Decreased association of HSP90 impairs endothelial nitric oxide synthase in fetal lambs with persistent pulmonary hypertension

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Konduri, Girija G., Jingsong Ou, Yang Shi, and Kirkwood A. Pritchard, Jr. Decreased association of HSP90 impairs endothelial nitric oxide synthase in fetal lambs with persistent pulmonary hypertension. Am J Physiol Heart Circ Physiol 285: H204–H211, 2003; 10.1152/ajpheart.00837.2002.—Persistent pulmonary hypertension of newborn (PPHN) is associated with decreased nitric oxide (NO) release and impaired pulmonary vasodilation. We investigated the hypothesis that decreased association of heat shock protein 90 (HSP90) with endothelial NO synthase (eNOS) impairs NO release and vasodilation in PPHN. The responses to the NO agonist ATP were investigated in fetal lambs with PPHN induced by prenatal ligation of ductus arteriosus, and in sham ligation controls. ATP caused dose-dependent vasodilation in control pulmonary resistance arteries, and this response was attenuated in PPHN vessels. The response of control pulmonary arteries to ATP was attenuated by L-nitro-arginine methyl ester (L-NAME), a NO antagonist, and geldanamycin, an inhibitor of HSP90-eNOS interaction. The attenuated response to ATP observed in PPHN was improved by pretreatment of vessels with L-NAME or 4,5-dihydroxy-1,3-benzene-disulfonate, a superoxide scavenger. Pulmonary arteries from PPHN lambs had decreased basal levels of HSP90 in association with eNOS. Association of HSP90 with eNOS and NO release increased in response to ATP in control pulmonary artery endothelial cells, but not in cells from PPHN lambs. Decreased HSP90-eNOS interactions may contribute to the impaired NO release and vasodilation observed in the ductal ligation model of PPHN.

Our laboratory’s previous studies (15–18) demonstrated that the purine nucleotide ATP contributes to birth-related pulmonary vasodilation in fetal lambs. ATP causes pulmonary vasodilation in fetal lambs in part by stimulation of NO release (17). ATP stimulates NO release from cultured vascular endothelial cells (8, 28). However, the mechanism of NO activation by ATP and its alteration in PPHN remain unknown.

Heat shock protein 90 (HSP90), a molecular chaperone, modulates the endothelial NO synthase (eNOS) activity (10, 12, 32) and balance of NO and superoxide release from eNOS (30) in response to several physiological stimuli. However, the role of this constitutively expressed chaperone in mediating vasodilation in the fetal pulmonary arteries and its role in mediating the effects of ATP on eNOS is unknown. We proposed the hypothesis that 1) association of HSP90 with eNOS facilitates the release of NO and vasodilation when fetal pulmonary arteries are stimulated by ATP and 2) dissociation of HSP90 from eNOS in PPHN impairs the ability of NO to release NO in response to stimulation by ATP. We investigated the hypothesis in fetal lambs with pulmonary hypertension induced by prenatal ligation of ductus arteriosus, an established model of PPHN. The objectives of our study were to investigate the following: 1) the role of HSP90-eNOS association in the vasodilator response to ATP in isolated intrapulmonary resistance arteries, 2) the HSP90-eNOS association in isolated pulmonary arteries, and 3) the effects of ATP on the HSP90-eNOS interaction and NO release from pulmonary artery endothelial cells.

