Nitric oxide-endothelin-1 interactions after surgically induced acute increases in pulmonary blood flow in intact lambs


Nitrergic changes in pulmonary blood flow (PBF) accompany the surgical repair of a number of congenital heart defects, including tetralogy of Fallot, pulmonary and tricuspid atresia, and other single ventricular heart defects. Dynamic changes in pulmonary vascular resistance (PVR) after surgery can be a significant source of morbidity and mortality, because the balance between systemic vascular resistance (SVR) and PVR is critical in maintaining adequate cardiac output and systemic arterial oxygen saturation in these patients (5, 30). Indeed, postoperative therapeutic interventions are often aimed at altering PVR to achieve an appropriate circulatory balance (4, 45). However, despite this clinical focus, little is known about the mechanisms that regulate pulmonary vascular tone after surgically induced alterations in PBF.

Mounting data demonstrate the importance of vasoactive factors, such as nitric oxide (NO) and endothelin-1 (ET-1), in the regulation of vascular tone in both health and disease (17). NO is produced in the vascular endothelium by the enzyme endothelial NO synthase (eNOS) from the precursor l-arginine (32). Once formed, NO diffuses into the adjacent smooth muscle cell, where it activates soluble guanylate cyclase, catalyzing the conversion of GTP to cGMP (25). Increased cGMP levels ultimately result in smooth muscle cell relaxation and vasodilatation. ET-1 is likewise produced in the vascular endothelium by the endothelin-converting enzyme (ECE-1) from the precursors preproET and proendothelin-1 (Big-ET) (51). ET-1-induced pulmonary vasoactive responses, which include both vasoconstriction and/or vasodilation, are mediated by at least two receptor populations: the ETA and ETB receptors. The ETA receptor is located on smooth muscle cells and mediates vasoconstriction. There are two ETb receptor subpopulations, one on endothelial cells that mediate vasodilatation via NO production and another, less well characterized, population on smooth muscle cells, that likely mediate vasoconstriction (3, 27, 39, 43). Alterations in NO and ET-1 signaling are known to contribute to the pathophysiology of a wide array of pulmonary hypertensive disorders, including congenital heart disease with increased PBF (2, 9, 10, 19, 21, 22, 46).

Alterations in NO and ET-1 during acute increases in PBF have been examined in the fetal lamb after mechanical constriction of the ductus arteriosus (1, 31). In this model, acute constriction of the ductus arteriosus is associated with an initial sharp increase in PBF, which is subsequently reversed by profound pulmonary vasoconstriction (1, 31). Recent studies indicate that this increase in pulmonary vascular tone is associated with increased ET-1 levels and ET-receptor-mediated decreases in NO signaling (31). However, the role of NO and ET-1 in regulating vascular tone after acute changes in PBF in the postnatal pulmonary circulation has not been determined. Therefore, the purpose of this study was to examine the role of NO, ET-1, and their potential interactions in regulating pulmonary vascular tone after surgically induced increases in PBF in the intact juvenile lamb. We hypothesized that acute increases in PBF would result in increased ET-1 signaling and in pulmonary circulation; congenital heart disease

