Heat shock protein 90 modulates endothelial nitric oxide synthase activity and vascular reactivity in the newborn piglet pulmonary circulation

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Aschner JL, Foster SL, Kaplowitz M, Zhang Y, Zeng H, Fike CD. Heat shock protein 90 modulates endothelial nitric oxide synthase activity and vascular reactivity in the newborn piglet pulmonary circulation. Am J Physiol Lung Cell Mol Physiol 292: L1515–L1525, 2007. First published March 2, 2007; doi:10.1152/ajplung.00252.2006.—Heat shock protein 90 (Hsp90) binding to endothelial nitric oxide synthase (eNOS) is an important step in eNOS activation. The conformational state of bound Hsp90 determines whether eNOS produces nitric oxide (NO) or superoxide (O2•−). We determined the effects of the Hsp90 antagonists geldanamycin (GA) and radicicol (RA) on basal and Ach-stimulated changes in vessel diameter, cGMP production, and Hsp90:eNOS coimmunoprecipitation in piglet resistance level pulmonary arteries (PRA). In perfused piglet lungs, we evaluated the effects of GA and RA on Ach-stimulated changes in pulmonary arterial pressure (Ppa) and perfusate accumulation of stable NO metabolites (NOx). The effects of GA and RA on Ach-stimulated O2•− generation was investigated in cultured pulmonary microvascular endothelial cells (PMVEC) by dihydroethidine (DHE) oxidation and confocal microscopy. Hsp90 inhibition with GA or RA reduced Ach-mediated dilation, abolished the Ach-stimulated increase in cGMP, and reduced eNOS:Hsp90 coprecipitation. GA and RA also inhibited the Ach-mediated changes in Ppa and NOx accumulation rates in perfused lungs. Ach increased the rate of DHE oxidation in PMVEC pretreated with GA and RA but not in untreated cells. The cell-permeable superoxide dismutase mimetic M40401 reversed GA-mediated inhibition of Ach-induced dilation in PRA. We conclude that Hsp90 is a modulator of eNOS activity and vascular reactivity in the newborn piglet pulmonary circulation. Uncoupling of eNOS with GA or RA inhibits Ach-mediated dilation by a mechanism that involves O2•− generation.

PULMONARY CIRCULATORY ADAPTATION at birth occurs, at least in part, through nitric oxide (NO)-dependent signaling mechanisms (1, 9, 18, 19, 29). Inhibition of nitric oxide synthase (NOS) activity in the late gestation fetus or newborn disrupts the fall in pulmonary vascular resistance and the 8- to 10-fold increase in pulmonary blood flow that is essential to postnatal gas exchange and survival (1, 18, 19, 36, 47). The generation of NO from endothelial nitric oxide synthase (eNOS) is a highly orchestrated cellular event, regulated both at the transcriptional level and by a number of posttranslational modifications, including dual acylation, cellular localization, and phosphorylation. The availability of substrate, arginine, and of the cofactor tetrahydrobiopterin (BH4) determines whether eNOS produces NO or generates O2•−, a process known as NOS uncoupling (25). Protein-protein interactions with calmodulin, caveolin-1, and heat shock protein 90 (Hsp90) are also important in the regulation of eNOS activity.

Hsp90 is a constitutively expressed molecular chaperone that coordinates the trafficking and regulation of diverse signaling proteins, including eNOS. Hsp90 association with eNOS facilitates eNOS activation (3, 24, 45). It has been suggested that the ATP/ADP conformational state of Hsp90 determines whether the catalytic product of eNOS is NO or O2•− (35). Geldanamycin (GA), an ansamycin antibiotic, and radicicol (RA), a macrocyclic antifungal, bind to the ATP binding site of Hsp90, uncoupling eNOS and driving the enzymatic reaction toward production of superoxide radical. The importance of the posttranslational modification of eNOS by Hsp90 in regulating perinatal pulmonary vascular tone and reactivity is poorly understood. Konduri et al. (30) demonstrated decreased Hsp90:eNOS interactions in isolated vessels from fetal lamb lungs with pulmonary hypertension compared with vessels from control fetal lungs. To date, there are no published studies in vascular preparations from the newborn postnatal lung or investigations of Hsp90:eNOS interactions in an intact lung preparation at any developmental stage.

The current study was designed to test the hypothesis that Hsp90 binding to eNOS regulates vascular tone and NO production in the newborn pulmonary circulation. Studies were performed with cannulated piglet pulmonary resistance arteries (PRA), in situ perfused lungs, and with cultured pulmonary microvascular endothelial cells (PMVEC) to determine whether inhibition of ATP binding to Hsp90 influences pulmonary vascular responses by altering the balance of NO and O2•− generation from eNOS.