METHODS

Creation of PPHN model. This study was approved by the Animal Studies Subcommittee of the Research and Development Committee of the Zablocki Veterans Affairs Medical Center (Milwaukee, WI). Ewes were obtained at 120 ± 2 days of gestation and were allowed to acclimate to the facility for 1 wk before the surgical procedure. The ewes were fasted for 24 h and were given general anesthesia with 1–2% isoflurane and oxygen for the procedure. The fetus was approached via a midline laparotomy incision and a hysterotomy incision on the uterus close to the fetal head. The fetal left thorax was exteriorized, and the fetal thorax was exteriorized...
and a left lateral thoracotomy was done to identify and ligate the ductus arteriosus with the use of umbilical tape. Control fetal lambs had thoracotomy performed without ligation of the ductus arteriosus. The fetus was then returned to the uterus, and pregnancy was allowed to continue for 8 days. Previous studies (26, 43) have demonstrated that fetal lambs develop hemodynamic and structural alterations of PPHN with this procedure. Blood samples were obtained from fetal arterial catheter for measurement of blood pH and blood gas tensions with the use of a blood gas analyzer (model ABL30, Radiometer). These samples were obtained during surgery just before and 10 min after occlusion of ductus arteriosus and once on each postoperative day. Control fetal lambs had blood gas tension measurements at the same time points. In addition, the fetal mean systemic and pulmonary arterial pressures were measured at the same time points with the appropriate pressure transducers (model PT 100, Grass Instruments) and physiograph (model 7D, Grass), as described previously (19). After 8 days of ductal constriction, the ewe was euthanized and laparotomy and hysterotomy incisions were quickly opened. The fetal chest was opened and the heart and lungs were removed en bloc.

**Isolation of pulmonary arteries and investigation of the response to ATP.** Pulmonary arteries were dissected into the lung parenchyma, with care taken not to damage the endothelium. Fifth- through seventh-generation intrapulmonary arterioles with an internal diameter of 50–120 μm were connected to two glass pipettes tapering to an outside diameter of 70 μm and were tied in place. Arterioles were superfused with Krebs buffer (37°C, equilibrated with 95% O2-5% CO2) and luminally pressurized to 25 mmHg. The pressure chosen reflects the transmural pressure for the resistance vessels in fetal lamb lungs (21, 31). Arterioles from control and PPHN lambs were studied at the same transmural pressure to normalize the conditions for these vessels. In addition, the increase in transmural pressure for resistance arterioles in PPHN is not expected to be >10 mmHg, on the basis of the 17-mmHg increase in pressure we observed in left pulmonary artery after ductal constriction and the expected 40% drop in pressure from conduit to resistance size arteries (31). Previous studies (6) have demonstrated an alteration in endothelium-dependent vasodilation when the transmural pressure was increased by twofold and not at small changes in transmural pressure. The internal diameter of the arterioles was monitored by using a stereomicroscope (Zeiss), charge-coupled device television system camera (9KP 130 AU, Hitachi), a monitor (CVM 1271, Sony), and a calibrated video measuring system (Colorado Video). After an equilibration period of 30 min, reactivity of the vessel was confirmed by constriction with 30 mM KCl. The KCl-containing buffer was then removed, and arterioles were preconstricted with 10⁻⁶ M norepinephrine before the dilator responses were tested. The presence of intact endothelium was confirmed by the evaluation of vasodilator response to acetylcholine (10⁻⁵ M). Treatment of the vessels with 10⁻⁵ M geldanamycin, 10⁻⁴ M N⁵-nitro-L-arginine (L-NAME), and 10⁻² M 4,5-dihydroxy-1,3-benzenedisulfonate (Tiron) was done with the super- fusion stopped, and the vessels were allowed to incubate with each drug for 20 min. The response to intraluminal application of 10⁻⁴–10⁻³ M ATP was determined with or without pretreatment of vessels with these agents. Our pilot studies demonstrated no vasodilator response to ATP at 10⁻⁸ M and plateau of the vasodilator response at 10⁻³–10⁻² M doses in control resistance pulmonary arteries. The doses we chose therefore are expected to reproduce the range of vasodilator responses of these arteries to ATP.

**Western analysis for eNOS protein.** Peripheral lung tissue and third- to fifth-generation pulmonary arteries were flash frozen in liquid nitrogen, pulverized, and placed in a cell lysis buffer (modified RIPA). The mixture was then homogenized, sonicated to break the cells, and the cell debris was removed by centrifugation. The sample was heated to 70°C for 10 min, and an aliquot of protein (50 μg) was removed and resolved by SDS-PAGE (10%). Separated proteins were transferred to nitrocellulose membranes and were blotted for eNOS with a specific antibody (9D10, Zymed) overnight at 4°C. Enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia) were used to visualize bands. The autoradiographs were imaged with Adobe Photoshop 5.5 software and relative band densities quantified by using NIH Image 1.62.