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Am J Physiol Heart Circ Physiol 290: H1922–H1932, 2006. First published December 9, 2005; doi:10.1152/ajpheart.01091.2005.—Several congenital heart defects require surgery that acutely increases pulmonary blood flow (PBF). This can lead to dynamic alterations in postoperative pulmonary vascular resistance (PVR) and can contribute to morbidity and mortality. Thus the objective of this study was to determine the role of nitric oxide (NO) and endothelin (ET)-1, and their interactions in the alterations of PVR after surgically induced increases in PBF. Twenty lambs underwent placement of an aortopulmonary vascular graft. Lambs were instrumented to measure vascular pressures and PBF and studied for 4 h. Before and after shunt opening, lambs received an infusion of saline without further changes thereafter. Plasma ET-1 levels increased 17.6% (P < 0.05), and total NOS activity decreased 61.1% (P < 0.05). These changes were associated with an increase in total NOS activity (+61.4%; P < 0.05) at 4 h. NO synthase inhibition (L-NNA) after shunt placement prevented the plateau of decreased PVR and decreased PBF by 117.8% and decreased PVR by 40.7% (n = 6), or Nω-nitro-l-arginine (l-NNA), a NO synthase (NOS) inhibitor (n = 5). In control lambs, shunt opening increased PBF by 117.8% and decreased PVR by 40.7% (P < 0.05) by 15 min, with no further changes thereafter. Plasma ET-1 levels increased 17.6% (P < 0.05), and total NOS activity decreased 61.1% (P < 0.05) at 4 h. ET-receptor blockade (tezosentan) prevented the plateau of decreased PBF and PVR, such that PBF was increased and PVR was decreased compared with controls at 3 and 4 h (P < 0.05). These changes were associated with an increase in total NOS activity (+61.4%; P < 0.05) at 4 h. NOS inhibition (l-NNA) after shunt placement prevented the sustained decrease in PVR seen in control lambs. In these lambs, PVR decreased by 15 min (P < 0.05) but returned to baseline by 2 h. Together, these data suggest that surgically induced increases in PBF are limited by vasoconstriction, at least in part by an ET-receptor-mediated increase in PVR. Thus NO appears to be important in maintaining a reduction in PVR after acutely increased PBF.
ET-receptor-mediated decreases in NO signaling, with a net result of increasing PVR over time.

To address this hypothesis, we established a model of increased PBF by placing an 8-mm aortopulmonary vascular graft in intact juvenile (4–6 wk old) lambs. Lambs were instrumented to measure hemodynamic variables and PBF and studied over a 4-h period. To determine potential alterations in NO and ET-1, we determined lung tissue NOS activity (both calcium dependent and independent), plasma ET-1 levels, ECE-1 activity, and protein levels of eNOS, inducible NOS (iNOS), neuronal NOS (nNOS), preproET-1, ECE-1, and ETA and ETB receptors before and 4 h after shunt placement. To elucidate potential differential roles of ET-1 and NO, these parameters were compared with two additional groups of lambs: one group treated with tezosentan, a combined ETA- and ETB-receptor antagonist, and a second group treated with Nω-nitro-L-arginine (L-NNA), a NOS inhibitor, both before and after aortopulmonary shunt placement. Finally, to elucidate potential changes related to the study preparation, an additional group of lambs was studied after a sham surgical procedure.

We found that surgically induced acute increases in PBF are accompanied by increases in plasma ET-1 levels, independent of changes in ECE-1 activity and protein levels, and by ET-receptor-mediated decreases in eNOS activity. These alterations correlate with increases in PVR and act to limit increases in PBF over time.

MATERIALS AND METHODS

Twenty-four juvenile lambs (4–6 wk of age) were fasted for 24 h, with free access to water. The lambs were then anesthetized with ketamine hydrochloride (15 mg/kg im). While the animals were under additional local anesthesia with 2% lidocaine hydrochloride, polyurethane catheters were placed in an artery and vein of each hind leg. These catheters were advanced to the descending aorta and the inferior vena cava, respectively. The lambs were then anesthetized with ketamine hydrochloride (∼0.3 mg·kg⁻¹·min⁻¹), diazepam (0.002 mg·kg⁻¹·min⁻¹), and fentanyl citrate (1.0 μg·kg⁻¹·h⁻¹), intubated with a 7.0-mm outer diameter cuffed endotracheal tube, and mechanically ventilated with a VIP Bird Sterling (Palm Springs, CA) time-cycled, pressure-limited ventilator. Pancuronium bromide (0.1 mg·kg⁻¹·dose⁻¹) was given intermittently for muscle relaxation. With the use of strict aseptic technique, a midsternotomy incision was then performed, and the pericardium was incised. The use of side-biting vascular clamps, an 8.0-mm Gore-Tex vascular graft then performed, and the pericardium was incised. With the use of strict aseptic technique, a midsternotomy incision was then temporarily closed with towel clamps. An intravenous flow probe (Transonic Systems, Ithaca, NY), using a continuous suture technique. With the use of a purse-string suture technique, polyurethane catheters were placed directly into the right and left atrium and main pulmonary artery. An ultrasonic flow probe (Transonsics Systems, Ithaca, NY) was placed around the left pulmonary artery to measure PBF. The midsternotomy incision was then temporarily closed with towel clamps. An intravenous infusion of lactated Ringer solution and 5% dextrose (75 ml/h) was begun and continued throughout the study period. Cefazolin (500 mg iv) and gentamicin (3 mg/kg iv) were administered before the first surgical incision. The lambs were maintained normothermic (39°C) with a heating blanket.