MATERIALS AND METHODS

Animals. Newborn piglets, 1–7 days of age, were killed with a lethal intraperitoneal injection (75–100 mg/kg) of pentobarbital sodium. Heart and lungs were removed en bloc and stored in cold, oxygenated physiological bicarbonate solution (PBS) before study (1–48 h). All experimental protocols were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals and approved by the Animal Care and Use Committee of Vanderbilt University Medical Center and Wake Forest University School of Medicine. These animal resource facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

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Isolation of PRA for measurement of pressurized diameter. Piglet PRA (80- to 300-μm diameter) were isolated, cannulated, and presurized for continuous measurement of diameter according to our previously published methods (2, 15–17, 23). Briefly, piglets were killed with an overdose of pentobarbital sodium (75–100 mg/kg ip), heart and lungs were removed en bloc, and PRA measuring <300 μm in diameter were dissected in oxygenated Krebs-Henseleit buffer with the following millimolar composition: 118 NaCl, 25 NaHCO3, 4.8 KCl, 1.2 MgSO4·7 H2O, 1.2 KH2PO4, 11 dextrose, and 2.0 CaCl2·2 H2O equilibrated with 5% O2, 5% CO2, balance N2 to maintain pH 7.4. The arteries were transferred to an arteriograph (Living Systems Instrumentation, Burlington, VT) where they were cannulated at one end, secured with a single strand of suture, gently flushed free of blood, and cannulated at the distal end. All side branches were tied with a single fiber of 10-0 braided nylon thread to achieve a leak-free preparation. The arteriograph was set on the stage of an inverted microscope (Nikon TMS) with a video camera (Sony XC 73) attached to the viewing tube. Intraluminal pressure was maintained at 15 mmHg by a pressure servo system connected to the proximal cannula. Prewarmed (37°C) buffer was circulated through the vessel chamber at a rate of 30 ml/min. The vessel image was projected on a television monitor, and the vessel diameter was continuously measured using a video dimension analysis system (Living Systems Instrumentation).

PRA study protocol. After a 30-min equilibration period with a stable diameter, vascular smooth muscle and endothelial function were assessed by contraction to 50 mM KCl or 10 mM U-46619 and dilation to the calcium ionophore A23187 (0.1 μM) or Ach (1 μM), respectively. Following another 30-min equilibration period, the effects of vehicle, GA (1 μM), RA (20 nM), or nitro-arginine (LNA) (0.1 mM) on baseline diameter were determined. Subsequently, PRA were constricted by cumulative addition of U-46619 (0.001–10.0 μM) or buffer to the reservoir through a bubble trap into the pulmonary arterial cannula, at a rate of 30 ml/min. The vessel image was projected on a television monitor, and the vessel diameter was continuously measured using a video dimension analysis system (Living Systems Instrumentation).

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cGMP enzyme immunoassay. cGMP concentrations were measured in dissected PRA using an enzyme immunoassay system and the recommended acetylation procedure (Cayman Chemical). Studies were performed in the presence of the cGMP phosphodiesterase inhibitor, IBMX. PRA homogenates were incubated with LNA, GA, RA, or buffer for 15 min before the addition of Ach (1 μM) or buffer control. Intracellular plus excreted cGMP was extracted by the addition of ice-cold 7.5% TCA in phosphate buffer. The TCA was removed by three extractions with water-saturated ether. The developed chromogen absorbance was measured at 405 nm using a VMAX 96-well plate spectrophotometer ( Molecular Devices). cGMP was calculated from a four-parameter fit, linear regression curve of standards (4–500 pg/ml). cGMP content was normalized for protein content of the sample, which was determined by the Pierce BCA method.

