Oxidant stress from uncoupled nitric oxide synthase impairs vasodilation in fetal lambs with persistent pulmonary hypertension

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Konduri GG, Bakhtashvili I, Eis A, Pritchard K Jr. Oxidant stress from uncoupled nitric oxide synthase impairs vasodilation in fetal lambs with persistent pulmonary hypertension. Am J Physiol Heart Circ Physiol 292: H1812–H1820, 2007. First published December 1, 2006; doi:10.1152/ajpheart.00425.2006.—Persistent pulmonary hypertension of newborn (PPHN) is associated with decreased NO release and impaired pulmonary vasodilation. We hypothesized that increased superoxide (O2•−) release by an uncoupled endothelial nitric oxide synthase (eNOS) contributes to impaired pulmonary vasodilation in PPHN. We investigated the response of isolated pulmonary arteries to the NOS agonist ATP and the NO donor 5-nitroso-N-acetylpenicillamine (SNAP) in fetal lambs with PPHN induced by prenatal ligation of ductus arteriosus and in sham-ligated controls in the presence or absence of the NOS antagonist nitro-L-arginine methyl ester (L-NAME) or the O2•− scavenger 4,5-dihydroxy-1,3-benzenedisulfonate (Tiron). ATP caused dose-dependent relaxation of pulmonary artery rings in control lambs but induced constriction of the rings in PPHN lambs. L-NAME, the NO precursor L-arginine, and Tiron restored the relaxation response of pulmonary artery rings to ATP in PPHN. Relaxation to NO was attenuated in arteries from PPHN lambs, and the response was improved by L-NAME and by Tiron. We also investigated the hypothesis that increased generation of O2•− from uncoupled eNOS and the resulting oxidative stress in the pulmonary arteries contribute to the impaired vasodilator responses to the physiological NOS agonist ATP and to NO, 2) the effect of ATP on NO and O2•− release from pulmonary artery endothelial cells (PAEC) and pulmonary artery segments with intact endothelium in control and PPHN lambs, 3) the effect of ATP on HSP90-eNOS interactions in control PAEC and their alteration in endothelial cells from PPHN lambs, and 4) evidence of tyrosine nitration of HSP90, a marker for oxidative stress, in pulmonary arteries in PPHN.

METHODS

Ewes were obtained at 120 ± 2 days of gestation and were allowed to acclimatize to the facility for a week before the surgical procedure. The detailed methodology for creation of the ductal ligation model was reported previously (16). Briefly, the fetal lambs underwent a left lateral thoracotomy under general anesthesia with 1–2% isoflurane administered to the ewe. The ductus arteriosus was identified and ligated with umbilical tape for creation of PPHN or left undisturbed for sham ligation, used as control. Eight fetal lambs each had either ligation of the ductus arteriosus or the sham procedure done. We previously reported (16) a sustained increase in pulmonary artery pressure with no change in systemic pressure and no significant change in acid-base status in this model. Fetal lungs were harvested after 8 days for isolation of pulmonary arteries and PAEC. Studies were done in isolated cultured PAEC to investigate the interactions of eNOS with HSP90 and release of NO and O2•− from eNOS. Parallel studies in the ductal ligation model of PPHN demonstrated an increase in superoxide (O2•−) release from NADPH oxidase in pulmonary arteries (5). Our previous studies in this model (16) demonstrated that PPHN is associated with decreased heat shock protein (HSP90-nitric oxide synthase (NOS) interactions and impaired NO synthesis in fetal lambs. Studies in bovine aortic endothelial cells demonstrated that inhibition of HSP90-endothelial NO (eNOS) interactions leads to uncoupling of eNOS and increased O2•− release from NOS (22, 23). However, the mechanism of decreased HSP90-eNOS interactions and a potential role for NOS as a source for O2•− generation in PPHN remains unknown. We investigated the hypothesis that increased generation of O2•− from uncoupled eNOS and the resulting oxidative stress in the pulmonary arteries contribute to the impaired pulmonary vasodilation in this model. We also proposed that tyrosine nitration of HSP90 as a result of oxidative stress leads to decreased HSP90-eNOS interactions and impaired eNOS function in PPHN. The objectives of our studies were to investigate 1) the role of NOS-derived O2•− in the impaired vasodilator responses to the physiological NOS agonist ATP and to NO, 2) the effect of ATP on NO and O2•− release from pulmonary artery endothelial cells (PAEC) and pulmonary artery segments with intact endothelium in control and PPHN lambs, 3) the effect of ATP on HSP90-eNOS interactions in control PAEC and their alteration in endothelial cells from PPHN lambs, and 4) evidence of tyrosine nitration of HSP90, a marker for oxidative stress, in pulmonary arteries in PPHN.