Experimental protocol. After a 30-min recovery period, baseline measurements of the hemodynamic variables (pulmonary and systemic arterial pressure, heart rate, left PBF, and left and right atrial pressures) and systemic arterial blood gases and pH were measured. Blood was collected from the femoral artery for plasma ET-1, and a peripheral lung wedge biopsy was obtained for NOS activity and eNOS, iNOS, nNOS, preproET-1, ECE-1, ETA- and ETB-receptor protein determinations. A side-biting vascular clamp was utilized to isolate peripheral lung tissue from a randomly selected lobe, and the incision was cauterized. Approximately 300 mg of peripheral lung were obtained for each biopsy.

In control (group 1, n = 9) and sham animals (group 2, n = 4), an infusion of normal saline (vehicle) was initiated and continued throughout the study period. In group 3 (n = 6), an infusion of tezosentan (a combined ETA- and ETB-receptor antagonist) was initiated at 0.5 mg·kg⁻¹·h⁻¹ and continued throughout the study period. The dose of tezosentan was determined from previous work that demonstrates physiologically significant blockade of ET-1-induced vascular alterations (18). In group 4 (n = 5), an infusion of L-NNA was initiated at 6 mg·kg⁻¹·h⁻¹ and continued throughout the study period. The dose of L-NNA was determined from previous studies that demonstrate complete blockade of acetycholine-induced vasodilatation (16).

One hour after the initiation of the infusion (vehicle, tezosentan, or L-NNA), the vascular clips were removed from the aortopulmonary graft during the shunt, in groups 1, 3, and 4. In the sham group (group 2), the procedure was identical, except that the graft was not opened. Hemodynamic variables were monitored continuously for 4 h. At 4 h after shunt opening, blood was again taken for plasma ET-1 determinations, and a lung biopsy was obtained for NOS activity and eNOS, iNOS, nNOS, preproET-1, ECE-1, and ETA- and ETB-receptor protein determinations.

At the end of the protocol, all lambs were killed with a lethal injection of pentobarbital sodium, followed by bilateral thoracotomy as described in Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). All protocols and procedures were approved by the Committee on Animal Research of the University of California, San Francisco.

Drug preparation. Tezosentan (molecular weight 649.6, Actelion Pharmaceuticals, Allschwil, Switzerland) was diluted in sterile 0.9% saline to a final concentration of 1 mg/ml and used immediately. L-NNA (Sigma Chemical, St Louis, MO) was diluted in sterile 0.9% saline to a final concentration of 2 mg/ml and used immediately.

Measurements. Pulmonary and systemic arterial pressures and right and left atrial pressures were measured using Sorensen neonatal transducers (Abbott Critical Care Systems, North Chicago, IL). Mean pressures were obtained by electrical integration. Heart rate was measured by a cardiotachometer triggered from the phasic systemic arterial pressure pulse wave. Left PBF was measured on an ultrasonic flowmeter (Transonic Systems). All hemodynamic variables were measured continuously with a Gould Ponomah Physiology Platform (version 4.2) and Acquisition Interface (model ACG-16; Gould, Cleveland, OH) and recorded with a Dell Inspiron 5160 computer (Dell, Round Rock, TX). Blood gases and pH were measured on a Radiometer ABL5 pH/blood gas analyzer (Radiometer, Copenhagen, Denmark). Hemoglobin concentration and oxygen saturation were measured by a co-oximeter (model 682, Instrumentation Laboratory, Lexington, MA). PVR was calculated using standard formulas. Body temperature was monitored continuously with a rectal temperature probe.

