Nitric oxide and superoxide generation from endothelial NOS: modulation by HSP90

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IN THE VASCULATURE, nitric oxide (NO), which acts as endothelium-derived relaxing factor, is derived from the oxidation of L-arginine, synthesized by the endothelial NO synthase (eNOS) (16, 18, 22, 31). Endothelium-derived NO is a critical regulator of cardiovascular homeostasis through its profound effects on blood pressure, vascular remodeling, platelet aggregation, and angiogenesis (17). Abnormal regulation of endothelial function is a characteristic feature in the pathophysiology of a number of pulmonary hypertensive disorders.

A number of factors regulate eNOS. These include post-translational phosphorylation (7); availability of cofactors and substrates such as tetrahydrobiopterin (BH4), FAD, FMN, L-arginine, and oxygen (38); its subcellular localization (10, 30); and protein-protein interactions such as binding to Ca2+ -dependent calmodulin and caveolin-1 (5, 8). Recent studies have suggested that efficient NO production requires the binding of heat shock protein 90 (HSP90) to eNOS, which augments eNOS enzymatic activity and regulates the release of NO and superoxide by eNOS in response to several physiological stimuli (6, 25, 27). Recently, we have found that there are differences in lung NO and superoxide generation from eNOS during the perinatal period of the lamb (18). However, the mechanisms underlying these differences have not been adequately elucidated. Thus the purpose of this study was to investigate the potential role of HSP90 in mediating the alterations in NO and superoxide derived from NO in the perinatal lamb lung.

Our results demonstrated that HSP90 levels were lower in pulmonary artery endothelial cells (PAECs) isolated from fetal compared with 4-wk-old lambs, in which we have previously shown eNOS to be partially uncoupled. Our studies demonstrated that HSP90 overexpression promoted eNOS coupling. Conversely, inhibiting HSP90 induced eNOS uncoupling in PAECs isolated from fetal lamb, in which we have previously shown eNOS to be partially uncoupled. Our studies demonstrated that HSP90 overexpression promoted eNOS coupling. Conversely, inhibiting HSP90 induced eNOS uncoupling in PAECs isolated from fetal lamb, in which we have previously shown to have a tightly coupled eNOS. Finally, we investigated the HSP90-eNOS interactions and release of NO and superoxide in the lamb model of pulmonary hypertension associated with increased blood flow (shunt) (25). Our data demonstrated that eNOS was uncoupled in 4-wk-old shunt lambs as evidenced by perturbed eNOS-HSP90 interactions, impaired NO synthesis, and higher eNOS-dependent superoxide generation. Together, our data indicate an important role for HSP90 in promoting NO generation and inhibiting superoxide generation by eNOS and suggest that the disruption of this interaction may be involved in the endothelial dysfunction associated with pulmonary hypertension.

pulmonary hypertension; endothelial dysfunction; oxidative stress; endothelial nitric oxide synthase

MATERIALS AND METHODS

Purification of recombinant human eNOS. The poly-His-pCWeNOS vector (a gift from P. Ortiz de Montellano, University of California,
San Francisco) was transformed into the protease-deficient Escherichia coli strain BL21 (DE3) plySs (Novagen). Cells were grown in Luria broth with 1% glycerol containing 200 μg/ml ampicillin and 40 μg/ml chloramphenicol. Cultures were grown at 28°C until an optical density (600 nm) of 0.8 was reached. Approximately 1 h before that, heme precursor δ-aminolevulinic acid (0.5 mM final concentration) was added. Cells were then induced by adding isopropylthiogalactoside (0.8 mM final concentration); 0.5 mM ATP and 3 μM riboflavin were also added, and the cells were then grown at 22°C for a further 48 h in the dark. Cells were then harvested by centrifugation (15 min at 4,000 g at 4°C). The cell pellet was resuspended in lysis buffer [40 mM N-(2-hydroxyethyl)piperazine-N-(3-propane sulfonic acid) (EPPS), pH 7.6, containing 1 mg/ml lysozyme, 150 mM NaCl, 0.5 mM L-arginine, 4 μM BH4, 2 μM FAD, and 10% glycerol], and protease inhibitor cocktail (Sigma) was added according to the manufacturer’s recommendation. The bacterial suspension was incubated with mild shaking at 4°C for 30 min to ensure complete cell lysis. Cells were broken by sonication using three 25-s pulses followed by three cycles of freezing and thawing. Cell debris was removed by centrifugation at 30,000 g for 30 min at 4°C. The supernatant was then applied to a Ni-NTA His-Bind Superflow (Novagen) column pre-equilibrated with buffer A (40 mM EPPS, pH 7.6, containing 150 mM NaCl, 10% glycerol, and 0.5 mM L-arginine). The column was washed with 5 bed volumes of buffer A followed by buffer B (buffer A with 25 mM imidazole). The bound protein was then eluted with buffer C (buffer A with 200 mM imidazole). The heme-containing fractions were pooled and concentrated using Centricon-100 YM-10 (Millipore). The concentrated proteins were dialyzed against three changes of buffer A containing 4 μM BH4 and 1 mM DTT. The proteins were further purified using a 2′,5′-ADP-Sepharose column equilibrated with buffer D (40 mM Tris buffer, pH 7.6, containing 1 mM L-arginine, 3 mM DTT, 4 μM BH4, 4 μM FAD, 10% glycerol, and 150 mM NaCl) and washed with buffer D containing 400 mM NaCl to prevent nonspecific binding. eNOS was then eluted with buffer E (buffer D with 5 mM 2′-AMP). The heme-containing fractions were pooled, concentrated, and dialyzed at 4°C against buffer D containing 1 mM DTT, 4 μM BH4, 4 μM FAD, and 10% glycerol and were stored at −80°C until used. The DTT, BH4, and FAD were removed by repeated buffer exchange using Centricon before use.