**Isolation and culture of endothelial cells.** Pulmonary arterioles were dissected into lung parenchyma up to the third-generation branches, which were ligated and cut distally. The main pulmonary artery was detached from the right ventricle and ductus arteriosus was ligated. The endothelial cells were separated from pulmonary arteries with the use of 0.25% collagenase type A (Roche) and cells were grown to confluence with endothelial growth media (endothelial SFM, Life Technologies). Cells for individual experiments were grown to confluence in 6-well plates for NOS activity assays or 100-mm flasks for immunoprecipitation studies at passage 4.

**Assay for nitrite.** Endothelial cells from control and ductal ligation lambs were grown to confluence in 6-well plates. NOS activity was assessed by accumulation of nitrite in the supernatant, detected by ozone-chemiluminescence method as described previously (30). Briefly, confluent endothelial cells were washed clean of media and were incubated in Dulbecco’s phosphate-buffered saline (PBS) for 30 min at 37°C in a tissue culture incubator. Cells in selected wells were treated with 10⁻⁴ M N⁵-nitro-L-arginine (L-NMMa), a NOS antagonist, during this period. We used l-NMMA and not L-NAME as a NOS antagonist for these assays because l-NAME contains a nitro group that is estimated as nitrite in ozone-chemiluminescence analyzer (30). The PBS in each well was aspirated and replaced with PBS containing 30 μM L-arginine with or without 10⁻⁶–10⁻⁵ M ATP. Cells were incubated for 15 min at 37°C, and the supernatant was aspirated for nitrite measurement by ozone chemiluminescence. The background nitrite levels in the solutions used for incubation of cells were also measured and subtracted from the values of test samples. Cell lysis buffer was added to each well and the protein concentration in each well was estimated by modified Bradford method to calculate the amount of nitrite per milligram of protein. The activity of NOS in PBS-treated wells was normalized to 100% and the percent change from this control was calculated for each treatment.

**Coprecipitation studies for eNOS and HSP90.** Confluent endothelial cells in 100-mm flasks were treated with either PBS or 10⁻⁵ M ATP in PBS for 10 min. The PBS was then aspirated and cells were lysed in modified RIPA buffer (12). The samples were sonicated, and cell debris was removed by centrifugation (14,000 g at 4°C for 10 min). A 500-μg aliquot of protein was used for immunoprecipitation (30) with H32 antibody for eNOS (BioMol). The immunoprecipitated proteins were separated by SDS-PAGE (7.5–15%) and transblotted to nitrocellulose membrane. The membranes were blocked in milk in PBS-1× Tween (0.1%) and then immunoblotted for eNOS, phosphorylated-eNOS, and HSP90 with the use of appropriate antibodies [9D10-Zymed (Ser1177) Cell Signaling.com and (H38220) Transduction Laboratories, respectively]. The bands were visualized with the appropriate anti-immunoglobulin horseradish peroxidase conjugate.
Table 1. Hemodynamic variables, arterial blood gas tensions, and blood pH in fetal lambs before and after ductal constriction