A RAPID AND SUSTAINED DECREASE in pulmonary vascular resistance (PVR) at birth facilitates initiation of gas exchange in the newborn infant (6, 7). Release of NO from pulmonary vascular endothelium plays a major role in this transition (1, 26, 29). Our previous studies (13–15) demonstrated that the purine nucleotide ATP is released in the pulmonary circulation in response to oxygen exposure at birth and activates NO release from endothelium. Failure of PVR to decrease in postnatal life results in persistent pulmonary hypertension of newborn (PPHN), a condition associated with decreased NO release and impaired pulmonary vasodilation (4, 27, 32, 33). Previous
studies were done in isolated pulmonary arteries to determine the functional effects of eNOS-derived $\text{O}_2^-$ in PPHN. The protocol for use of animals in this study was approved by the Institutional Animal Care and Use Committees of the Medical College of Wisconsin and Zablocki Department of Veterans Affairs Medical Center.

Isoflation and study of pulmonary arteries from lungs. Details of methods for isolation and study of pulmonary arteries from fetal lambs in tissue bath were reported previously (16). Third- to fifth-generation intrapulmonary arteries with an internal diameter of 300–500 μm were dissected and isolated from the lung. The arteries were cut into rings 1-mm in length, suspended with stainless steel hooks in water-jacketed chambers, and connected to force displacement transducers (FT03, Grass Instruments). The artery rings were bathed in 2 mL of physiological salt solution (PSS) kept at 37°C and aerated to maintain normal acid-base status and oxygenation of tissue. They were allowed to equilibrate for 45 min, stretched to a passive tension of 0.8 g, and preconstricted with $10^{-6}$–$10^{-7}$ M norepinephrine. The tension reached with norepinephrine constriction for each ring was normalized to 100%, and the percent change from this tension with each dose of ATP or $\text{N}$-nitro- $\text{N}$-acetylpenicillamine [SNAP (NO donor); Sigma, St. Louis, MO] was expressed as mean (SD). Relaxation responses to $10^{-8}$–$10^{-10}$ M doses of ATP and SNAP were determined, and the responses were compared with rings treated with PSS alone. Separate rings were pretreated with $10^{-4}$ M nitro-l-arginine methyl ester (L-NAME), a NOS inhibitor, l-arginine, NOS substrate, sepiapterin, a tetrahydrobiopterin (BH$_4$) analog, and 4.5-dihydroxy-1,3-benzenedisulfonate (Tiron), a $\text{O}_2^-$ scavenger (all from Sigma). Relaxation responses to ATP and NO were then studied after 15 min of incubation with these agents.

Isolation of PAEC. PAEC were isolated and characterized with techniques described previously (16). Briefly, the right and left pulmonary arteries were dissected up to the third-generation branches in the lung. The branches were tied and cut distally, and endothelial cells were isolated with 0.1% collagenase. PAEC were grown in six-well plates for measurement of NO, 100-mm flask for immunoprecipitation studies, and eight-well chamber glass slides (Lab-Tek II, Nalgene, Naperville, IL) for assessment of $\text{O}_2^-$ release with dihydroethidium (DHE) fluorescence. We previously reported (16) that PAEC from PPHN lambs continue to show a decrease in HSP90-eNOS interactions and impaired NO release with NOS agonists compared with control cells up to the fourth passage. We therefore studied PAEC from control and PPHN lambs up to passage 4 to investigate altered eNOS biology in PPHN.

Measurement of NO release. PAEC in six-well plates were placed in serum-free medium overnight and washed with Hank's balanced salt solution (HBSS). NOS activity was assessed by accumulation of cGMP in the cells, detected with an enzyme immunoassay (EIA) method as described previously (34). Cells in selected wells were treated with the NOS inhibitor L-NAME ($10^{-4}$ M) for 30 min. PAEC were then exposed to HBSS with or without $10^{-6}$–$10^{-5}$ M ATP for 15 min. The reaction was stopped by addition of cell lysis buffer, and cGMP content of the lysate was measured with an ELISA method (Amersham). The protein concentration in each well was estimated to calculate the amount of cGMP per milligram of protein. The cGMP in control, unstimulated cells was normalized to 100%, and the percent change from the baseline was compared between different treatments.