Cell culture. Primary cultures of ovine fetal PAECs were isolated as described previously (18). Cells were maintained in DMEM containing phenol red supplemented with 10% fetal calf serum (Hyclone, Logan, UT), antibiotics, and antimitotics (MediaTech, Herndon, VA) at 37°C in a humidified atmosphere with 5% CO2-95% air. Cells were utilized between passages 3 and 10, seeded at ~50% confluence, and utilized when fully confluent. All experiments comparing PAECs isolated from fetal and 4- to 8-week-old lambs were carried out using passage-matched cells to account for potential phenotypic alterations during culturing. For the experiments where HSP90 activity was inhibited, PAECs isolated from fetal lambs were incubated with radicicol (20 μM) for 30 min.

Shear stress. Laminar shear stress was applied using a cone-plate viscometer that accepts six-well tissue culture plates, as described previously (3, 35, 37). This method achieves laminar flow rates that represent physiological levels of laminar shear stress in the major human arteries, which is in the range of 5–20 dyn/cm2 with localized increases to 30–100 dyn/cm2.

eNOS immunoprecipitation and Western blotting. Serum-starved PAECs (16 h) were solubilized with a lysis buffer containing 1% Triton X-100, 20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Pierce, Rockford, IL). Insoluble proteins were precipitated by centrifugation at 13,000 rpm for 10 min at 4°C, and the supernatants were then incubated overnight with the anti-eNOS antibody (2 μg) at 4°C, followed by incubation in protein G plus protein A agarose (Calbiochem, La Jolla, CA) for 2 h. The immune complexes were precipitated by centrifugation, washed three times with lysis buffer, boiled in SDS sample buffer, subjected to SDS-PAGE on 4–12% polyacrylamide gels, and transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween (TBST). The primary antibody, anti-HSP90 (1:1,000; BD Transduction Laboratories), was then added and incubated for 1 h at room temperature. The membrane was then washed three times with TBST (10 min) and then incubated with a secondary antibody coupled to horseradish peroxidase. The membrane was then washed three times with TBST as described above. Reactive bands were visualized using the SuperSignal West Femto maximum sensitivity substrate kit (Pierce) and Kodak 440CF image station (Kodak, New Haven, CT). The intensity of the reactive bands was quantified using Kodak 1D software. The efficiency of each immunoprecipitation was normalized by reprobing the membrane with the immunoprecipitating antibody (eNOS).

HSP90 overexpression: construction and purification of recombinant HSP90 adenovirus. An adenovirus expressing HSP90 was generated by using the procedure of He et al. (15). Coding sequence for full-length human HSP90β (14) was subcloned into a pAd-Track-CMV shuttle vector. The pAd-Track-CMV-HSP90 construct was linearized with PmeI and was cotransformed with an adenoviral backbone plasmid, pAdEasy-1, into E. coli BJ5183 cells. Recombinants were selected using kanamycin resistance and confirmed using restriction enzyme digestion. Recombinant plasmids were then transfected into HEK-293 cells. Viral production was monitored over 7–10 days by visualization of green fluorescent protein expression. Virus was then harvested and purified by banding on a CsCl gradient. The purified virus was dialyzed and stored at −80°C. PAECs were infected with HSP90 adenoviral construct. Cells were infected at a multiplicity of infection of 100:1. Verification of expression was performed using Western blot analysis as described above, and the blot was probed with anti-HSP90 antibody (1:1,000; BD Transduction Laboratories) and normalized using anti-β-actin antibody (1:1,000, Sigma).