Com munoprecipitation. Dissected PRA (20–600 μm) were immediately placed in HEPES buffer on ice until quantities of PRA sufficient to perform the coimmunoprecipitation protocols were obtained. The dissected PRA were incubated in HEPES at 37°C for 20 min in the presence of either GA (1 μM), RA (20 nM), or vehicle (DMSO or EtOH) before the addition of Ach (10 μM) or HEPES buffer for an additional 1 min. The supernatants were discarded, and the vessels were snap frozen in liquid nitrogen and stored at −80°C until assayed. The vessels were homogenized on ice in lysis buffer (20 mM Tris·HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100 mM NaCl, 1.0 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). Protein concentrations of the cell lysates were determined using the Pierce BCA protein assay. To reduce nonspecific binding of proteins, 500 μg of detergent-soluble protein was precleared with pansorbin and then incubated for 6 h at 4°C with anti-Hsp90 monoclonal antibody (1 μg/ml total cell protein; Transduction Laboratories, San Diego, CA). Immune complexes were precipitated by overnight incubation at 4°C with protein G-Sepharose. The next morning, the beads were washed in lysis buffer and pelleted in a microcentrifuge to remove all unbound protein. The immunoprecipitated samples were heated at 80°C for 15 min in Laemmli loading buffer, and proteins resolved by SDS-PAGE on an 8% acrylamide gel. Proteins were electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked in 0.1% Tween PBS with 5% nonfat milk and then incubated with the primary antibodies (Hsp90 1:2,000, eNOS 1:500). Blots were probed with secondary antibody (1:2,500, goat anti-mouse IgG) labeled with horseradish peroxidase and visualized using a chemiluminescin substrate (ECL, Amersham Pharmacia Biotech, Little Chalfont, England).

Isolated, perfused lungs with measurement of perfusate NO metabolites and exhaled NO. Piglets were preanesthetized with a mixture of intramuscular ketamine (10 mg/kg) and acepromazine (2 mg/kg), anesthetized with pentobarbital (10 mg/kg iv), given heparin (1,000 IU/kg iv), and then exsanguinated. The piglet lungs were isolated and perfused in situ with Krebs-Ringer bicarbonate (KRB) solution containing 5% dextran, mol wt 70,000 (KRB) at 37°C, at a rate of 50 ml/min−1·kg−1 for measurement of pulmonary arterial pressure (Ppa) according to our previously published methods (12–14). Briefly, the piglet lungs were connected to a rotary pump-controlled perfusion circuit in which perfusate was continuously circulated from a reservoir through a bubble trap into the pulmonary arterial cannula, through the lungs to the left atrial cannula, and back to the reservoir. Pulmonary arterial, left atrial, and airway pressures were continuously monitored. The lungs were perfused for 0.5–1 h to establish stability of the Ppa followed by perfusion for an additional 60-min baseline period after which GA (1 μM), RA (20 nM), or vehicle (DMSO; 30–40 μl) was added to the reservoir. After an additional 20–30 min of perfusion, Ach (10 μg/min) was infused into the pulmonary arterial cannula for 30 min. Perfusate samples were collected every 10 min throughout perfusion for measurement of nitrite and nitrate (NOx−) using a chemiluminescence technique as described below. In addition, gas from the exhalation limb of the ventilator was continuously monitored throughout perfusion for determination of exhaled NO output as described below.

Exhaled NO output measurement. Throughout perfusion, the expiratory gas was sampled from the tracheal tube and passed through a chemiluminescence analyzer (model 280 NOA; Sievers, Boulder, CO) to measure NO concentration, as previously described (50). The NO analyzer was calibrated daily with authentic NO (45 ppm in N2; Matheson, Chicago, Ill.) The NO detection limit was 1 ppb.

Per fusate accumulation of NOx− in isolated lungs. A chemiluminescence analysis, described previously (50), was used to determine perfusate NOx− concentration (nmol/ml) at each collection time. Perfusate (20 μl) was injected into the reaction chamber of a chemiluminescence NO analyzer (model 170B NOA, Sievers). The reaction chamber contained vanadium(III) chloride in 1 M HCl heated to 90°C to reduce nitrite and nitrate to NO gas. The NO gas was carried into the analyzer using a constant flow of N2 gas via a gas bubble trap containing 1 M NaOH to remove HCl vapor. A standard curve was generated by adding known amounts of NaNO3 to distilled water and assaying as described for the perfusion samples. The perfusate NOx− concentration (nmol/ml) was calculated for each collection time by multiplying the perfusate concentration of NOx− at that sample collection time by the volume of the system (perfusion circuit + reservoir) at the sample collection time plus the amount of NOx− removed with all previous samples. The amount of...
Measurement of dihydroethidium oxidation by confocal microscopy. Dihydroethidium (DHE) oxidation was measured by confocal microscopy (Zeiss LSM 510) for the detection of superoxide production from cultured PMVEC. PMVEC were loaded with the fluorescent probe, DHE, which is taken up by cells, and in the presence of superoxide is converted to ethidium, which intercalates with nuclear DNA, giving a fluorescence signal that is proportional to the amount of superoxide present. Bovine PMVEC were obtained fromVEC Technologies (Albany, NY). Studies were performed with passage 9 or lower near-confluent cells grown on Nunc Lab Tek chambered coverglass slides (Nunc, Rochester, NY). On the day of study, cultures were washed and incubated in HEPES buffer in the presence or absence of GA (1 μM), RA (20 nM), LNA (100 μM), or vehicle for 30 min. The chambers were set on the stage of the Zeiss LSM 510 confocal microscope. Excitation wavelength was set at 488 nm and emissions filters at 565–615 nm. Autofluorescence was measured at time 0 and at 2 min, following which DHE (10 μM) was added to the cell cultures. Images were collected at 2-min intervals for the next 10 min for calculation of basal DHE oxidation, following which ACh (1 μM) or control buffer was added, and images were collected at 2-min intervals for an additional 10 min. The average fluorescence intensity at each time point was calculated from 10 cells per culture using the LSM Image Browser software.