Measurement of $\text{O}_2^-$ release from PAEC. PAEC from control and PPHN lambs were grown to confluence in eight-well chamber glass slides. PAEC in selected wells were pretreated with $10^{-4}$ M L-NAME (NOS antagonist) or superoxide dismutase (SOD, 1,000 U/ml; Sigma) for 30 min. The PAEC were then treated with HBSS alone or with $10^{-5}$ M ATP and DHE ($10^{-5}$ M) to detect increases in intracellular $\text{O}_2^-$ generation (23). After 15-min incubation with ATP or HBSS, supernatant was aspirated and cells were rinsed with ice-cold HBSS. Slides were coverslipped, visualized, and imaged with a fluorescent microscope (NIKON Eclipse 600) equipped with a SPOT RT Slide camera and software (Diagnostic Instruments). DHE fluorescence from PAEC and pulmonary artery segments was quantified with MetaVue software (Universal Imaging, Downingtown, PA).

DHE fluorescence in pulmonary artery segments with intact endothelium. The second-generation branches of pulmonary arteries were dissected fresh from the lungs of control and PPHN lambs. A 1-mm length of the artery was cut open to expose endothelium and placed in six-well plates with 1 mL of HBSS. The pulmonary artery segments were incubated in HBSS alone or with $10^{-4}$ M L-NAME or 1,000 U/ml SOD for 30 min. DHE ($10^{-5}$ M) and HBSS with or without $10^{-5}$ M ATP were then added (23). After 15 min, pulmonary artery segments were washed, placed in microwells of chamber glass slides, and coverslipped. DHE fluorescence of endothelial tissue was visualized and quantified as described above.

Immunoprecipitation studies. Confluent PAEC in 100-mm flasks were treated with either HBSS or $10^{-5}$ M ATP in HBSS for 10 min. The supernatant was then aspirated, and cells were lysed in modified radioimmunoassay (RIA) buffer (8). eNOS was immunoprecipitated from cell lysate with an eNOS antibody (H32, Transduction Laboratories). The immunoprecipitate was immunoblotted for eNOS and HSP90 (16) with appropriate antibodies [9D10 (Zymed) and H38220 (Transduction Laboratories), respectively]. Bands were visualized with the appropriate anti-immunoglobulin horseradish peroxidase conjugate (Sigma), ECL reagents (Amersham Pharmacia), and Kodak X-OMAT film. An aliquot of protein (1 mg) from control and PPHN PAEC was used to perform Western blots for eNOS and soluble guanylate cyclase (sGC) to assess for potential changes in the expression of these proteins in PPHN. Autoradiograms were imaged with Adobe Photoshop version 5.5 software, and relative band densities were quantified with NIH Image 1.62.

Third- to fifth-generation pulmonary arteries were dissected clear of surrounding parenchyma, flash frozen in liquid nitrogen, pulverized, and placed in modified RIPA buffer (16). The mixture was homogenized and sonicated to break the cells, and cell debris was removed by centrifugation. An aliquot (1 mg) of the protein was removed for immunoprecipitation with HSP90 antibody (Transduction Laboratories). The immunoprecipitate was immunoblotted for HSP90, and the HSP90 blot was probed with antibody for nitrotyrosine (Upstate) as described above. In some studies, nitrotyrosine was immunoprecipitated from protein lysate with appropriate antibody (Upstate) and the immunoprecipitate was immunoblotted for HSP90 as described above. Expression of HSP90 was assayed by Western blotting for this protein in samples from control and PPHN pulmonary arteries, and the ratio of nitrated HSP90 to HSP90 was calculated for the samples from control and PPHN lambs.

Statistical analysis. Data are shown as means (SD). Changes in vascular ring tension with incremental doses of ATP and with different blockers were analyzed by two-way ANOVA (37). Comparison of cGMP and DHE fluorescence values with different treatments was also done by two-way ANOVA. When a significant difference ($P < 0.05$) was found, a Duncan’s multiple-range test was done to determine which means were different. Comparison of densitometric data for eNOS, HSP90, and nitrated HSP90 from control and pulmonary hypertension groups was done by unpaired $t$-tests.