Detection of NOx in pulmonary arterial endothelial cells. NO generated by PAECs in response to shear or generated in vitro by eNOS was measured using an NO-sensitive electrode with a 2-mm-diameter tip (ISO-NOP sensor; WPI) connected to an NO meter (ISO-NOMark II; WPI) as described previously (40). The NO meter was calibrated with PmeI and was cotransformed with an adenoviral backbone plasmid, pAdEasy-1, into E. coli BJ5183 cells. Recombinants were selected using kanamycin resistance and confirmed using restriction enzyme digestion. Recombinant plasmids were then transfected into HEK-293 cells. Viral production was monitored over 7–10 days by visualization of green fluorescent protein expression. Virus was then harvested and purified by banding on a CsCl gradient. The purified virus was dialyzed and stored at −80°C. PAECs were infected with HSP90 adenoviral construct. Cells were infected at a multiplicity of infection of 100:1. Verification of expression was performed using Western blot analysis as described above, and the blot was probed with anti-HSP90 antibody (1:1,000; BD Transduction Laboratories) and normalized using anti-β-actin antibody (1:1,000, Sigma).

Electron paramagnetic resonance spectroscopy and spin trapping. To detect superoxide generation in intact cells, we performed electron paramagnetic resonance (EPR) measurements as described previously (40) using the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrroline-HCl (CMH; Alexis Biochemicals, San Diego, CA). Following overnight serum starvation of the cells, 20 μl of spin-trap stock solution consisting of CMH [20 μM in Dulbecco’s PBS (DPBS) plus 25 μM desferrioxamine (Calbiochem)] and 5 μM diethyldithiocarbamate (Alexis Biochemicals, Lausen, Switzerland) plus 2 μl of DMSO were added to each well before shear stimulation. Adherent cells were trypsinized and pelleted at 500 g after a 45-min incubation at 37°C following shear to allow entrapment of superoxide by the spin trap. Cell pellet was washed and suspended in a final volume of 35 μl of DPBS (plus desferrioxamine and diethyldithiocarbamate), loaded into a 50-μl capillary tube, and analyzed with a MiniScope MS200 EPR (Magnettech, Berlin, Germany) at a microwave power of 40 mW, modulation amplitude of 3,000 mG, and modulation frequency of 100 kHz. EPR spectra were analyzed measured for amplitude using ANALYSIS software (version 2.02; MagneTech), and experimental groups were compared using the statistical analysis described below. In the experiments examining the role of HSP90 in modulating superoxide production, the cells were preincubated with the HSP90 inhibitor radicicol 30 min before shear activation.

Effect of HSP90 on NOx and superoxide generation by eNOS in vitro. The in vitro reaction was conducted in 50 μl of buffer containing 50 mM HEPES, pH 7.4, 1 mM NADPH, 100 μM L-arginine, 1 mM Ca2+, 10 μg/ml calmodulin, and 1 μg of eNOS. To
determine the effect of BH₄ in eNOS, we carried out coupling experiments in both the presence and absence of 4 μM BH₄. Endothelial NOS-dependent superoxide generation in vitro was performed in 50 μl of reaction mix containing 50 mM HEPES, pH 7.4, 1 mM NADPH, 1 mM Ca²⁺, 10 μg/ml calmodulin, 4 μM BH₄, 1 μg of eNOS, and CMH hydrochloride. In the experiments determining the effect of HSP90, eNOS and HSP90 were incubated for 15 min before measurements were made. NO and superoxide generation were then determined as described above. To quantitate catalytic activity of eNOS protein, we also performed a standard reaction of the superoxide-generating enzyme xanthine oxidase in the presence of xanthine and CMH. A reaction curve was generated by adding 1 U/ml xanthine oxidase (0.5 U/mg; Sigma) into 500 μM xanthine (Sigma) solution in buffered PBS, pH 7.4, in the EPR reaction buffer described above. Reactions were allowed to proceed at 25°C for up to 20 min. Following incubation, ~35 μl of each reaction mixture were loaded and the EPR spectra were analyzed as described above. Given that 1 unit of xanthine oxidase (~2 mg of protein) converts 1 μmol of xanthine per minute at 25°C, on the basis of this standard curve we calculate the coefficient of 840.6 EPR amplitude units/μmol of superoxide produced in the reaction. We then utilized this to convert the waveform amplitude generated by eNOS in the presence and absence of HSP90 into nanomoles of superoxide converted per minute per microgram of eNOS protein.

Surgical preparations and care. Twelve mixed-breed Western pregnant ewes (137–141 days of gestation, term ~145 days) were operated on under sterile conditions with the use of local anesthesia (2% lidocaine hydrochloride) and inhalational anesthesia (1–3% isoflurane). A midline incision was made in the ventral abdomen, and the pregnant horn of the uterus was exposed. Through a small uterine incision, the left fetal forelimb and chest were exposed, and a left lateral thoracotomy was performed in the third intercostal space. Additional fetal anesthesia consisted of local anesthesia with 1% lidocaine hydrochloride and ketamine hydrochloride (20 mg im). With the use of side-biting vascular clamps, an 8.0-mm Gore-Tex vascular graft (~2 mm long; W.L. Gore and Associates, Milpitas, CA) was anastomosed between the ascending aorta and main pulmonary artery with 7.0 prolene (Ethicon, Somerville, NJ), using a continuous suture technique. The thoracotomy incision was then closed in layers. This procedure was previously described in detail (25).