Calculations and statistical analysis. Results are graphically expressed as means ± SE. For all analyses, n refers to the number of piglets studied. Between-group differences in mean percent change in baseline lumen diameter were examined by analysis of variance with a Scheffé adjustment for multiple comparisons. Dilation responses in PRA are expressed as percent reversal of the U-46619-induced constriction. Average differences in dilation between groups over the range of ACh concentrations were examined by repeated measures analysis of variance with a Scheffé adjustment for multiple comparisons. Analyses were performed using SAS version 9.2 (SAS, Carey, NC).

For the isolated perfused lung studies, a one-way ANOVA with post hoc multiple comparison test was used to compare changes in Ppa and exhaled NO between lungs treated with GA, RA, or vehicle. The NOx− accumulation (nmol) was the amount of NOx− at each collection time minus the amount of NOx− at time 0. The amount of NOx− at time 0 was determined from the y-intercept of a linear regression line fit to the amount of NOx− in the perfusate vs. time for the first 60 min of perfusion.

RESULTS

Figure 1 depicts the effects of the Hsp90 antagonists, GA and RA, and the nonselective NOS inhibitor, LNA, on baseline PRA diameter. GA and RA caused significant constriction, similar in magnitude to the constriction induced by addition of LNA. Vehicle control (methanol or DMSO) had no significant effect on vessel diameter.

Figure 2A depicts the effects of Hsp90 antagonism and NOS inhibition on the concentration-dependent dilation responses to the endothelial-dependent vasodilator, ACh. Cannulated PRA preconstricted with the thromboxane mimetic U-46619 demonstrated a concentration-dependent dilation to ACh that was significantly and similarly inhibited by pretreatment for 15 min with LNA, GA, and RA (Fig. 2A). Similar results were obtained when studies were performed in the presence or absence of the cyclooxygenase inhibitor, indomethacin. For example, in the presence of GA, maximal dilation to ACh in the presence and absence of indomethacin was 37.3% and 38.8%, respectively. Therefore, results from study protocols performed in the presence and absence of indomethacin were combined.

Figure 2B summarizes the effects of Hsp90 antagonism on the concentration-dependent responses to SNAP, an agent that does not require NOS activation to cause dilation. Pretreatment with the Hsp90 antagonist, GA, had no effect on dilation to SNAP.

Basal and ACh-stimulated cGMP concentrations in dissected PRAs are depicted in Fig. 3. Basal cGMP concentrations were not statistically different among the treatment groups. In control PRA, ACh stimulation caused a 2.4-fold increase in cGMP concentration. Pretreatment of PRAs with GA, RA, or LNA completely inhibited the ACh-stimulated increase in cGMP.

Figure 4 summarizes the results from coimmunoprecipitation assays in isolated PRA. Upon stimulation with ACh for 1 min, the amount of eNOS that coprecipitated with Hsp90 increased in vehicle-treated vessels (Fig. 4A). In contrast, when