RESULTS

Response of pulmonary artery rings to ATP. Pulmonary artery rings kept in PSS alone maintained their tension after preconstriction with norepinephrine for the duration of the study (data not shown). ATP caused a dose-dependent relaxation of the control pulmonary artery rings (Fig. 1A). The response to ATP was attenuated by the NOS antagonist L-NAME and not significantly altered by the NO precursor L-arginine (Fig. 1A). The BH$_4$ analog sepiapterin had no effect on the relaxation response to ATP, whereas the SOD mimicic...
Tiron significantly enhanced the response to ATP (Fig. 1B). ATP caused active constriction of pulmonary artery rings from the ducal ligation lambs (Fig. 1C). Pretreatment with l-arginine and L-NAME changed the effects of ATP to relaxation of rings in hypertensive pulmonary arteries (Fig. 1C). Sepiapterin prevented the constrictor response to ATP, and Tiron enhanced the relaxation response to ATP (Fig. 1D). These data indicate that NOS-dependent oxidative stress contributes to impaired relaxation response to ATP in PPHN induced by ductal ligation in fetal lambs.

**Response of pulmonary artery rings to NO.** The control pulmonary artery rings relaxed well to SNAP, an NO donor, and the response was not altered by L-NAME or Tiron (Fig. 2A). In contrast, the relaxation response to SNAP was attenuated in pulmonary artery rings from PPHN lambs. The response to SNAP was significantly improved by the NOS antagonist L-NAME and the superoxide (O$_2^•$) scavenger 4,5-dihydroxy-1,3-benzenedisulfonate (Tiron; Tir, B). L-Arginine and the tetrahydrobiopterin analog sepiapterin (Sep) had no effect on the response to ATP (B). ATP caused a dose-dependent constriction of PA rings in PPHN lambs (C). In contrast, ATP caused relaxation of PA rings in the presence of L-NAME and l-arginine (C). The relaxation response was markedly enhanced in the presence of the O$_2^•$ scavenger Tiron (D). Sepiapterin attenuated the constrictor response but did not enhance the relaxation response to ATP in PPHN.
impairs the response of the guanylate cyclase-cGMP system in vascular smooth muscle to NO.

Effect of ATP on NO release. ATP caused a dose-dependent increase in cGMP levels in control but not in PPHN endothelial cells (Fig. 3, A and B). The increase in cGMP levels in control cells was inhibited by L-NAME, suggesting that an increase in NO release occurs in control but not in PPHN cells with ATP (Fig. 3, A and B). The sGC expression in the endothelial cells in control and PPHN lambs was comparable (Fig. 3, C and D), suggesting that the lower cGMP levels in PPHN cells are due to decreased NO release.

Effect of ATP on \( \text{O}_2^\cdot \) release. There was no increase in DHE fluorescence with ATP in control cells (Fig. 4), and no changes were noted with L-NAME or with SOD. In contrast, PAEC from ductal ligation lambs had increased DHE fluorescence at baseline and in response to stimulation with ATP (Fig. 4). The increase in DHE fluorescence observed in hypertensive PAEC with ATP was attenuated by the NOS antagonist L-NAME and by SOD (Fig. 4). Pulmonary artery segments harvested from control lambs had no changes in DHE fluorescence with ATP or with L-NAME and SOD (Fig. 5). Pulmonary arteries from PPHN lambs showed increased DHE fluorescence in the endothelium in response to ATP exposure (Fig. 5). The increase in DHE fluorescence with ATP was attenuated by L-NAME and by SOD, suggesting an increase in ROS-precipitated \( \text{O}_2^\cdot \) release in response to ATP in PPHN.

Immunoprecipitation studies. ATP stimulated an increase in HSP90-eNOS interactions in control PAEC (Fig. 6, A and B). In contrast, ATP did not alter the HSP90-eNOS interactions in hypertensive PAEC (Fig. 6, A and B) as we reported previously (16). The expression of eNOS in PAEC was similar between control and PPHN lambs (Fig. 6, C and D). The immunoprecipitate of HSP90 from control pulmonary arteries showed a faint nitrotyrosine signal, and this was significantly increased in hypertensive pulmonary arteries (Figs. 7A and 8A). The immunoprecipitate of nitrotyrosine from hypertensive pulmonary arteries showed increased levels of nitrated HSP90 compared with control pulmonary arteries (Figs. 7B and 8B), verifying that increased tyrosine nitration of HSP90 occurs in PPHN. The expression of HSP90 was similar in control and PPHN pulmonary artery segments, indicating that the increase in nitrated HSP90 signal in PPHN is not due to increased HSP90 expression in PPHN (Fig. 7, C and D).

DISCUSSION

Our study provides evidence that NOS-derived reactive oxygen species (ROS) contribute to the impaired relaxation response of pulmonary arteries in PPHN. PPHN is associated with uncoupled eNOS function and increased \( \text{O}_2^\cdot \) /decreased NO release in response to ATP, a physiological agonist. The increase in \( \text{O}_2^\cdot \) appears to impair the vasodilator response to both endothelium-dependent vasodilator ATP and -independent vasodilator NO. These data suggest that ROS contribute to vascular adaptation in both endothelium and vascular smooth muscle in PPHN.