Four weeks after spontaneous delivery, all lambs were killed with a lethal injection of pentobarbital sodium, followed by bilateral thoracotomy as described in the NIH Guide for the Care and Use of Laboratory Animals. The lungs were then harvested for study. Lung pieces were snap frozen in liquid nitrogen and stored at −80°C. In addition, 30 ml of arterial blood was collected in iced Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing ethylenediaminetetraacetic acid (7.5 mM). The samples were centrifuged (4,000 g for 15 min) at 4°C, and the resulting plasma was snap frozen in liquid nitrogen and then stored in polypropylene storage tubes at −80°C.

All protocols and procedures were approved by the Committee on Animal Research of the University of California, San Francisco and the Medical College of Georgia.

Measurement of superoxide levels in peripheral lung tissue. Approximately 0.2 g of peripheral lung tissue was sectioned from fresh frozen tissue and immediately immersed in either normal EPR buffer [PBS supplemented with 5 μM diethylidithiocarbamate (Sigma-Aldrich) and 25 μM desferrioxamine (Sigma-Aldrich)] or EPR buffer supplemented with 100 U/ml polyethylene glycol-superoxide dismutase (PEG-SOD; Sigma), 100 μM 2-ethyl-2-thiopseudourea (ETU; Sigma), an inhibitor of human NO synthases (9), or 10 μM apocynin, an inhibitor of NADPH oxidase (36). Samples were incubated for 30 min on ice. During incubation, samples were analyzed for protein content using Bradford analysis (Bio-Rad). Sample volumes were then adjusted with EPR buffer plus 25 mg/ml CMH hydrochloride (Axxora) to achieve equal protein content and a final CMH concentration of 5 mg/ml. Samples were homogenized for 30 s with a WWR PowerMAX AHS 200 tissue homogenizer, incubated for 60 min on ice, and then centrifuged at 14,000 g for 15 min at room temperature. Supernatant (35 μl) was loaded into a 50-μl capillary tube and analyzed for superoxide generation as described above.

Measurement of NOx levels in lung tissue. To quantify bioavailable NO, we measured NO and its metabolites in the peripheral lung tissue from shunt and age-matched control lambs at 4 wk of age. In solution, NO reacts with molecular oxygen to form nitrite and with oxyhemoglobin and superoxide anion to form nitrate. Nitrite and nitrate are reduced using vanadium(III) and hydrochloric acid at 90°C. NO is purged from the solution, resulting in a peak of NO for subsequent detection by chemiluminescence (NOA 280; Sievers Instruments, Boulder CO). The detection limit is 1 nM/ml of nitrate.

Statistical analysis. Statistical calculations were performed using GraphPad Prism version 4.01 software. The means ± SD or SE were calculated for all samples, and significance was determined using either the unpaired t-test or ANOVA. A value of P < 0.05 was considered significant.

RESULTS

Total HSP90 levels in PAECs isolated fetal and 4-wk-old lambs. We first examined total HSP90 levels in passage-matched PAECs isolated from fetal and 4-wk-old lambs. Western blot analysis revealed significantly higher expression of HSP90 in PAECs isolated from fetal compared with 4-wk-old lambs (P < 0.05, Fig. 1).

Effect of HSP90 on superoxide and NO production by eNOS in vitro. To investigate the potential role of HSP90 on eNOS coupling, we initially utilized recombinant human eNOS and HSP90. Our data indicate that superoxide production from eNOS was significantly diminished (Fig. 2A) in the presence of HSP90, whereas NO production was significantly enhanced (Fig. 2B). Interestingly, although the presence of HSP90 could significantly reduce superoxide production from eNOS in the

![Fig. 1. Comparison of total heat shock protein 90 (HSP90) protein in pulmonary arterial endothelial cells (PAECs) isolated from fetal and 4-wk-old lambs. A: total protein extracts (25 μg) prepared from PAECs isolated from fetal and 4-wk-old lambs were separated on a 4–20% denaturing polyacrylamide gel, electrophoretically transferred to nitrocellulose membranes, and analyzed using a specific antiseraum raised against HSP90 protein. The blot was then reprobed for β-actin to normalize loading. A representative image is shown. B: there was a significant decrease in the densitometric value for HSP90, relative to β-actin, in the lysates prepared from PAECs isolated from 4-wk-old lambs compared with those from fetal PAECs. Values are means ± SE; n = 3. *P < 0.05 vs. PAECs isolated from fetal lambs.](http://ajplung.physiology.org/)
absence of BH4 (Fig. 2A), it could not substitute for the requirement of BH4 in NO generation (Fig. 2B). The addition of HSP90 appeared to decrease total catalytic activity of eNOS, calculated as the total nanomoles of NO and superoxide per minute per microgram of eNOS protein (Fig. 2C). However, the amount of NO generated as a percentage of total catalytic activity was significantly higher in the presence of HSP90 (Fig. 2D), whereas the amount of superoxide generated was significantly lower (Fig. 2D).