![Fig. 1. Effects of heat shock protein 90 (Hsp90) and nitric oxide synthase (NOS) antagonists on basal tone in pulmonary resistance arteries (PRA) from newborn piglets. The y-axis is the percent constriction from baseline diameter. Geldanamycin (GA; 1.0 μM; n = 12) and radicicol (RA; 20 nM; n = 12) caused a significant constriction, similar in magnitude to the constriction caused by nitro-L-arginine (LNA; 100 μM; n = 10). Methanol (n = 5) or DMSO (n = 5), the vehicles used to dissolve GA and RA, respectively, had no effect on baseline tone (n = 10). *P < 0.05 different from baseline diameter and from vehicle control.](http://ajplung.physiology.org/) by 10.220.33.6 on June 19, 2017
significant effect on the SNAP-mediated dilation response. SNAP (n) control PRA, preconstricted to 30 – 60% of baseline diameter with the thromboxane mimetic U-46619, demonstrated a concentration (conc)-dependent dilation to ACh (n = 9). Pretreatment for 15 min with 1 μM GA (n = 8), 20 nM RA (n = 6), or 100 μM LNA (n = 7) significantly inhibited the ACh-mediated dilation response. *P < 0.05 different from vehicle control. B: effects of GA on S-nitroso-N-acetylpenicillamine (SNAP)-mediated dilation in PRA from newborn piglets. Vehicle-treated control PRA, preconstricted to 30–60% of baseline diameter with the thromboxane mimetic U-46619, demonstrated a concentration-dependent dilation to SNAP (n = 5). Pretreatment for 15 min with 1 μM GA (n = 5) had no significant effect on the SNAP-mediated dilation response.

vessels from the same lungs were treated with GA (Fig. 4B) or RA (Fig. 4C), ACh failed to increase Hsp90:eNOS coprecipitation. When basal (unstimulated) samples were run on the same gel, we observed that GA and RA not only failed to disrupt existing complexes between Hsp90 and eNOS but, in fact, increased Hsp90:eNOS coprecipitation (data not shown).

As Hsp90/client protein interactions have not been previously reported in the intact lung, we determined whether Hsp90 antagonism modulates Ppa in perfused lungs of piglets. Table 1 and Figs. 5 and 6 summarize these results. The baseline conditions of perfusion were similar for all three groups of lungs (Table 1). Baseline Ppa increased similarly after addition of GA or RA (Fig. 5A) but did not increase significantly after addition of vehicle. During ACh infusion, Ppa decreased more than twice as much in vehicle-treated lungs than in either GA-treated or RA-treated lungs (Fig. 5B).

Figure 6A shows the change in NOx− accumulation rate in all three groups during ACh infusion. The magnitude of increase in NOx− accumulation was significantly greater during ACh infusion in the vehicle-treated than in the GA-treated or RA-treated lungs. Notably, there was a similar increase in exhaled NO amounts during ACh infusion in all three groups of lungs (Fig. 6B).

Figures 7A and 8A summarize results of DHE oxidation by confocal microscopy in cultured PMVEC. Representative confocal images are shown in Figs. 7B and 8B. The vehicle time control (Fig. 7A) demonstrates that DHE oxidation gradually increased over time in the absence of exposure to an agonist, such as ACh. Nearly identical time control profiles were obtained in the presence of GA (results not shown) and RA (results in Figs. 8, A and B). Addition of ACh at time 12 min did not change the rate of increase in DHE oxidation and produced a curve that was not different from any of the time controls. In contrast, the rate of increase in DHE oxidation abruptly changed following addition of ACh in PMVEC pretreated with GA. Notably, the rate of increase in DHE oxidation was not affected by ACh in PMVEC pretreated for 15 min with LNA in the absence of addition of GA. The fluorescence profile of cells treated with LNA in the presence or absence of ACh was not different from the profile for the time control studies (data not shown).

Similar results to those with GA were obtained when the Hsp90 antagonist, RA, was used (Fig. 9, A and B). Compared with the RA time control (cells pretreated with RA in the absence of ACh), the addition of ACh to RA-pretreated PMVEC caused a significant increase in DHE fluorescence. To verify the specificity of the DHE oxidation signal for O2•−, we used the SOD mimetic, M40401. Figure 9B shows that pretreatment with M40401 (3 μg/ml) blocked the DHE oxidation signal and abolished both the time-dependent as well as the ACh-stimulated increase in DHE oxidation following ACh addition, demonstrating the ability of M40401 to rapidly dismutate O2•−.

To determine whether the vascular effects of Hsp90 antagonism were mediated by O2•− production, we examined the effect of M40401 on baseline luminal diameter of cannulated
PRA (Fig. 9A) and on ACh-stimulated dilation (Fig. 9B) in the presence and absence of GA. Figure 9A illustrates the change in baseline diameter when PRAs were treated with either vehicle, GA, native (cell-impermeable) superoxide dismutase (SOD), the cell-permeable SOD mimetic, M40401, or the combination of GA and M40401. PRA diameter was minimally altered by vehicle and native SOD. Baseline diameter decreased in response to GA and increased in response to M40401. Furthermore, in the presence of M40401, GA had no effect on PRA diameter. Figure 9B shows that the GA-mediated inhibition of ACh-induced dilation is restored in the presence of the SOD mimetic, M40401.