Assay for NOS activity. NOS activity was determined by using the conversion of L-[³H]arginine to L-[³H]citrulline as described by Bush et al. (12). Briefly, peripheral lung tissues were homogenized in NOS assay buffer [50 mM Tris·HCl (pH 7.5) containing 0.1 mM EDTA and 0.1 mM EGTA] with a protease inhibitor cocktail. Enzyme reactions were carried out at 37°C in the presence of total lung protein extracts (500 μg), 1 mM NADPH, 14 μM tetrahydro-biopterin, 100 μM FAD, 1 mM MgCl₂, 5 μM unlabeled L-arginine, 15 nM L-[³H]arginine, calmodulin (25 units), and 5 mM calcium to produce conditions that drive the reaction at maximal velocity. Duplicate assays were run in the presence of the NOS inhibitor L-NNA to detect nonspecific production of [³H]citrulline. This value was then sub-

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tracted to obtain the final activity value. Assays were incubated for 60 min at 37°C such that no more than 20% of the \(^{[3]H}\)arginine was metabolized to ensure that the substrate was not limiting. The reactions were stopped by the addition of ice-cold stop buffer (20 mM sodium acetate (pH 5), 1 mM L-citrulline, 2 mM EDTA, and 0.2 mM EGTA) then applied to columns containing 1 ml of Dowex AG50W-X8 resin, Na\(^+\) form, preequilibrated with 1 N NaOH. L-\(^{[3]H}\)citrulline was then quantitated by scintillation counting. All activities were normalized to the amount of protein in each lysate. To determine the potential contribution of iNOS to total NOS activity, assays were repeated without calcium supplementation.

**Plasma ET-1 determinations.** Three milliliters of systemic arterial blood were collected and placed in iced polypropylene tubes containing 330 \(\mu\)l aprotinin and 100 \(\mu\)l EDTA. The tubes were immediately centrifuged at 4,000 \(\times\) g for 20 min. Collected plasma was treated with equal volumes of 0.1% trifluoroacetic acid and stored at \(-70^\circ\)C. The acidified supernatant was centrifuged at 1,000 \(\times\) g for 20 min and loaded on a 3 \(\times\) 18 C18 Sep-Pak column (Peninsula Laboratories, Belmont, CA) equilibrated with 0.1% trifluoroacetic acid. The adsorbed material was eluted with 3 ml of 0.1% trifluoroacetic acid-60% acetonitrile. The eluant was dried in a Savant speed vac and stored at \(-70^\circ\)C or assayed immediately for immunoreactive ET-1. ET-1 standard, \(^{125}\)I-labeled ET-1, anti-ET antibody, and secondary antibody were purchased from Peninsula Laboratories. Cross-reactivity for measured human and bovine ET-1 antiserum is 100% for human ET-1, 7% for bovine ET-1, and 0.03% for bovine ET-2 and ET-3. Inter- and intra-assay variabilities were 10 and 4%, respectively. Each sample was assayed in duplicate. This assay was modified from a previously published method (50).

**Assay for ECE activity.** Tissues (snap frozen and stored at \(-80^\circ\)C) were minced in 5x of extraction buffer [25 mM sodium phosphate buffer (pH 6.8) and 0.1% Triton X-100] and then sonicated for 10 s on ice at 35% amplitude using a High Intensity Ultrasonic Processor (Autotune series A; Daigger, Vernon Hills, IL). The samples were centrifuged at 20,000 \(\times\) g for 10 min at 4°C, and the supernatants were collected, and the protein concentration was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). The samples were then applied to columns containing 1 ml of Dowex AG50W-X8 resin, Na\(^+\) form, preequilibrated with 1 N NaOH. The eluant was dried in a Savant speed vac and stored at \(-70^\circ\)C or assayed immediately for immunoreactive ET-1. ET-1 standard, \(^{125}\)I-labeled ET-1, anti-ET antibody, and secondary antibody were purchased from Peninsula Laboratories. Cross-reactivity for measured human and bovine ET-1 antiserum is 100% for human ET-1, 7% for human ET-2 and ET-3, and 0% for bovine ET-2 and ET-3. Inter- and intra-assay variabilities were 10 and 4%, respectively. Each sample was assayed in duplicate. This assay was modified from a previously published method (50).