Overexpression of HSP90 in PAECs isolated from 4-wk-old lambs increases the association of eNOS and HSP90. To begin to determine the significance of the decrease in HSP90 expression in PAECs isolated from 4-wk-old lambs in eNOS uncoupling, we overexpressed HSP90 using an adenoviral construct. The expression of HSP90 was initially confirmed by Western blot analysis. HSP90 expression was approximately twofold higher in HSP90-transduced cells compared with uninfected cells ($P < 0.05$, Fig. 3). To examine the effect of increasing HSP90 expression on eNOS-HSP90 interactions, we utilized immunoprecipitation from unstimulated and shear-stimulated control and HSP90-overexpressing PAECs. Western blotting for HSP90 on the immunoprecipitated eNOS complex revealed that shear stimulation led to higher association of HSP90 with eNOS in both HSP90-overexpressing and control PAECs ($P < 0.05$, Fig. 4). However, the HSP90-eNOS association was found to be significantly higher in HSP90-transduced compared with control cells under both static and shear conditions ($P < 0.05$, Fig. 4).

Fig. 2. Effect of HSP90 on superoxide and nitric oxide production by recombinant endothelial nitric oxide synthase (eNOS). Purified human eNOS (1 μg), NADPH (1 mM), Ca2+ (1 mM), and calmodulin (10 μg/ml) were incubated with or without recombinant HSP90 (2 μg) or tetrahydrobiopterin (BH4; 4 μM), and the superoxide (O2•−) and NO generated were measured over a 15-min period. A: the presence of HSP90 significantly decreased superoxide generation from eNOS even in the absence of BH4. B: HSP90 also significantly increased NO generation, but only in the presence of BH4. Values are mean ± SE; $n = 6$. *$P < 0.05$ vs. no HSP90. C and D: in addition, although HSP90 decreased total catalytic activity of eNOS (calculated as total NO and O2•− generated per minute per microgram; C), there was a significant increase in the NO generated as a percentage of the total catalytic activity (D). Values are means ± SE; $n = 3$. *$P < 0.05$ vs. NO generated by eNOS alone. †$P < 0.05$ vs. O2•− generated by eNOS alone.

Fig. 3. Overexpression of HSP90 in PAECs isolated from 4-wk-old lambs. PAECs isolated from 4-wk-old lambs were transduced with adenovirus expressing either green fluorescent protein (AdGFP) or HSP90 (AdHSP90). Cells were then harvested after 48 h and subjected to Western blot analysis. A: total protein extracts (25 μg) prepared from PAECs isolated from fetal and 4-wk-old lambs were separated on a 4–20% denaturing polyacrylamide gel, electrophoretically transferred to nitrocellulose membranes, and analyzed using a specific antiserum raised against HSP90 protein. The blot was then reprobed for β-actin to normalize loading. A representative image is shown. B: there was a significant increase in densitometric value for HSP90, relative to β-actin, in the lysates prepared from PAECs transduced with AdHSP90 compared with those transduced with AdGFP. Values are means ± SE; $n = 3$. *$P < 0.05$ vs. GFP transduced.
from 4-wk-old lambs. PAECs isolated from 4-wk-old lambs were transduced with either AdGFP or AdHSP90. After 48 h, they were then exposed or not to laminar shear stress (20 dyn/cm², 15 min), and the association of eNOS with HSP90 was determined using immunoprecipitation analyses. A: PAECs isolated from 4-wk-old lambs were harvested for immunoprecipitation (IP) by using a specific antibody to eNOS. The levels of HSP90 associated with eNOS were determined by probing the membrane with an anti-HSP90 antibody. The membrane was then reprobed with eNOS (to normalize for loading). A representative image is shown. IB, immunoblot. B: the association of eNOS with HSP90 was significantly increased under both basal (no shear) and shear stress conditions when PAECs were transduced with AdHSP90. Values are means ± SE; n = 3. *P < 0.05 vs. control. †P < 0.05 vs. shear alone.

**Effect of HSP90 overexpression on NO production and eNOS-dependent superoxide generation in PAECs isolated from 4-wk-old lambs.** We next determined the effect of HSP90 overexpression on NO production under both unstimulated and shear-stimulated conditions. Shear stimulation significantly increased NO generation in both unstimulated and shear-stimulated conditions (Fig. 5A). However, the NO generation was found to be significantly higher in HSP90-transduced compared with control cells under both static and shear conditions (P < 0.05, Fig. 5A). We next determined the effect of HSP90 overexpression on eNOS-dependent superoxide production under both unstimulated and shear-stimulated conditions. Shear stimulation significantly increased eNOS-dependent superoxide generation only in control PAECs (P < 0.05, Fig. 5B). Superoxide generation was found to be unchanged between HSP90-overexpressing and control cells under unstimulated conditions (Fig. 4B), but eNOS-dependent superoxide levels were significantly lower compared with control cells when exposed to shear stress (P < 0.05, Fig. 5B).