**DISCUSSION**

Important new findings in this study are that Hsp90 antagonists impair ACh-dependent vasodilation and reduce cGMP production in resistance level pulmonary arteries of newborn piglets. This is the first study to demonstrate the functional relevance of Hsp90:eNOS interactions in the pulmonary circulation in the immediate postnatal period. In addition, we are the first to show that the effects of Hsp90 antagonists in the intact lung are similar to those found in isolated vessels, further validating the physiological significance of the interaction between eNOS and its chaperone protein, Hsp90.
There is a growing awareness that posttranslational modification of eNOS by Hsp90 could be involved in modulating reactivity of various vascular beds, including the pulmonary circulation. A number of in vitro studies have provided evidence that coupling between Hsp90 and eNOS is important for eNOS activation and NO generation (7, 21, 24, 26, 33, 35, 38, 48). Yet, the functional impact of Hsp90:eNOS interactions on vascular reactivity has received limited attention. GA, an ansamycin antibiotic, has been a useful tool in prior investigations of Hsp90 function. GA binds to the ATP-binding site of Hsp90 and thereby alters the conformational state in which Hsp90 binds to its client proteins, which include eNOS (27, 37). For example, treatment with GA attenuated vasorelaxation to ACh in aortic rings (24) and the mesenteric circulation of adult rats (41). Likewise, pulmonary arteries from fetal lambs showed decreased dilation to ATP when pretreated with GA (30). In addition, flow-induced dilation was diminished in GA-treated rat middle cerebral arteries (53). Thus, in agreement with our findings, these other studies provide evidence that pharmacological inhibition of Hsp90 signaling blocks vascular responses mediated by agonist-induced and shear stress-induced NO production. To our knowledge, we are the first to strengthen the functional evidence by showing that RA, an agent structurally unrelated to GA that also inhibits Hsp90 signaling (37, 40), attenuates pulmonary vascular responses to a NO agonist in a similar fashion as does GA.

Hsp90 antagonists, such as GA and RA, have the potential to modify agonist-induced activity of any protein that interacts with Hsp90, which includes, but is not limited to eNOS (5, 22, 46, 49, 55). In this light, it is important to note that we assessed the influence of Hsp90 antagonists on parameters that reflect NO production including cGMP in isolated PRA and perfusate

### Table 1. Isolated perfused lung baseline conditions

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<th>Ppa (cmH2O)</th>
<th>NOx− (nmol/min)</th>
<th>exNO ppb</th>
<th>pH</th>
<th>PO2 (Torr)</th>
<th>PCO2 (Torr)</th>
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<tr>
<td>DMSO</td>
<td>24.5±1.5</td>
<td>0.9±0.4</td>
<td>5.2±1.3</td>
<td>7.43±0.01</td>
<td>142±2</td>
<td>49±5</td>
</tr>
<tr>
<td>Geldanamycin</td>
<td>22.7±1.6</td>
<td>1.5±0.6</td>
<td>5.5±0.8</td>
<td>7.40±0.01</td>
<td>143±1</td>
<td>47±2</td>
</tr>
<tr>
<td>Radicicol</td>
<td>21.9±2.0</td>
<td>1.7±0.4</td>
<td>6.7±1.5</td>
<td>7.41±0.01</td>
<td>146±5</td>
<td>49±1</td>
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Values are means ± SE. The baseline conditions of perfusion, including baseline Ppa, baseline NOx−, baseline exhaled NO (exNO), pH, PO2, and PCO2, were similar for all 3 groups of lungs. Ppa, pulmonary arterial pressure; NOx−, nitric oxide metabolites.

Fig. 5. Effects of Hsp90 antagonism on baseline and ACh-mediated changes in pulmonary artery pressure (Ppa) in newborn piglet perfused lungs. A: GA (n = 12) and RA (n = 7) infusion induced a similar increase in baseline Ppa; infusion of vehicle (DMSO; n = 11) had no significant effect on baseline Ppa. *P < 0.05 different from vehicle-treated lungs. B: ACh infusion caused a significantly greater decrease in Ppa in vehicle-treated lungs (n = 11) than in either GA-treated (n = 12) or RA-treated lungs (n = 7). *P < 0.05 different from vehicle-treated lungs.