**Preparation of protein extracts and Western blot analysis.** Lung protein extracts were prepared by homogenizing peripheral lung tissues in Triton lysis buffer [50 mM Tris-HCl (pH 7.6), 0.5% Triton X-100, and 20% glycerol] containing a protease inhibitor cocktail. Extracts were then clarified by centrifugation (15,000 \(\times\) g for 10 min at 4°C). Supernatant fractions were then assayed for protein concentration using the Bradford reagent (Bio-Rad, Richmond, CA) and blocked in PBS containing 0.5% Tween. The samples were then incubated at room temperature with a monoclonal antibody raised to \(\beta\)-actin (1:10,000 dilution; Sigma). After the membranes were washed, the protein bands were visualized with chemiluminescence using a Kodak Digital Science Image Station (NEN) and analyzed using the KED-1 software. All captured and analyzed images were determined to be in the dynamic range of the system.

The eNOS, nNOS, and iNOS antiserum was obtained from Transduction Laboratories (Lexington, KY). The ETA-receptor antiserum was generated as previously described (7). The ETA-receptor antiserum was obtained from Affinity Bioreagents (Golden, CO). The specificity of the preproET-1 antibody was verified with a preincubation step with purified ET-1 (50 ng ET-1/15 \(\mu\)l of antiserum) protein. The purified ET-1 was purchased from Sigma. ECE-1 \(\alpha\) antiserum were generated as previously described (29).

Positive controls were run to demonstrate antibody specificity. The methodology and exposure times used were those that we previously demonstrated to be within the linear range of the autoradiographic film and able to detect changes in lung protein expression (7, 9). In addition, to ensure that equal protein was loaded in each lane for the experiments in which preproET-1, ECE-1\(\alpha\), ETA, and ETB receptor expression was investigated, all blots were stripped and reprobed for \(\beta\)-actin. Blots were stripped in 100 mM \(\beta\)-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.8) for 30 min at 50°C. The membranes were blocked again by using 5% nonfat dry milk in TBS containing 0.1% Tween and then incubated at room temperature with a monoclonal antibody to \(\beta\)-actin (1:10,000 dilution; Sigma). After being washed with TBS containing 0.1% Tween, the blots were incubated with a goat anti-mouse IgG-horseradish peroxidase conjugate and the protein bands were visualized as described above.

All data from the Western blot analysis are reported as the ratio of densitometric value of the protein of interest divided by the densitometric metric value of the \(\beta\)-actin band to generate a relative expression for each protein.

**Immunohistochemistry for iNOS and nNOS.** Snap frozen lung tissue samples stored at \(-80^\circ\)C were included in Tissue-Tek OCT Compound (Sakura Finetek USA, Torrance, CA), cryosectioned at 5 \(\mu\)m, collected onto Superfrost Plus slides (VWR Scientific, West Chester, PA), allowed to air-dry at room temperature, and stored at \(-80^\circ\)C until needed. For staining, slides were removed from \(-80^\circ\)C and blocked in PBS-T; goat anti-mouse Alexafluor-546 in PBS-T/NGS (1:1,000, 1:500 for preproET-1, ECE-1\(\alpha\), ETA, and ETB receptor expression was investigated, all blots were stripped and reprobed for \(\beta\)-actin). Blots were stripped in 100 mM \(\beta\)-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.8) for 30 min at 50°C. The membranes were blocked again by using 5% nonfat dry milk in TBS containing 0.1% Tween and then incubated at room temperature with a monoclonal antibody to \(\beta\)-actin (1:10,000 dilution, Sigma). After being washed with TBS containing 0.1% Tween, the blots were incubated with a goat anti-mouse IgG-horseradish peroxidase conjugate and the protein bands were visualized as described above.