<table>
<thead>
<tr>
<th>Variable</th>
<th>Study Group</th>
<th>Preocclusion</th>
<th>Postocclusion</th>
<th>24 h After Occlusion</th>
<th>Day 3 of Occlusion</th>
<th>Day 7 of Occlusion</th>
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<tbody>
<tr>
<td>PA pressure, mmHg</td>
<td>PPHN</td>
<td>54 ± 7</td>
<td>71 ± 6*</td>
<td>72 ± 7*</td>
<td>70 ± 6*</td>
<td>72 ± 8*</td>
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<td>54 ± 6</td>
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<td>50 ± 5</td>
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<tr>
<td>Systemic pressure, mmHg</td>
<td>PPHN</td>
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<td>53 ± 8</td>
<td>52 ± 8</td>
<td>53 ± 7</td>
<td>52 ± 6</td>
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<tr>
<td></td>
<td>Control</td>
<td>51 ± 8</td>
<td>52 ± 7</td>
<td>52 ± 7</td>
<td>50 ± 6</td>
<td>48 ± 6</td>
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<td>pH</td>
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<td>7.44 ± 0.02*</td>
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<tr>
<td></td>
<td>Control</td>
<td>7.38 ± 0.03</td>
<td>7.37 ± 0.04</td>
<td>7.43 ± 0.04*</td>
<td>7.44 ± 0.04*</td>
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<tr>
<td>PaCO2, mmHg</td>
<td>PPHN</td>
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<td>20 ± 8</td>
<td>21 ± 6</td>
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Values are means ± SD. PA, pulmonary arterial; PPHN, persistent pulmonary hypertension of newborn. Pre- and postocclusion vascular pressures and blood gases were obtained during surgery, just before and 10 min after occlusion of the ductus arteriosus. In the control group, ductus arteriosus was exposed but not ligated, and vascular pressures and blood gases were obtained 10 min after exposure of ductus arteriosus. *P < 0.05 from preocclusion baseline.

RESULTS

Eight of the thirteen fetal lambs (61%) with constriction of ductus arteriosus and eight of the nine fetal lambs with sham ligation (control) survived for 8 days and were used for the studies. Four lambs in each group were used to harvest pulmonary arteries for studies in a tissue bath and for immunoprecipitation and Western blot analysis. Four lambs in each group were used for isolation of endothelial cells.

Constriction of ductus arteriosus in fetal lambs was accompanied by an increase of 17 ± 4 mmHg in pulmonary artery pressure and no significant change in systemic pressure. The increased gradient between systemic and pulmonary artery pressures in the fetus was maintained for the duration of ductal constriction (Table 1). The arterial pH, Pco2, and PaO2 did not change immediately after constriction of the ductus arteriosus (Table 1). Serial measurements of arterial blood gas values during the 8-day period of ductal constriction demonstrated a 20% decrease in PaO2 over time, accompanied by an increase in pH and no change in PaCO2 (Table 1). The control fetal lambs also had an increase in pH, 24 h after procedure, but no changes in PaO2 and PaCO2 after the procedure (Table 1).

ATP caused a dose-dependent vasodilation of intrapulmonary resistance arteries harvested from control lambs (Fig. 1). The maximum increase in luminal diameter was observed at 10⁻³ M dose of ATP. Both L-NAME and geldanamycin attenuated the relaxation response to ATP, with a decrease in maximal response to ATP. The attenuation of the vasodilator response to ATP by geldanamycin was similar to the inhibition caused by NOS antagonist L-NAME (Fig. 1). The O₂⁻ scavenger Tiron did not affect the vasodilator response to ATP in control studies, but improved the response to ATP by 10.2 ± 0.3 µm at day 7 of occlusion.
ATP in geldanamycin-treated vessels (Fig. 1). These data suggest that release of NO plays a significant role in mediating the vasodilator response to ATP in normal pulmonary arteries. Geldanamycin, an inhibitor of HSP90-eNOS interactions appears to cause O₂⁻ dependent impairment of vasodilator response to ATP in the intrapulmonary resistance arterioles.

The vasodilator response to ATP was attenuated in the intrapulmonary resistance arterioles isolated from fetal lambs with ductal ligation (Fig. 2). The vasodilator response to ATP was also attenuated in the PPHN arteries, as reported by Steinhorn et al. (36) (data not shown). Pretreatment of these vessels with the NOS antagonist L-NAME or the O₂⁻ scavenger Tiron improved the response to ATP (Fig. 2). These data suggest that a dysfunctional NOS and an increased release of O₂⁻ contribute to the impaired vasodilator response to ATP in pulmonary hypertension.