Fig. 6. A: effects of Hsp90 antagonism on the percent change in NO metabolite (NOx−) accumulation rate during ACh infusion in newborn piglet perfused lungs. ACh infusion induced a greater percent change in NOx− accumulation rate in vehicle-treated than in the GA-treated or RA-treated lungs. *P < 0.05 different from NOx− accumulation rate in vehicle-treated lungs. B: effects of Hsp90 antagonism on the percent change in exhaled NO during ACh infusion in newborn piglet perfused lungs. Exhaled NO amounts increased similarly during ACh infusion in vehicle-treated, GA-treated, and RA-treated lungs.
accumulation of the stable metabolites of NO in whole lung. Our findings that both GA and RA reduce ACh-induced NO production by pulmonary arteries and the intact lung strongly support the notion that these pharmacological Hsp90 antagonists altered pulmonary vascular responses by inhibiting NOS activity. Furthermore, we provide evidence that the physical association of Hsp90 with eNOS in intact resistance level pulmonary arteries is modified by two structurally distinct Hsp90 antagonists, in that the ACh-induced increase in coimmunoprecipitation of eNOS with Hsp90 was inhibited by both GA and RA. Together, our findings show that Hsp90:eNOS interactions contribute to regulation of pulmonary vascular reactivity in the newborn piglet in the first week of life, as has been suggested for other vascular beds in other species and developmental stages (24, 30, 41, 53).

In addition to modulating agonist-induced reactivity, Hsp90:eNOS interactions could influence baseline vascular tone. We know of only one study, performed with adult canine cerebral...
arteries, that shows the functional importance of Hsp90:eNOS interactions in modulating basal tone in any vascular bed (28). Our findings show that pharmacological Hsp90 antagonism with both GA and RA influenced baseline diameter of neonatal PRA in a fashion similar to that of NOS inhibition. Using GA and RA as tools to inhibit Hsp90 chaperone function, these data suggest that an interaction between Hsp90 and a client protein, presumably eNOS, regulates resting tone in newborn PRA.

Of interest, unlike the study with cerebral arteries (28), we did not find that either GA or RA reduced basal cGMP levels in pulmonary arteries. Nor did GA or RA disrupt preexisting complexes between Hsp90 and eNOS. In fact, we found that Hsp90 binding to eNOS is augmented by GA or RA under basal conditions. This observation has also been reported by others (33, 35). In light of the failure of GA or RA to reduce basal amounts of cGMP, the “uncoupling” of NOS by the Hsp90 antagonists is a likely alternative explanation for the observed change in baseline tone in PRA exposed to GA or RA. As suggested others, the capacity of Hsp90 to change its conformation (by binding ATP) is fundamental to the mechanisms by which Hsp90 enhances NO production and limits

Fig. 8. A: in the RA time control (addition of HEPES buffer at 12 min to PMVEC pretreated with 20 nM RA), there was a gradual, steady increase in DHE oxidation over time (n = 2 different PMVEC cultures). Addition of ACh at time 12 min caused an abrupt increase in DHE oxidation in PMVEC pretreated with RA (20 nM, n = 2 different cultures; ○). Pretreatment with the superoxide dismutase (SOD) mimetic, M40401, quenched the fluorescent signal and abolished the increase in DHE oxidation that occurred gradually over time as well as the abrupt increase in DHE oxidation following ACh stimulation in PMVEC treated with GA (n = 2 PMVEC cultures in both M40401 groups). *P < 0.05 different from both M40401 groups and from each other. B: representative confocal images captured at time 2 min, 12 min, and 22 min, corresponding to the treatment groups shown in A.
eNOS-dependent $O_2^{•−}$ production (33, 35). In the presence of GA or RA, ongoing basal NOS activity would thereby result in an altered balance of NO and $O_2^{•−}$, with $O_2^{•−}$ predominating, since the stoichiometry for arginine-coupled NOS activity indicates that two molecules of $O_2^{•−}$can be generated by uncoupled activity compared with one molecule of NO for coupled activity (35). Increased $O_2^{•−}$ generation from uncoupled NOS is the most likely explanation for the seemingly contradictory findings of enhanced basal Hsp90/eNOS association, no net change in basal cGMP levels, but vasoconstriction from baseline tone in the presence of GA and RA. It is also important to be mindful that different conditions were used for the functional studies in cannulated arteries and the biochemical assays for cGMP. The functional studies were performed with pressurized vessels, whereas the biochemical assays were not. The presence of a transmural wall pressure may have activated NOS, such that in the presence of GA or RA, $O_2^{•−}$ generation was exaggerated leading to constriction.