PPHN affects 0.2% of full-term infants during their transition to postnatal life (35). Prenatal ductal constriction from in utero exposure to nonsteroidal anti-inflammatory drugs is associated with PPHN (3). Prenatal ductal constriction in fetal
Lambs reproduces the hemodynamic and structural features of PPHN (2, 18). Although decreases in eNOS expression and NO release occur in this model, they do not fully account for the impaired pulmonary vasodilation in PPHN. Lambs with PPHN show impaired pulmonary vasodilator response to inhaled NO (28) and enhancement of this response with exogenous SOD (28). These data suggest that oxidant stress impairs pulmonary vasodilation in PPHN. The source of ROS in PPHN was not clear from previous studies. NADPH oxidase activity and expression are increased in pulmonary arteries in lambs with PPHN (5). However, a potential role for eNOS also as a source of O$_2^-$ was suggested by our previous observation of decreased HSP90-eNOS interactions in the pulmonary arteries in this model (16). Altered HSP90-eNOS interactions are associated with uncoupling of eNOS activity and increased O$_2^-$ release (22, 23) in cultured endothelial cells. Our present studies provide in vivo evidence that eNOS uncoupling from decreased HSP90-eNOS interaction impairs pulmonary vascular responses to vasodilator stimuli. We observed that both cultured PAEC and endothelium in intact pulmonary artery seg-

Fig. 4. Effect of ATP on dihydroethidium (DHE) fluorescence observed in control and PPHN endothelial cells. A: representative micrographs of DHE fluorescence from control (A–D) and PPHN (E–H) lambs: untreated (A and E), ATP alone (B and F), l-NAME+ATP (C and G), and superoxide dismutase (SOD)+ATP (D and H). Fluorescence in untreated PPHN cells was higher than in control cells and increased with ATP exposure. The increased fluorescence with ATP was attenuated by l-NAME and SOD. B: summarized data as means (SD) for 8 experiments each for control and PPHN cells. $P < 0.05$: *from untreated control, † from untreated PPHN cells, ‡ from ATP alone in PPHN cells. ATP had no effect on DHE fluorescence in the control PAEC. PAEC from PPHN lambs had higher DHE fluorescence at baseline and increased fluorescence with ATP that was attenuated by l-NAME and SOD. These results suggest stimulation of NOS-derived O$_2^-$ by ATP in PAEC from PPHN lambs.
ments demonstrate increased NOS-derived $O_2^-$ in PPHN. The mechanism of eNOS uncoupling in PPHN remains unclear. Previous studies demonstrated that ductal constriction leads to increased pulmonary artery pressure without a change in flow in fetal lambs (2, 16). Murata et al. (19) reported that a decrease in HSP90-eNOS interactions occurs in hypoxia-induced pulmonary hypertension in rats. Increased pressure load on endothelial cells in pulmonary hypertension may therefore result in decreased HSP90-eNOS interactions occurring in hypoxia-induced pulmonary hypertension in rats. The mechanism of decreased HSP90-eNOS interaction with the pressure load on endothelium remains unclear. HSP90 associates with cytoskeleton proteins actin and tubulin when the cells are stressed and experience a decrease in intracellular ATP levels (10). We observed that the PPHN cells stimulated with ATP showed a trend for decreased HSP90-eNOS interactions, associated with a significant increase in oxidative stress. The potential targeting of HSP90 to cytoskeleton to stabilize the endothelial cell in the presence of oxidative stress in pulmonary hypertension requires further investigation.

Previous studies have suggested that the association of HSP90 with eNOS is facilitated by tyrosine phosphorylation of HSP90 (21, 31). We observed that ATP promotes association of HSP90 with eNOS in control PAEC. In contrast, hypertensive PAEC did not show this response to ATP. We observed increased tyrosine nitration of HSP90 in the pulmonary arteries.
Fig. 6. A and B: representative immunoblots (IB, A) and summarized data (B) for the effect of $10^{-5}$ M ATP on the heat shock protein (HSP)90-eNOS interactions in endothelial cells from control and PPHN lambs. Data are means (SD) for 9 experiments each for control and PPHN cells. *$P < 0.05$ from untreated cells. ATP stimulated the association of HSP90 with eNOS and increased HSP90-to-eNOS ratio significantly in control but not in PPHN cells. IP, immunoprecipitation.

