), eNOS (forward 5’-CCTCACCGCTACAAC-3’ and
reverse 5’-GCACAGCCAGGTTGATCT-3’). Real-time RT-PCR was performed using the
iQ5 multicolor real-time PCR detection system (Bio-Rad) as described above.

**ATP quantification:** Lung tissue samples were homogenized in ATP releasing agent
using ATP extraction kit by Sigma-Aldrich. Lysates were analyzed for ATP concentration
using firefly luciferase bioluminescence method. ATP levels in each sample were
expressed in nanomoles/milligram of protein.

**Measurement of SOD2 activity:** Lung tissue samples were homogenized in HEPES
buffer and SOD2 specific activity was quantified using colorimetric method as described
previously. SOD2 specific activity was obtained by treatment of cell lysates with
potassium cyanide (KCN) to inhibit cytosolic and extracellular SOD isoforms. One unit of
SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation
of the O$_2^-$, and SOD2 activity was corrected against protein content measured using
BCA method.

**Co-immunoprecipitation studies:** eNOS-HSP90 interaction and eNOS serine$^{1179}$
phosphorylated fraction of total eNOS protein were assessed in lung tissue using
immunoprecipitation as previously described.$^2$ eNOS was immune-precipitated from lung
tissue. The immune-precipitate was blotted for eNOS, HSP90 and serine$^{1179}$
phosphorylated eNOS.

**Endothelial NOS knockdown:** We used small-interfering RNA (siRNA, Santa Cruz
Biotechnology) to knockdown eNOS expression in PAEC. Control PAECs were seeded
in antibiotic-free medium supplemented with 20% FCS. 10µl of eNOS siRNA duplex was
diluted in 1ml of Opti-MEM1 and 10µl of Lipofectamine RNAiMax, (Invitrogen, Carlsbad, CA). After 15 min at room temperature, cells suspended in 4ml of antibiotic free culture medium were added to the diluted siRNA mixture and incubated at 37°C in a CO2 incubator. After 24h of incubation at 37°C in a CO2 incubator, medium was replaced with fresh 1x normal regular growth medium and incubated for another 24 h before the cells were used for experiments. PAEC transfected with non-silencing RNA (sc-37007) were used as controls.

Assessment of mitochondrial O$_2^-$ levels: We used mitochondrial targeted hydroethidine epifluorescence (MitoSOX, Molecular Probe, Sigma) to quantify O$_2^-$ levels in the mitochondria. Control PAECs were grown to 50–60% confluence in eight-well chamber slides. Control cells in selected wells were transduced with siRNA targeted to eNOS. After 24h of siRNA transduction, growth medium was replaced with 1x normal regular growth medium. Control PAEC transduced with eNOS siRNA and PPHN PAEC were incubated with or without 200nM detaNONOate for 18h. After treatments, cells were incubated with 10µM Mito-HE for 30 min to detect mitochondrial O$_2^-$ levels as described previously. Fluorescence signals were quantified using fluorescence detection microscopy and analyzed with MetaView software.

Assessment of mitochondrial function: The effects of NO-induced changes in mitochondrial bioenergetics were assessed using Seahorse Bioscience XF24 technology (Seahorse Bioscience; N. Billerica, MA). Prior to the studies, empirical optimization of cell density, doses of oligomycin, FCCP and antimycin for PAEC were determined. PAEC, 3×10$^4$ in count, from control and PPHN were grown in 24-well plates. Specified wells containing control and PPHN cells were treated with 200nM detaNONOate for 18h in normal culture medium. Just before the experiment, culture medium was replaced with Seahorse media and left to equilibrate at 37°C for 1-2 h. Baseline O$_2$ consumption rate (OCR) was then established and measured. Oligomycin was then injected to measure...
OCR due to proton leak. Maximum OCR was measured after injection of FCCP. Non-
mitochondrial O₂ consumption was measured after antimycin was injected. Cells were
washed in HBSS and protein was quantified using BCA method. Results were
normalized to protein content.