Pulmonary arteries isolated from lambs with ductal ligation had decreased HSP90 in association with eNOS compared with control pulmonary arteries (Fig. 3). Western blots for eNOS in peripheral lung tissue and in pulmonary arteries demonstrated a 50% decrease in eNOS protein levels in ductal ligation lambs (Fig. 4), consistent with decreased expression of eNOS previously reported in this model (5, 35, 41).

ATP increased the nitrite levels in the supernatant of control pulmonary artery endothelial cells (Fig. 5). Pretreatment of endothelial cells with the NOS antagonist, L-NMMA prevented the increase in nitrite levels in response to ATP, suggesting that increased NOS activity was the source of increased nitrates in the supernatant (Fig. 5). ATP failed to increase nitrite release from the hypertensive endothelial cells (Fig. 5).

ATP increased the Ser1177 phosphorylation of eNOS and the association of HSP90 with eNOS in control endothelial cells (Fig. 6). Exposure of the pulmonary artery endothelial cells from ductal ligation lambs to ATP resulted in an increase in phosphorylation of the enzyme, but a decrease in the association of HSP90 with eNOS (Fig. 7).

Fig. 2. Effect of persistent pulmonary hypertension (PPH) on the vasodilator response to ATP (A) and the effects of L-NAME and Tiron on the response of hypertensive vessels to ATP (B). Data are means ± SD for 6 vessels for each study. Pulmonary arteries from ductal ligation lambs showed attenuated response to ATP (A). The response of hypertensive arteries to ATP was improved by pretreatment with L-NAME and the superoxide scavenger Tiron. *#P < 0.05 from control ATP response and from the PPH group.

Fig. 3. Representative immunoblot (IB) showing heat shock protein 90 (HSP90)-endothelial nitric oxide synthase (eNOS) association in pulmonary artery segments from one control lamb and two newborn lambs with PPHN (top). Relative HSP90/eNOS ratios in pulmonary artery segments from 4 control and 4 PPHN lambs shown as means ± SD with control as 1 (bottom). *P < 0.05, significant difference by unpaired t-test from control. The HSP90/eNOS ratio was significantly lower in pulmonary arteries from PPHN lambs.

Fig. 4. Representative Western blot for eNOS protein in pulmonary artery segments and peripheral lung tissue from one control and one PPHN lamb (top) and relative density for eNOS protein from four control and four PPHN lambs shown with controls as 1 (bottom). eNOS protein levels decreased in both pulmonary arteries and peripheral lung tissue in PPHN lambs. IOD, integrated optical density. *P < 0.05, significant difference from control by unpaired t-test.
DISCUSSION

Our study demonstrated that association of HSP90 with eNOS facilitates the release of NO and vasodilation in response to ATP in fetal pulmonary arteries. The impaired NO release and vasodilation in response to ATP in pulmonary hypertension was associated with decreased interaction of HSP90 with eNOS. A decrease in this association may result in both a decreased release of NO and an increased release of \( \text{O}_2^\bullet \) (30), contributing to impaired vasodilator response in this model of pulmonary hypertension. To the best of our knowledge, this is the first report of an alteration in HSP90-NOS signaling in PPHN.

PPHN affects ~0.2% of full-term newborn infants during their transition to postnatal life (42). The affected infants have persistent elevation of pulmonary vascular resistance, resulting in a failure to establish oxygenation during early postnatal life (3). Although the pathophysiology of PPHN remains unclear, the constriction of ductus arteriosus in the fetus is known to be associated with this disease. Thus PPHN has been reported (22, 29) in infants born to mothers that had exposure to indomethacin and aspirin, cyclooxygenase inhibitors that cause constriction of fetal ductus arteriosus. Administration of nonsteroidal anti-inflammatory drugs to pregnant animals causes PPHN in the offspring (13). We recently reported (4) a high incidence of exposure to nonsteroidal anti-inflammatory drugs in infants with PPHN. Although prenatal ductal constriction may not occur in all infants with PPHN, it has been used in animal models because it reproduces the hemodynamic and structural alterations of PPHN (3, 26, 43). After an 8-day period of ductal constriction, a decrease or loss of postnatal pulmonary vasodilation is seen in fetal lambs (3, 26). Lambs delivered at term after a 10-day period of prenatal ductal ligation have persistence of high PVR, hypoxemia, and structural changes in pulmonary arteries that mimic those seen in infants with severe PPHN (26, 43). A significant impairment of vasodilator response to several physiological and pharmacological stimuli occurs after ductal ligation in fetal lambs (36, 38). The altered reactivity includes a rapid and sustained loss of vasodilator response to oxygen, a major stimulus for birth-related pulmonary vasodilation (24, 38). However, a potential alteration in the response of pulmonary arteries to ATP in the ductal ligation model of PPHN was previously unknown.