Statistical analysis. The means \pm SD and SE were calculated for the baseline hemodynamic variables, systemic arterial blood gases and pH, and ET-1 concentrations. The general hemodynamic variables and systemic arterial blood gases and pH were compared within each group over time by ANOVA for repeated measures. Student-New-
Hemodynamic changes after aortopulmonary shunt opening in vehicle-treated (control) lambs before and 4 h after shunt opening, were compared by the paired t-test. Plasma ET-1 levels, Values are means ± SE; n = 9. *P < 0.05 vs. baseline.

RESULTS

There were no differences in gestational age, weight (mean 14.5 ± 3.3 kg), sex distribution, or baseline hemodynamic variables between control, sham, tezosentan-treated, or L-NNA-treated lambs (data not shown).

In control lambs, opening of the aortopulmonary vascular graft resulted in a rapid increase in PBF (from 37.7 ± 11.7 to 82.1 ± 21.83 ml·min⁻¹·kg⁻¹ at 15 min; P < 0.05) and decrease in left PVR (LPVR; from 0.27 ± 0.12 to 0.16 ± 0.06 mmHg·ml⁻¹·min⁻¹·kg at 15 min; P < 0.05), which did not change further over the subsequent 4-h study period (Fig. 1). Pulmonary artery pressure increased, and mean and diastolic systemic arterial pressure decreased at 15 min after shunt opening and did not change further over the 4-h study period (P < 0.05; Table 1). Left atrial pressure increased over baseline at 1 h after shunt opening (P < 0.05; Table 1). Opening of the shunt was not associated with changes in systemic arterial pressure, heart rate, or right atrial pressure (Table 1). Systemic arterial pH (from 7.43 ± 0.06 to 7.39 ± 0.04), PCO₂ (from 33.0 ± 7.0 to 39.1 ± 6.7 Torr), and PaO₂ (from 87.1 ± 18.7 to 80.1 ± 17.1 Torr) did not change.

Four hours after shunt opening, plasma ET-1 levels increased (from 9.33 ± 1.0 to 11.00 ± 2.1 pg/ml; P < 0.05). This increase was independent of changes in ECE-1 activity (0.165 ± 0.04 vs. 0.160 ± 0.01 pmol·min⁻¹·µg⁻¹) and protein levels (relative to β-actin) of ECE (0.167 ± 0.063 vs. 0.192 ± 0.042), PreproET (0.203 ± 0.125 vs. 0.133 ± 0.062), and ETA (0.203 ± 0.120 vs. 0.266 ± 0.147) and ETB (0.099 ± 0.052 vs. 0.1 ± 0.056) receptors (data expressed as mean densitometric values ± SD for baseline vs. 4 h after shunt opening). Furthermore, total NOS activity decreased by 61.1% compared with baseline at 4 h after shunt opening (P < 0.05; Fig. 2A). Before shunt opening, calcium-independent NOS activity consisted of 18.9% of total NOS activity and decreased by 36.8% at 4 h after shunt opening (P < 0.05). The levels of eNOS protein were unchanged over the 4-h study period (Fig. 2, B and C). Detected iNOS protein levels were minimal, and nNOS protein levels were not detectable (Fig. 2, C and D). Because Western blot analysis did not detect significant iNOS and nNOS protein levels, we performed immunohistochemistry. As seen in Fig. 3, low levels of both iNOS and nNOS were detected in the lamb lungs, without significant changes noted after 4 h of shunt opening.