**Statistical analyses:** Data are shown as mean ± SE. Student’s t-test was used for
normally distributed data, and Mann-Whitney U-test was used for data that did not pass
the normality test for comparison between two groups. One-way ANOVA with Student-
Newman-Keuls post hoc test was used when more than two groups were compared.

Data were analyzed with Graph Pad Prism (Graph Pad software, Inc., La Jolla, CA). A p
value < 0.05 was considered significant.

**Results**

*Mitochondrial biogenesis is impaired in the lungs of PPHN fetal lambs:* Copy
numbers for ND1 and ND2 DNA, components of mitochondrial electron transport chain
complex -I, were decreased at baseline in the lungs of unventilated PPHN fetal lambs
compared to gestation-matched normal fetal lamb controls (Fig 1A). This observation
demonstrates that mtDNA copy number was decreased in PPHN. Mitochondrial electron
transport chain protein subunit proteins were also decreased in PPHN, accompanied by
reduced ATP levels in PPHN fetal lambs when compared with controls (Fig 1B and C).
These findings are consistent with our previous observation that AMPK function and
ATP levels are decreased in PPHN PAEC.², ³⁸

*Effects of mechanical ventilation and hyperoxia with or without iNO on systemic
oxygenation in PPHN fetal lambs:* Seven lambs with PPHN were ventilated without
iNO and the FiO₂ was kept at 1.0 for the entire duration of 24 h ventilation. Seven other
lambs with PPHN were ventilated with addition of iNO after 6h of age and the FiO₂ was
weaned, based on arterial blood gases to maintain arterial PO₂ in the target range.
PPHN lambs had severe hypoxemia with OI of 37±7 at birth. There was no difference in the baseline OI between the two groups at birth. Administration of iNO resulted in a significant reduction in the mean airway pressure (MAP) and FiO2, based on improved oxygenation, compared to PPHN lambs ventilated with oxygen alone (Table 1). The arterial pCO2 was significantly lower and pH was higher in PPHN lambs that received iNO compared to ventilated PPHN lambs with O2 alone (Table 1).

**Ventilation with 100% O2 exacerbates the mitochondrial dysfunction in PPHN:** In comparison with unventilated PPHN fetal lambs, ventilation with 100% O2 for 24 h induced a further decrease in ETC subunits (Fig. 1B); however, we observed no significant change in ATP levels from unventilated PPHN lambs (Fig 1C). This suggests that an extra-mitochondrial source of ATP, such as aerobic glycolysis, is induced to maintain the ATP levels in these lambs.

**Inhaled nitric oxide with weaning FiO2 restores mitochondrial function in PPHN.** INO treatment for 12-18 h in ventilated PPHN fetal lambs resulted in a decrease in mean FiO2 to <0.7 and mean OI to <30 within 2-6 h of iNO initiation (Table 1). This strategy was associated with increased mitochondrial DNA copy number and ETC subunits (Fig. 1A and B). Since increased mitochondrial number and protein content does not imply functional integrity of new mitochondria, we measured ATP levels in these samples. Compared to ventilation with 100% O2 alone, iNO + lower FiO2 use was also associated with higher ATP levels (Fig 1C). These data suggest improved mitochondrial function in the group assigned to iNO use and FiO2 wean in PPHN.

**Ventilation with 100% oxygen impairs and iNO restores PGC1α function in PPHN.** The decreased mitochondrial copy number and ETC were associated with decreases in levels of PGC1α, a transcription co-factor that regulates mitochondrial biogenesis and phospho-AMPKα and Sirt1, which regulate the function of PGC-1α, in PPHN. The
protein level of AMPKα did not change with PPHN in the absence of ventilation (Fig 2A).

Ventilation with 100% O₂ led to decrease in AMPKα and further decreases in p-AMPKα, PGC1α and Sirt-1 protein levels compared to unventilated PPHN samples (Fig 2B and C). These data suggest that ventilation with 100% induces downregulation of transcriptional activators that regulate mitochondrial biogenesis.