Fig. 7. A and B: representative Western blot (C) and summarized data (D) for eNOS protein levels in control and PPHN cells from 6 lambs each shown as means (SD). eNOS expression was similar in PAEC from control and PPHN lambs.
and its reaction with NO from eNOS to form ONOO⁻ can potentially lead to tyrosine nitration of HSP90 and decrease its interaction with eNOS in PPHN. The potential role of ONOO⁻ in causing tyrosine nitration of HSP90 in the endothelial cell and the specific tyrosine residues nitrated by ONOO⁻ require further investigation.

We observed that scavenging O₂⁻ is more effective than NOS inhibition in decreasing DHE fluorescence in PAEC and improving the relaxation response to ATP in PPHN. This may be due to increased bioavailability of endogenous NO or contribution of O₂⁻ from sources other than eNOS in PPHN (5). The enhancement of pulmonary vasodilator response to ATP by t-NAME may facilitate the initiation of vasodilator response to ATP by these NO-independent mechanisms. Similarly, t-NAME also enhanced the response to the NO donor SNAP, suggesting that NOS-derived O₂⁻ may impair sensitivity of sGC on smooth muscle to NO (17). The mechanism of decreased response of the guanylyl cyclase-cGMP system in PPHN remains unclear. ONOO⁻ has been shown to decrease the sensitivity of guanylyl cyclase to NO in vitro (17). Expression of sGC is decreased in the pulmonary arteries of PPHN lambs (4, 30). Hydrogen peroxide, a product of oxidant stress, has been shown to decrease the guanylyl cyclase expression and NO-generated cGMP levels in cultured fetal pulmonary artery smooth muscle cells (36). However, it is unclear whether endothelium-derived O₂⁻ or ONOO⁻ is transported to the vascular smooth muscle. The SOD mimetic Tiron enhanced the relaxation response to ATP in the control pulmonary arteries, suggesting that O₂⁻ modulates the response of pulmonary artery rings to ATP. Although we did not observe an increase in O₂⁻ release in control pulmonary artery segments or endothelial cells exposed to ATP, some O₂⁻ release may have occurred in the pulmonary arteries under the tissue bath conditions in vitro.

We observed that the eNOS protein levels in the endothelial cells are similar in control and PPHN lambs. Previous studies reported a decrease in eNOS protein levels in pulmonary arteries in PPHN. However, these studies were done in protein lysate from homogenized isolated pulmonary arteries and lung tissue (16, 27, 32) in PPHN. The apparent increase in eNOS expression in the immunoblots shown for PAEC from PPHN lambs is probably related to differences in the proliferative state of the cells. We attempted to minimize the variation in immunoprecipitation technique by performing the assays on control and PPHN cells on the same day. Previous studies reported higher eNOS expression in proliferating endothelial cells compared with confluent cells (22).

Studies in mice with hyperphenylalanine phenotype (hph-I mice) have reported a role for BH₄ depletion in eNOS uncoupling and pulmonary hypertension (11, 20). We did not measure BH₄ levels in the lungs or PAEC in our model. However, the BH₄ analog sepiapterin had minimal effect on the relaxation response to ATP in our studies. We previously reported (16) that alteration of HSP90-NOS interactions by geldanamycin impairs the vasodilator response to ATP in fetal pulmonary arteries. These data suggest that in fetal pulmonary hypertension HSP90-NOS interactions play an important role in the modulation of pulmonary vascular tone. Whether oxidized BH₄ plays a role in the altered HSP90-eNOS interactions or eNOS uncoupling in the ductal ligation model of PPHN remains unknown.

In conclusion, our studies provide evidence that eNOS is uncoupled and serves as a source of oxidative stress in pulmonary hypertension induced by ductal ligation in fetal lambs. NOS-derived oxidant stress contributes to impaired pulmonary vasodilation during transition of the fetus to postnatal life in PPHN. The mechanisms of eNOS uncoupling in PPHN require further investigation.

Fig. 8. Summary data for the relative IOD ratios of nitrotyrosine to HSP90 (A) and nitrated HSP90 to HSP90 (B) for PA segments from 4 control and 4 PPHN lambs. Data from control PA are normalized to 1. *P < 0.05 from control. The ratios of nitrotyrosine to HSP90 and nitrated HSP90 to HSP90 are significantly higher in PA segments from PPHN lambs.
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UNCOUPLING OF eNOS IN PPHN

GRANTS
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