**Effect of HSP90 inhibition on eNOS-HSP90 interactions in PAECs isolated from fetal lambs.** To examine the effect of inhibiting HSP90 activity on eNOS-HSP90 interactions in a cell line in which eNOS is tightly coupled, we treated PAECs isolated from fetal lambs with radicicol (20 μM) for 30 min and then exposed PAECs to shear. We then immunoprecipitated eNOS from unstimulated and shear-stimulated PAECs. Western blotting for HSP90 and eNOS on the immunoprecipitated eNOS complex revealed that shear stimulation led to higher association of HSP90 with eNOS only in the PAECs not exposed to radicicol (P < 0.05, Fig. 6). In addition, the HSP90-eNOS association was found to be significantly lower in radicicol-treated compared with control cells under static conditions (P < 0.05, Fig. 6).

**Effect of HSP90 inhibition on shear-induced NO production and eNOS-dependent superoxide generation in PAECs isolated from fetal lambs.** To confirm that inhibiting HSP90 attenuates NO production, we incubated PAECs isolated from fetal lambs with or without radicicol (20 μM) for 30 min and then exposed PAECs to shear stress. Shear stimulation significantly increased NO generation only in cells not exposed to radicicol (P < 0.05, Fig. 7A). In addition, the NO generation was found to be significantly lower in radicicol-treated compared with control cells under static conditions (P < 0.05, Fig. 7A). We next determined the effect of HSP90 inhibition on eNOS-dependent superoxide production under both unstimulated and shear-stimulated conditions. Shear stimulation had no significant effect on eNOS-dependent superoxide generation in PAECs isolated from fetal lambs (Fig. 7B). Similarly, NOS-dependent superoxide generation was unchanged in the radicicol-treated cells under static conditions (Fig. 7B). However, NOS-dependent superoxide levels were significantly increased in radicicol-treated cells compared with untreated cells when exposed to shear stress (P < 0.05, Fig. 7B). In addition, we...
found that the increased superoxide generation was NOS dependent, since apocynin (an NADPH oxidase inhibitor) pre-treatment did not decrease the increased superoxide generation in radicicol-exposed PAECs isolated from fetal lambs and subjected to shear stress (Fig. 7C).

eNOS-HSP90 interactions are decreased in peripheral lung tissue prepared from 4-wk-old shunt compared with age-matched control lambs. Endothelial NOS protein levels were increased in shunt lambs above control levels at 4 wk, whereas HSP90 levels were unchanged (Fig. 8A). However, there was a significant decrease in eNOS-HSP90 interactions in 4-wk-old shunt compared with control lambs (P < 0.05, Fig. 8B).

NOS-dependent superoxide anion production is higher and NO generation lower in peripheral lung tissue prepared from 4-wk-old shunt compared with age-matched control lambs. Utilizing EPR, we found that superoxide generation was significantly higher in peripheral lung lysates prepared from 4-wk-old shunt compared with control lambs (P < 0.05, Fig. 8C). Also, in the shunt lambs, pretreatment with PEG-SOD decreased the amplitude to control levels, suggesting specific quenching of superoxide anions by PEG-SOD (Fig. 8D). Furthermore, pretreatment with the NOS inhibitor ETU significantly reduced the amplitude in shunt lambs (P < 0.05, Fig. 8D), indicating that NOS is a significant source of superoxide anions in the shunt lambs. Pretreatment of control lysates with apocynin had no effect on the waveform amplitude (Fig. 8E).

Finally, we determined relative NO generation in shunt lambs compared with age-matched control lambs at 4 wk of age. To calculate this, we quantified NOx levels in peripheral lung tissue and then divided by total NOS protein levels (determined by Western blot analysis) in the extracts. Despite the increase in NOS expression in shunt lambs (Fig. 8A), there was a significant decrease in relative NO generation (P < 0.05, Fig. 8F).

Fig. 6. Effect of radicicol on the association of eNOS with HSP90 in PAECs isolated from fetal lambs. PAECs isolated from fetal lambs were exposed or not to the HSP90 inhibitor radicicol (20 μM, 30 min), and then the association of eNOS with HSP90 was determined using immunoprecipitation analyses. A: PAECs isolated from fetal lambs were harvested for IP by using a specific antibody to eNOS. The level of HSP90 associated with eNOS was determined by probing the membrane with an anti-HSP90 antibody. The membrane was then reprobed with eNOS (to normalize for loading). A representative image is shown. B: the association of eNOS with HSP90 was significantly decreased when PAECs were exposed to radicicol in both static and shear conditions. Values are means ± SE; n = 4. *P < 0.05 vs. control. †P < 0.05 vs. shear alone.