INO treatment for 12-18 h in ventilated PPHN fetal lambs resulted in an increase in PGC1α, as well as p-AMPKα, AMPKα and Sirt1 expression when compared to ventilation with 100% O₂ alone and to un-ventilated PPHN fetal lambs (Fig 2B and 2C).

**NO-induced mitochondrial biogenesis improves cellular respiration in PPHN**

**PAEC:** We recently reported that PPHN-PAEC have lower ATP/higher ADP levels, suggesting uncoupling of respiration and ATP synthesis.² We tested the impact of NO-induced mitochondrial biogenesis on cellular respiration by measuring oxygen consumption and utilization for ATP synthesis in PPHN-PAEC. Previous studies reported that 1µM detaNONOate releases 4nM NO in the medium with a half-life of 22 h.⁵ Our data shows that addition of 200nM detaNONOate releases ~ 3nM of NO over 18h consistently into the culture medium, which will then be available to the PAEC (Fig 3A).

At baseline, basal oxygen consumption rate and extra-mitochondrial O₂ consumption rate were higher in PPHN PAEC when compared to normotensive PAEC (control). In contrast, ATP-linked O₂ consumption rate was lower in PPHN-PAEC. In normotensive PAEC, treatment with detaNONOate for 18h decreased the mitochondrial biogenesis and ATP synthesis (data not shown). In contrast, treatment with detaNONOate induced an increase in ATP-linked O₂ consumption, which was associated with a decrease in OCR, extra-mitochondrial O₂ consumption and proton leak in PPHN-PAEC (Fig 3B). These data suggest that PPHN is associated with decreased mitochondrial biogenesis, uncoupling of respiration and decreased ATP synthesis. These changes were reversed by treatment with an NO donor.
INO and DetaNONOate improve Redox balance in PPHN PAEC: Previous studies have shown that PGC-1α overexpression upregulates mitochondrial biogenesis and SOD2 function in cultured neurons. In this study, we observed that iNO increased SOD2 mRNA and protein and SOD2-specific activity in the lungs of PPHN fetal lambs when compared to un-ventilated PPHN fetal lambs (Fig 4A, B and C). In contrast, ventilation with 100% O2 alone had no significant impact on SOD2 expression and activity when compared with non-ventilated PPHN fetal lambs. NO-induced increase in oxidative capacity and ATP synthesis could potentially lead to increased mitochondrial $O_2^{\cdot-}$ levels as a by-product of oxidative phosphorylation. We therefore, investigated the effects of NO on mitochondrial $O_2^{\cdot-}$ levels. Treatment for 18 h with detaNONOate decreased mitoSOX fluorescence intensity in PPHN-PAEC when compared with untreated PPHN-PAEC (Fig 5).

Role of eNOS and NO in mitochondrial biogenesis and Redox regulation:

Knockdown of eNOS in PAEC from control fetal lambs decreased eNOS protein level by ~80% and markedly reduced ETC subunits and SOD2 expression when compared to non-silencing RNA transfected cells (Fig 6A and B). Knockdown of eNOS in control cells also increased mitoSOX fluorescence intensity when compared to controls (Fig 5B). This indicates the critical role of eNOS in the regulation of mitochondrial redox balance and function (Fig 5). Upregulation of SOD2 function may help attenuate the mitochondrial $O_2^{\cdot-}$ from NO-induced increase in oxidative phosphorylation.

INO restores eNOS function in PPHN fetal lambs: Decreased NO bioavailability is a key factor in the development of PPHN. Previous studies have shown that impaired eNOS interactions with HSP90 increased $O_2^{\cdot-}$ formation instead of NO. Consistent with these reports, we observed a decrease in eNOS mRNA, protein, HSP90/eNOS ratio and serine phosphorylated eNOS in the unventilated PPHN lung samples (Fig 7 A-D). Ventilation of PPHN lambs with 100% O2 led to 2-fold increase in eNOS mRNA and no
increases in eNOS protein levels, HSP90/eNOS ratio and serine-1179 phosphorylation when compared to unventilated PPHN fetal lambs (Fig 7A-D). Use of iNO with weaning of FiO2 was associated with an 8-fold increase in eNOS mRNA, 3-fold increase in protein levels and increases in eNOS-HSP90 association and serine-1179 phosphorylation, compared to ventilation with 100% O2 alone (Fig 7A-D). These data demonstrate that administration of iNO and weaning of FiO2 is associated with conditions that favor a coupled function of eNOS in the lung.