Our studies with cultured endothelial cells and intact pulmonary arteries support the foregoing possibility. Our findings suggest that pulmonary endothelial cell $O_2^{•−}$ production increases when eNOS is activated in the presence of the antagonists GA or RA. Furthermore, the functional impact from this paradigm is illustrated by our cannulated artery studies showing that Hsp90 inhibition failed to impair dilator responses to the NO agonist, ACh, when $O_2^{•−}$ was dismutated to $H_2O_2$ by the SOD mimetic, M40401, a small-molecular-weight, man-ganese-containing compound that is cell permeable, stable in vivo, and selective for $O_2^{•−}$ (10). It is of interest that, consistent with our findings, the $O_2^{•−}$ scavenger, Tiron, improved dilator responses to the NO agonist, ATP, in fetal lamb pulmonary arteries (30). The ability of M40401 to restore dilator responses in our studies could be due to the removal of both intracellular and extracellular $O_2^{•−}$ (10). Regardless, findings from both of these studies indicate that in the perinatal pulmonary circulation, interference with the ATP conformational state of Hsp90 causes $O_2^{•−}$-dependent impairments of vasodilator responses to agonists known to activate eNOS.

It is important to keep in mind that the role of NOS in regulating either baseline vasomotor tone or reactivity has been shown to differ between species, vascular beds, and with postnatal age (31, 32, 34, 42, 51). Thus, it is possible that Hsp90:eNOS might not influence both baseline tone and vascular reactivity in all vascular beds and/or at all developmental stages. For example, although GA increased cerebral vascular baseline tone, it failed to alter responses to constrictors or dilators (28). Thus, further investigation is merited to more clearly delineate which vascular beds, at which developmental stages, and under which physiologically relevant conditions NOS activity is modulated by Hsp90. This information is important because of potential therapeutic implications. Specifically, in conditions shown to be associated with impaired NO signaling, new therapies designed to improve NO function by augmenting Hsp90 function and/or reducing $O_2^{•−}$ production may prove efficacious.

Our findings in isolated perfused lungs merit additional comment. In particular, it is notable that GA and RA inhibited ACh-induced increases in lung perfusate NO$^{-}$ accumulation but had no effect on ACh-induced increases in exhaled NO production. The exact anatomic sites that contribute to exhaled NO production are unclear. Both we (50) and other investigators (43, 44) have previously shown that NOS is expressed in airway epithelial cells of newborn animals, so it is logical that exhaled NO reflects, at least in part, airway NO production. However, whether or not exhaled NO reflects pulmonary vascular endothelial cell NO production is not yet certain (4, 8, 39). One possible explanation for our findings is that in our model perfusate, NO$^{-}$ reflects vascular NO production, whereas exhaled NO reflects airway NO production. In studies performed in the isolated perfused lung, the Hsp90 antagonist was infused directly into the pulmonary circulation where it would be in direct contact with the endothelium and primarily target vascular eNOS. We would not expect NO produced in the airway to be affected by this mode of administration. Regardless, our findings strongly support the notion that pharmacological Hsp90 antagonists alter pulmonary vascular pressure in the intact lung by inhibiting pulmonary vascular NOS activity.

Some of the limitations of our study merit comment. For example, we used GA as a pharmacological antagonist of Hsp90 function. GA has been shown to cause oxidant production by redox cycling independent from its action on Hsp90 (6,
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11). However, RA causes no such autooxidation (6, 33). Thus our use of more than one pharmacological Hsp90 antagonist strengthens the probability that our results are due to Hsp90-mediated effects. Another limitation of this study is the use of commercially available adult bovine pulmonary microvascular endothelial cells in the DHE oxidation studies. Further studies are needed to confirm that the Hsp90 antagonists stimulate O$_2^••$ production in newborn resistance level pulmonary arteries and endothelial cells. Another consideration is that eNOS function is regulated by several factors other than its association with Hsp90 (20, 25). In fact, Hsp90 may act as a scaffold for the recruitment of other regulatory molecules, including kinases, phosphatases, and guanylate cyclase (5, 7, 21, 22, 52, 54, 55) that may influence eNOS function. Further studies are needed to evaluate the physiological importance in the postnatal pulmonary circulation from these other modulators of eNOS regulation, some of which may involve additional influences from Hsp90 signaling.

In summary, our study shows that coupling between Hsp90 and eNOS influences normal eNOS coupling may result in elevated O$_2^••$ production. We speculate that manipulating the Hsp90:eNOS relationship may have important physiological consequences, including the development of novel therapies for neonates suffering from conditions associated with impaired pulmonary vascular NO function.

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