Fig. 7. Effect of radicicol on NO and superoxide generation in PAECs isolated from fetal lambs. PAECs isolated from fetal lambs were exposed or not to the HSP90 inhibitor radicicol (20 μM, 30 min) or the NOS inhibitor ETU (100 μM, 30 min). Cells were then exposed or not to laminar shear stress (20 dyn/cm², 15 min), and the levels of NO and superoxide generated were determined. A: HSP90 inhibition significantly decreased shear-mediated NO production. B: HSP90 inhibition significantly increased eNOS-dependent superoxide generation. C: NADPH oxidase inhibition (apocynin) did not significantly alter superoxide generation induced by HSP90 inhibition. Values are means ± SE; n = 4. *P < 0.05 vs. control. †P < 0.05 vs. shear alone. ‡P < 0.05 vs. radicicol treated + shear.
DISCUSSION

Our group (21) has previously investigated the developmental alteration in NO and superoxide level from pulmonary artery vessels and endothelial cells isolated from fetal and juvenile (4 wk old) lambs. Our previous results demonstrated that PAECs isolated from fetal lambs produce significant levels of NO and small amounts of superoxide upon shear stimulation, whereas PAECs isolated from 4-wk-old lambs produce significant amounts of both NO and superoxide. These data suggested that eNOS is uncoupled to a certain extent in PAECs during postnatal development. In those studies (21), we observed that BH4 levels were significantly reduced in PAECs isolated from 4-wk-old lambs. This reduction in BH4 could lead to eNOS coupling. However, there are a number of other pathways that can lead to eNOS uncoupling, including HSP90. Thus the aim of the present study was to determine whether there are differences in HSP90 expression and, if so, to determine the role of HSP90 in eNOS coupling in the perinatal period of the lamb. To achieve this goal, we again utilized PAECs isolated both from fetal lambs, in which eNOS is tightly coupled, and 4-wk-old lambs, in which eNOS is uncoupled (21). Our initial Western blot analyses detected higher HSP90 expression in PAECs isolated from fetal compared with 4-wk-old lambs. Thus we wanted to further investigate the importance of HSP90 in eNOS coupling.
Using purified eNOS and HSP90, we first demonstrated that HSP90 increases NO generation by eNOS in vitro. This is in agreement with recent studies revealing that HSP90 positively regulates eNOS (33). Because HSP90 is important for mediating eNOS-dependent NO generation, we sought to investigate whether HSP90 reduced eNOS superoxide generation in vitro. We have demonstrated for the first time that HSP90 attenuates superoxide production, as determined by spin-trapping methodology, from recombinant eNOS. This is in accordance with a recent report demonstrating that HSP90 decreases superoxide generation from neuronal NOS (33). Interestingly, we also found that HSP90 is able to decrease superoxide generation in the absence of BH4 but is not able to stimulate NO generation under BH4-free conditions. These data suggest that HSP90 may be able to decrease eNOS-derived superoxide independently of its stimulatory effect on NO generation. This would act to suppress eNOS-derived superoxide generation in conditions where BH4 is limiting, as our group (12) has shown previously in the 4-wk-old shunt lambs, and to limit the potential increase in oxidative stress from an uncoupled eNOS. However, further studies are required to investigate this possibility. Interestingly, we also found that HSP90 appears to decrease catalytic turnover in eNOS. This would appear to be at odds with the work of García-Cardena et al. (6), who found that HSP90 stimulated eNOS activity. However, in this study, eNOS activity was determined as the conversion of 3H-labeled L-arginine to L-citrulline, to determine NO activity in COS cell lysates or in purified eNOS. Since the activation of molecular oxygen to form superoxide can be independent of the metabolism of L-arginine, a decrease in catalytic activity would not be readily apparent when eNOS activity is measured by L-arginine to L-citrulline conversion.

Leading on from this in vitro study, we wanted to determine whether the decreased HSP90 expression we observed in PAECs isolated from 4-wk-old lambs was an important mediator of eNOS coupling in the perinatal period. Overexpressing HSP90 in PAECs isolated from 4-wk-old lambs resulted in enhanced association of eNOS with HSP90 under both basal and shear-activated conditions. We also detected significantly higher basal and shear-activated NO generation. This was associated with increased superoxide production, but only under shear conditions. These findings suggest that eNOS becomes more coupled when HSP90 expression is increased. This is consistent with studies showing that an increase in the association of HSP90 with eNOS increases production and activity of NO in response to growth factor stimulation (6).

Next, we disrupted eNOS-HSP90 interactions in the PAECs isolated from fetal lambs, in which eNOS is tightly coupled, to determine whether this was sufficient to lead to eNOS uncoupling. To achieve HSP90 inhibition, we used radicicol, a macrocyclic antifungal that binds to the NH2-terminal domain of the HSP90 (29). Immunoprecipitation of eNOS, followed by immunoblotting for eNOS and HSP90, revealed that exposure to radicicol decreased HSP90 interactions with eNOS. There also was a reduction in both basal and shear-dependent NO production. Moreover, there was an increase in superoxide generation, indicating that inhibiting HSP90 is sufficient to drive eNOS into an uncoupled state. Furthermore, our data indicate that the increased superoxide generation induced by radicicol was not due to NADPH oxidase activation, since pretreatment with apocynin failed to reduce the superoxide generated. These data are consistent with other studies indicating that inhibiting HSP90 function uncouples eNOS activity (6, 24).