Discussion

Our studies demonstrate that mitochondrial biogenesis and ATP synthesis are impaired in PPHN. The decrease in ATP level is associated with decreased eNOS activity and impaired pulmonary vasodilation, which was previously reported in PPHN.2, 35 This finding may be central to the metabolic alterations in PPHN and may have important therapeutic implications. Ventilation with high FiO2 exacerbated the mitochondrial metabolic abnormalities in PPHN. We observed that iNO treatment, which allowed decreased FiO2 use, restored mitochondrial biogenesis and Redox balance in PPHN. These data suggest that mitochondrial redox state is a critical part of the complex adaptive responses during postnatal transition. PPHN represents a failure of normal cardiopulmonary adaption at birth.10, 28 The ductal constriction model of PPHN simulates a known mechanism for PPHN and also reproduces the hemodynamic and structural alterations observed in neonates with PPHN.23 PPHN is frequently associated with parenchymal lung diseases such as meconium aspiration, sepsis and RDS, causing ventilation-perfusion (V/Q) mismatch and severe hypoxemia.9,10. Use of high inspired oxygen concentrations in the affected infants may worsen the pulmonary vascular dysfunction.23 Although O2 is known to stimulate eNOS activity and NO production,18 mechanical ventilation and hyperoxia...
increase ROS formation in a synergistic and cumulative manner. We observed that decreases in mitochondrial ETC subunit proteins in PPHN were associated with an increase in both mitochondrial and extra-mitochondrial oxygen consumption in PPHN. NO regulates mitochondrial oxygen consumption via reversible inhibition of cytochrome C oxidase (complex IV) enzyme. Our studies suggest that decreased NO levels led to loss of this inhibitory effect of NO on cellular oxygen consumption in PPHN. Furthermore, in PPHN, the ETC subunit proteins may be dysfunctional, which may lead to increased electron leak during electron transport in oxidative phosphorylation. This can lead to an increase in mitochondrial superoxide anion formation inside mitochondria in PPHN PAEC. The observed decrease in oxidative phosphorylation, coupled with increases in mitochondrial O$_2^-$ levels in PPHN suggests a potential increase in oxidative stress during exposure to hyperoxia in PPHN. Since most infants with PPHN are not diagnosed prenatally, initiation of iNO therapy is often delayed. Our data demonstrate that ventilation with 100% O$_2$ worsens the mitochondrial function in PPHN. This may lead to poor response to iNO and worsening of the vascular dysfunction. These observations suggest a potential mechanism for a lack of response to iNO in some PPHN infants.