Constriction of ductus arteriosus leads to an initial increase in the pulmonary flow and a more sustained increase in pressure and resistance in the fetal pulmo-
nary circulation (1, 24). We found that the increase in pulmonary artery pressure is maintained for the duration of ductal constriction in our model. In addition, we observed a 20% decrease in PaO₂ in the fetal lambs with ductal constriction. We speculate that the decrease in PaO₂ was due to increased right to left shunting of blood across foramen ovale in response to the increase in pulmonary artery pressure. This admixture of blood and hypoxemia were previously reported (3, 26) in lambs with prenatal ligation of ductus arteriosus. The elevated pressure and hypoxemia are associated with impaired release of NO (24, 27, 35, 38), a major regulator of pulmonary vascular tone. Previous studies (5, 35, 41) have demonstrated attenuated NO-dependent vasodilation and decreased NOS activity and expression in the lung in this model. Though a decrease in eNOS protein levels alone partly account for the decreased NO release, impaired NOS activity also occurs in PPHN. An increase in fetal pulmonary artery pressure is associated with rapid decreases in eNOS activity and NO-dependent vasodilation in the fetal lamb (27, 38). The decrease in eNOS activity occurs before a decrease in eNOS expression (27). Villamor et al. (41) demonstrated that immunoreactive eNOS persists in the vascular endothelium, 10 days after ductal constriction. Thus an impairment of NOS activity appears to contribute significantly to the impaired NO release after ductal ligation. However, the mechanism of the impaired NOS activity after constriction of ductus arteriosus remains unknown.

Our previous studies demonstrated that release of ATP contributes to perinatal pulmonary vasodilation in fetal lambs. The plasma levels of ATP increased during exposure of fetal lambs to oxygen, alone or accompanied by lung distension (15, 18). Infusion of ATP and its metabolites into fetal pulmonary artery reproduced the pulmonary vasodilation that occurs at birth in fetal lambs (19). Inhibition of ATP synthesis (16) or receptors for ATP and its metabolite adenosine (15, 18) attenuated the birth-related pulmonary vasodilation in fetal lambs. ATP causes vasodilation in fetal lambs, partly by NO-dependent mechanism and partly by its direct effect on vascular smooth muscle (17). The potential alteration in the vasodilator response to ATP in fetal pulmonary hypertension was previously unknown. Our data suggest that in the intrapulmonary resistance vessels, ATP causes vasodilation primarily by NO-dependent pathway. The attenuation of the vasodilator response to ATP in pulmonary arteries from lambs with pulmonary hypertension was greater than what we observed with L-NAME pretreatment in control vessels. These data suggest that in pulmonary hypertension, lack of vasodilation was due to both loss of NO release and alteration in other mechanisms that contribute to vasodilation in response to ATP. In addition, both L-NAME and Tiron have improved the vasodilator response to ATP in PPHN vessels. These data suggest that inhibition of NOS-dependent O₂⁻ allows these vessels to relax in response to ATP by NO-independent mechanisms. Our previous studies (33) in isolated rabbit pulmonary arteries suggested that ATP causes NO-independent vasodilation by activation of voltage-dependent K⁺ channels (Kᵥ) on vascular smooth muscle. O₂⁻ has been shown to inhibit smooth muscle Kᵥ channels in coronary arteries (20). These studies suggest that a decrease in O₂⁻ may allow ATP to cause vasodilation by stimulation of smooth muscle Kᵥ channels in PPHN.