Emerging evidence suggests the most important consequences of uncoupled NOS may be impairment of endothelium-dependent vasodilation, a hallmark of endothelial dysfunction (19, 20). Thus, in this study, we also determined the role of HSP90 in eNOS coupling in our established lamb model of pulmonary hypertension that mimics congenital heart disease with increased pulmonary blood flow (25). Previously, our group has demonstrated an upregulation of NOS expression in these shunted lambs (2) but that these lambs still had an impairment of endothelium-dependent vasodilation (1). Our previous studies also demonstrated that in shunt pulmonary arteries, relaxations in response to A-23187 and zaprinast were attenuated and that superoxide was at least partially responsible for the impairment in endothelium-dependent relaxations displayed in shunted lambs, since pretreatment of vessels with membrane-permeable superoxide dismutase enhanced relaxations to A-23187 in pulmonary arteries from shunt but not from control lambs (34). In support of this, we have demonstrated in the current study increased eNOS-dependent superoxide production in the shunt lambs and decreased NO generation compared with total protein expression. The findings of reduced NO synthesis and higher superoxide production in shunt lambs suggest that eNOS is uncoupled. Since 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 (6, 24, 27), we also analyzed eNOS-HSP90 interactions in shunt and age-matched control lambs. Our data show a strikingly reduced association of HSP90 with eNOS. This finding suggests that perturbed interaction of eNOS with HSP90 might be leading to uncoupled eNOS in shunt lambs. Overall, our data show that decreased HSP90-eNOS interactions in shunt lambs correlate with a decrease in tissue NOx levels and increased eNOS-dependent generation of superoxide in shunt lambs, all of which are indicative that eNOS is uncoupled. This is consistent with recent studies that have demonstrated decreased association of HSP90 with eNOS in fetal lambs with persistent pulmonary hypertension (19) and in hypoxia-induced pulmonary hypertension (23). Together, our data and these previous studies suggest that failure to maintain optimum HSP90 interaction with eNOS results in an inefficient coupling of enzyme activity to L-arginine metabolism and an increase in eNOS-dependent superoxide generation. Furthermore, the loss of eNOS-HSP90 interactions may be an important early event in the endothelial dysfunction associated with the development of pulmonary hypertension. It also should be noted that derangements in NOS signaling have been identified in other models of pulmonary hypertension induced by chronic hypoxia. However, the data obtained have been conflicting, with the exposure having opposing effects on lung NOx levels, being reduced in piglets (4) but increased in rats (28), although NOx levels were reduced in both cases. Similarly, cell culture studies have yielded conflicting data with reduced (39) and increased NO expression (13) as well as increased (32) and decreased NO production being observed (39). However, studies examining the potential for hypoxia to uncouple eNOS have not been resolved either in vitro or in vivo using animal models.
It also should be noted that the eNOS-HSP90 interaction may be only one component of the etiology underlying NOS uncoupling in shunt lambs, since our group has previously demonstrated both decreased L-arginine levels (26) and alterations in BH4 metabolism in shunt lambs (11, 26). In addition, we have found that BH4 levels are decreased in PAECs isolated from 4-wk-old compared with fetal lambs and that increasing BH4 levels led to an increase in NO and a decrease in superoxide generation from eNOS upon stimulation (21). Together, these findings suggest that the uncoupling of the eNOS enzyme in the perinatal period may be a complex process and that systems other than HSP90 and BH4 may be involved. For example, future studies could investigate the potential role of alterations in L-arginine and the endogenous inhibitor asymmetric dimethylarginine. Furthermore, it should be noted that NOS-derived superoxide is not the sole mechanism for increased oxidative stress in shunt lambs. Our present data utilizing EPR indicate that NADPH oxidase is also a significant source of superoxide in shunt lambs. However, the effect on superoxide generation of the NADPH oxidase inhibitor apocynin in peripheral lung tissue was not as great as with the NOS inhibitor ETU, suggesting that NOS-derived superoxide may be dominant in 4-wk-old lambs. It also should be noted that the EPR data presented confirm our previously published studies utilizing dihydroethidium oxidation to estimate superoxide generation (12).

In conclusion, overexpression of HSP90 promotes coupled eNOS activity, increasing NO generation and decreasing superoxide generation. Conversely, HSP90 inhibition decreases eNOS-HSP90 associations and results in a shift in the balance of NO and superoxide, favoring increased superoxide release and resulting in uncoupled eNOS activity. Furthermore, decreases in eNOS-HSP90 interactions correlate with uncoupled eNOS in a lamb model of pulmonary hypertension with increased pulmonary blood flow. Together, our data indicate that stimulating HSP90 activity may be a potential therapeutic approach for treating pulmonary hypertension.

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