Inhaled NO use led to improved oxygenation and reduced inspired oxygen concentrations and restored the mitochondrial biogenesis and Redox balance in ventilated PPHN lambs. The relative contribution of iNO and decreased exposure to O2 in restoring mitochondrial biogenesis is not clear from our study. Previous studies have shown that NO can decrease DNA damage in yeast caused by ROS. Randomized controlled trials have shown that earlier initiation of iNO therapy decreases ECMO rates in infants with PPHN. Our data on decreased ETC protein subunits and SOD2 expression and function following eNOS knockdown support the role of eNOS in the regulation of mitochondrial biogenesis and Redox homeostasis. Our findings are also
consistent with previous observations in eNOS $^{-/-}$ mice, which demonstrated that endogenous NO regulates mitochondrial biogenesis in vivo. We observed that iNO increased mitochondrial oxidative capacity and ATP synthesis in PPHN. In addition to increasing eNOS activity, ATP plays important roles in facilitating transport of substances across cell membranes. We reported previously that HSP70 chaperones SOD2 to the mitochondria after synthesis in the cytosol via ATP dependent mechanisms. $^2$ ATP increases SOD2 interactions with HSP70 and facilitates SOD2 import to the mitochondria, where it is activated and scavenges $\text{O}_2^-$. Inhaled NO increased SOD2 at both transcriptional and posttranslational levels. An increase in mitochondrial biogenesis from NO exposure can increase ATP synthesis by oxidative phosphorylation. In the absence of iNO, it was difficult to wean the FiO2 from 1.0 while maintaining PaO2 in the target range in our PPHN lambs (Table 1). INO use allowed us to wean the FiO2 and mean airway pressure, which also potentially contributed to favorable effects on mitochondrial redox state and biogenesis. PGC1α appears to play a critical role in iNO-induced mitochondrial biogenesis and Redox balance. We observed decreases in the levels of AMPKα, Sirt-1, pAMPKα, and eNOS proteins when PPHN fetal lambs were exposed to mechanical ventilation with hyperoxia. Expression of PGC1α and mitochondrial number and function were also decreased during ventilation with 100% O2. Increasing NO levels either by treatment with detaNONOate in cultured PAEC or iNO therapy in lambs increased PGC1α levels and mitochondrial biogenesis. We speculate that increased ROS formation in hyperoxia contributes to depletion of NO, thereby attenuating PGC1α. INO treatment restored eNOS expression and activity in PPHN. Previous studies have shown that eNOS expression, activity and eNOS-HSP90 interaction are decreased in PPHN. $^{16, 21}$ We observed that iNO treatment in ventilated PPHN fetal lambs increased eNOS mRNA and protein levels, serine phosphorylation and eNOS interactions with...
HSP90. In order to verify that these effects are due to NO, we investigated the effect of NO donor, detaNONOate on endothelial cells from PPHN lambs. These studies demonstrated that NO donor alone can restore eNOS expression and function in PAEC, consistent with the in vivo effects observed in ventilated lambs. Our data suggest that NO has an independent effect in improving eNOS function and mitochondrial biogenesis. There are some limitations to this study. We cannot determine whether ventilation with iNO, weaning to lower inspired oxygen concentrations or reduced volume and barotrauma from weaning MAP or a combination of the three factors resulted in the observed beneficial effects on mitochondrial biogenesis and eNOS activity. Our attempts to ventilate PPHN lambs with lower levels of inspired oxygen without iNO led to poor survival due to severe hypoxemia (data not shown). We have previously shown that ventilation with iNO and 100% oxygen increases eNOS expression by 2-fold in PPHN lambs. In the current study, we demonstrate that iNO and weaning inspired oxygen increase eNOS expression 4-fold. We speculate that iNO and weaning inspired oxygen may have an additive effect on eNOS expression. Another limitation to our study is that we did not measure pulmonary blood flow and pulmonary arterial pressures in our ventilated lambs. Therefore, we are unable to verify whether increased mitochondrial biogenesis in iNO treated lambs is associated with improved pulmonary vasodilation. A thoracotomy and probe placement for monitoring pulmonary hemodynamics significantly increased morbidity leading to increased mortality in PPHN lambs after 6-12 hours of ventilation.

In conclusion, PPHN is associated with impaired mitochondrial biogenesis and function and decreased ATP synthesis in pulmonary arteries in our fetal lamb model. Ventilation with 100% O₂ may worsen this vascular dysfunction in PPHN. Administration of iNO allowed for a rapid wean of FiO₂ and MAP. This strategy restored mitochondrial biogenesis and improved redox balance. Further studies are needed to determine
whether early initiation of iNO therapy in patients with PPHN restores vascular function and improves vasodilation and metabolic balance in the affected infants.