Regulation of NOS activity in the endothelial cell is a complex and dynamic process. NOS has both reductase and oxygenase domains and occurs as a dimer under physiological conditions (25). Activation of NOS is followed by its phosphorylation at Ser1177 site, which increases the electron flow through the enzyme (23). The balance of NO/O₂⁻ released by activated NOS depends on its conformation, which is regulated by cofactors such as HSP90 (30). HSP90 association with eNOS facilitates NO release in response to a number of physiological stimuli, such as shear stress, vascular endothelial growth factor, and estrogen (7, 12, 32). The absence of HSP90 in the NOS complex (10, 32) or inhibition of the HSP90 conformational change (30) impairs NO release and increases O₂⁻ release from NOS (10, 30) in response to agonist stimulation. Our studies demonstrated that ATP, a mediator of NO release in response to shear stress (14) and oxygen (15, 16), promoted the association of HSP90 with eNOS in control endothelial cells. Although ATP increased phosphorylation of eNOS in hypertensive cells, it failed to increase HSP90 association with eNOS and NO release. The increase in electron flow, which occurs with phosphorylation of eNOS, increases the release of NO or O₂⁻ from NOS (23). Our data suggest that activation of NOS is uncoupled from NO synthesis in pulmonary hypertension. Steinhorn et al. (37) reported an increase in O₂⁻ in pulmonary arteries from lambs with ductal ligation and observed an improvement in NO-mediated vasodilation when these vessels are treated with superoxide dismutase and catalase. We observed an improvement in vasodilator response to ATP by L-NAME and O₂⁻ scavenger Tiron in these pulmonary arteries. These observations suggest that O₂⁻ release from uncoupled NOS in pulmonary hypertension contributes to impaired vasodilation.

Pulmonary arteries harvested from lambs with ductal ligation showed a decrease in basal association of HSP90 with eNOS, suggesting an altered regulation of NOS function in these arteries. Although the total amount of NOS protein in ductal ligation lambs showed a decrease by Western blot, our immunoprecipitation procedure ensured equal loading of eNOS in samples from different arteries. Because immunoprecipitation is done with a limited amount of eNOS antibody relative to eNOS, it is not quantitative with respect to total amount of eNOS protein in the sample. However, the technique is useful to evaluate the relative amounts of associated proteins that coprecipitate with eNOS. The striking decrease in HSP90 coprecipitated with eNOS therefore indicates an alteration in the HSP90/NOS interaction. Because this ratio is an important determinant of the NO/O₂⁻ balance coming from NOS, these data support our concept of uncoupled
NOS contributing to impaired pulmonary vasodilation in PPHN.

The limitation of our study is that we did not address the alterations in other mechanisms that contribute to pulmonary vasodilation during the perinatal period. Previous studies (36) have demonstrated that ductal ligation is associated with a decreased sensitivity of vascular smooth muscle to NO with potential alterations in mechanisms downstream from release of NO. In addition, NOS function is regulated by several factors other than its association with HSP90 (25). Potentially, an alteration in its function caused by these factors may contribute to impaired NO release and pulmonary vasodilation in PPHN. We also observed a small but significant decrease in $P_{aO_2}$ by day 7 of ductal ligation. Whether the decrease in HSP90-NOS interaction is secondary to the effects of decreased $P_{aO_2}$ (39) or increased pulmonary artery pressure remains unknown.

In conclusion, our study provides evidence that decreased association of HSP90 with eNOS occurs in pulmonary hypertension induced by prenatal ductal ligation in fetal lambs. A decrease in this interaction is associated with significant impairment of NO release and vasodilator response to ATP, an important mediator of perinatal pulmonary vasodilation. The mechanisms involved in the decreased HSP90-eNOS interactions in pulmonary hypertension require further investigation.

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