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**Figure Legends**

**Figure 1:** Mitochondrial biogenesis is impaired in the lungs of fetal lambs with persistent pulmonary hypertension (PPHN). (A) Copy number for NADH dehydrogenases 1 and 2 (ND1 and ND2), components of the mitochondrial DNA (mtDNA) and (B) mitochondrial electron transport chain (ETC) complexes I-V are significantly decreased in lungs of PPHN fetal lambs when compared to gestation matched normotensive fetal lambs (control). This is accompanied by a decrease in ATP levels (C). Ventilation with 100% O2 caused a larger decrease in ETC complexes I-V, with no significant additional change in ND1 and ND2 DNA copy numbers in the lungs of PPHN fetal lambs. Addition of inhaled nitric oxide (iNO) in ventilated PPHN fetal lambs significantly increased ND1 and ND2 DNA copy numbers and ETC complex protein levels when compared to unventilated PPHN lambs and PPHN lambs ventilated with 100% O2 alone (N=6, p<0.05). # indicates p<0.05 from normal fetal lambs, Ω indicates p<0.05 from unventilated PPHN lamb and * p<0.05 from PPHN fetal lambs ventilated with 100% O2 alone.

**Figure 2:** Inhaled nitric oxide (iNO) restores mitochondrial biogenesis and oxidative phosphorylation by increasing the expression levels of PGC1α in ventilated PPHN fetal lambs. (A) In lung lysates of PPHN fetal lambs without ventilation, expression of SIRT 1, PGC1α as well as p-AMPKα were decreased. Ventilation with 100% O2 alone led to a further decrease in p-AMPKα and SIRT1 protein levels and PGC1 α protein and mRNA levels (B) and a decrease in AMPKα in PPHN lambs. Inhaled NO significantly increased the levels of p-AMPKα, AMPKα, SIRT1, as well as PGC1α in ventilated PPHN fetal lambs compared to PPHN lambs with or without ventilation. (N=6 p<0.05). # indicates p<0.05 from normal fetal lambs, Ω from unventilated PPHN lamb and * from PPHN fetal lambs ventilated with 100% oxygen alone.
Figure 3: DetaNONOate increased the coupling of respiration and oxidative phosphorylation in PPHN PAEC. In PPHN-PAEC, the basal O₂ consumption rate (OCR), proton leak, as well as extra-mitochondrial oxygen consumption rate were higher when compared to normotensive PAEC (controls), but the ATP-linked O₂ consumption was lower in PPHN-PAEC. Treatment with 200nM detaNONOate, an NO donor, for 18 h released ~3nM NO (Fig. 2A) and decreased OCR, proton leak and extra-mitochondrial O₂ consumption rates in PPHN-PAEC, while specifically increasing the ATP-linked OCR in PPHN PAEC (N=6, p<0.05). # indicates p<0.05 from normal PAEC and * p<0.05 from untreated PPHN PAEC.

Figure 4: Inhaled nitric oxide improves mitochondrial Redox balance in PPHN. SOD2 protein and mRNA levels were unchanged in the lungs of PPHN fetal lambs, whereas, SOD2 activity was significantly decreased in PPHN compared to the age-matched normal fetal lambs. Ventilation with 100% O₂ alone further decreased SOD2 mRNA, protein and activity levels in PPHN fetal lambs. Treatment with iNO increased SOD2 mRNA, protein and activity levels compared to PPHN fetal lambs ventilated with 100% O₂ alone or unventilated PPHN lambs (N=6, p<0.05). # indicates p<0.05 from control lambs and * p<0.05 from PPHN fetal lambs ventilated with 100% O₂ alone.

Figure 5: DetaNONOate decreased mitochondrial oxidative stress in PPHN-PAEC. Representative micrograph showing mitoSOX fluorescence in (A) control PAEC, (B) eNOS knockdown of control PAEC, (C) PPHN-PAEC and (D) detaNONOate treated PPHN-PAEC. MitoSOX fluorescence intensity was increased in eNOS knockdown control PAEC and in PPHN PAEC when compared to controls. DetaNONOate significantly decreased mitoSOX fluorescence in PPHN PAEC when compared to untreated PPHN-PAEC (N=4, p<0.05). # indicates p<0.05 from control PAEC, * from eNOS knockdown and from untreated PPHN PAEC.
Figure 6: Endothelial nitric oxide synthase (eNOS) and NO regulate mitochondrial biogenesis and Redox balance in the PAEC: (A) siRNA mediated eNOS knockdown decreased SOD2 protein level (A) and electron transport chain (ETC) complexes I-V (B) in control PAEC when compared to non-silencing (NS) siRNA (N=12, p<0.05). * indicate p<0.05 from non-silencing siRNA (controls).

Figure 7: Inhaled nitric oxide (iNO) increased endothelial nitric oxide synthase (eNOS) expression in PPHN fetal lambs. eNOS mRNA (A) and protein levels (B) were decreased in PPHN lungs. Ventilation of PPHN fetal lambs with 100% oxygen increased eNOS mRNA, but not protein levels. Inhaled NO given with the weaning of FiO₂ increased both eNOS mRNA and protein levels (A&B) (N=4, p<0.05). HSP90-eNOS association (C) and serine¹¹⁷⁹ phosphorylation of eNOS (D) were decreased in the lungs of PPHN lambs when compared to gestation-matched control lambs and did not change further during ventilation with 100% O₂. Addition of iNO was associated with increases in HSP90/eNOS association (C) and levels of Phos-serine¹¹⁷⁷ eNOS in PPHN lambs (D). (N=4, p<0.05). # indicates p<0.05 from control, Ω from unventilated PPHN lungs and * from ventilation with 100% oxygen.

Table 1: Changes in the arterial blood gases and ventilator mean airway pressure and (MAP in cmH₂O), and fractions of inspired oxygen concentrations (FiO₂) in PPHN fetal lambs ventilated with oxygen alone or oxygen plus iNO.
Figure 1
Figure 2

Panel A: Western blot analysis of AMPKα, p-AMPKα, Sirt-1, and PGC-1α expression in different experimental groups. The expression levels were normalized to β-actin.

Panel B: Bar graph showing the fold change in PGC-1α mRNA expression in control, PPHN, PPHN + Vent +100%O2, and PPHN + Vent + O2 +iNO groups. The bars indicate mean ± SD, and statistical significance was determined using ANOVA followed by Tukey's post-hoc test.

Legend:
- Control
- PPHN
- PPHN + Vent +100%O2
- PPHN + Vent + O2 +iNO

Statistical symbols:
- * p < 0.05 compared to control
- # p < 0.05 compared to PPHN
- Ω p < 0.05 compared to PPHN + Vent +100%O2

Figure 2
Figure 3

A) NO concentrations in nM/L over time (h)

B) Oxygen consumption/mg of protein

- Control
- PPHN
- PPHN + DetaNONOate

Legend:
- OCR
- ATP-linked O2 consump
- Proton leak
- Extra-mitochondrial O2 consumption

Statistical significance:
- * p < 0.05
- # p < 0.01
Figure 4

A. Western blot showing SOD2 protein expression normalized to β-actin under different conditions: Control, PPHN, PPHN + iNO, and PPHN + MV + O2. The blot shows a significant increase in SOD2 expression in the PPHN + iNO group compared to other groups. 

B. SOD2 activity measured in μg of protein. The graph shows a significant increase in SOD2 activity in the PPHN + iNO group compared to Control and PPHN + MV + O2 groups. 

C. Fold change in SOD2 mRNA expression. The graph shows a significant increase in SOD2 mRNA expression in the PPHN + iNO group compared to Control and PPHN + MV + O2 groups.
Figure 5
Figure 6

(A) Western blot analysis showing the expression of SOD2 and eNOS in NS-siRNA and eNOS siRNA groups. The bars represent the SOD2-eNOS/actin ratio.

(B) Western blot analysis showing the expression of ETC complexes I-V in NS-siRNA and eNOS siRNA groups. The bars represent the ETC complexes I-V/actin ratio.* Indicates significant difference.
**Figure 7**

A. Fold eNOS mRNA expression

B. eNOS/β-actin ratio

C. HSP90/eNOS ratio

D. P-eNOS/eNOS ratio