Table 1. Hemodynamic changes after aortopulmonary shunt opening in vehicle-treated (control) lambs

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>15</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
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<tr>
<td>MPAP, mmHg</td>
<td>15.8 ± 5.0</td>
<td>20.2 ± 3.2*</td>
<td>21.3 ± 3.1*</td>
<td>21.6 ± 3.4*</td>
<td>22.8 ± 3.3*</td>
<td>21.6 ± 2.2*</td>
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<td>LA, mmHg</td>
<td>8.3 ± 3.8</td>
<td>8.2 ± 2.9</td>
<td>9.7 ± 3.0*</td>
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<td>5.5 ± 1.3*</td>
<td>5.0 ± 1.8</td>
<td>4.8 ± 1.7</td>
</tr>
<tr>
<td>Systolic SAP, mmHg</td>
<td>92.69 ± 18.3</td>
<td>99.49 ± 26.1</td>
<td>93.41 ± 22.4</td>
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<tr>
<td>MSAP, mmHg</td>
<td>69.3 ± 18.7</td>
<td>66.4 ± 13.2</td>
<td>64 ± 11.9*</td>
<td>58.1 ± 9.1*</td>
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</tr>
<tr>
<td>Diastolic SAP, mmHg</td>
<td>58.12 ± 16.1</td>
<td>45.41 ± 14.5*</td>
<td>39.06 ± 8.1*</td>
<td>34.51 ± 7.8*</td>
<td>34.69 ± 10.3*</td>
<td>34.36 ± 16.9*</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>146 ± 25.9</td>
<td>149 ± 22.2</td>
<td>146 ± 27.7</td>
<td>146 ± 26.0</td>
<td>145 ± 23.3</td>
<td>141 ± 18.0</td>
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</table>

Values are means ± SD; n = 9 lambs. MPAP, mean pulmonary arterial pressure; LA, left atrial pressure; RA, right atrial pressure; SAP, systemic arterial blood pressure; MSAP, mean SAP; HR, heart rate. *P < 0.05 vs. baseline (ANOVA).
The infusion of tezosentan resulted in a decrease in diastolic and mean systemic arterial pressure (P < 0.05; Table 2). There were no changes in PBF, LPVR, heart rate, systolic systemic arterial pressure, or right and left atrial pressure.

In tezosentan-treated lambs, opening of the vascular graft resulted in a rapid increase in PBF (from 41.03 ± 10.9 to 71.4 ± 21.83 ml·min⁻¹·kg⁻¹ at 15 min; P < 0.05) and decrease in LPVR (from 0.36 ± 0.15 to 0.2 ± 0.06 mmHg·ml⁻¹·min⁻¹·kg⁻¹ at 15 min; P < 0.05; Fig. 4). However, unlike control lambs, PBF and LPVR did not reach a plateau but rather continued to change from 15 min to 4 h after shunt opening (ANOVA; P < 0.05; Fig. 4). Furthermore, by 3 h, PBF was higher and LPVR was lower than in vehicle-treated control lambs (P < 0.05; Fig. 4). Pulmonary artery pressure and left atrial pressure increased, mean and diastolic systemic arterial pressure decreased, and right atrial pressure did not change after shunt opening. Heart rate increased and systolic systemic arterial pressure decreased by 4 h after shunt opening (P < 0.05; Table 2). Systemic arterial pH (from 7.38 ± 0.09 to 7.37 ± 0.10), PCO₂ (from 39.7 ± 7.0 to 44.3 ± 10.0 Torr), and PaO₂ (from 77.5 ± 4.5 to 67.5 ± 13.5 Torr) did not change.

Interestingly, total NOS activity increased by 61.4% (from 0.34 ± 0.14 to 0.49 ± 0.14 pmol·min⁻¹·mg protein⁻¹; P < 0.05) at 4 h after shunt opening, as opposed to the decrease in control animals (Fig. 5A). Like control lambs, calcium-independent NOS activity consisted of 21.0% of total NOS activity, and decreased by 57.1% (from 0.07 ± 0.02 to 0.03 ± 0.02 pmol·min⁻¹·mg protein⁻¹; P < 0.05) at 4 h after shunt opening (P < 0.05). Similar to control lambs, these changes in NOS activity were independent of changes in eNOS protein levels (Fig. 5B).

The infusion of l-NNA resulted in an increase in systemic systolic, diastolic, and mean arterial pressures (P < 0.05; Table 3). Pulmonary arterial pressure, right and left atrial pressure, PBF, LPVR, and heart rate did not change.

In l-NNA-treated lambs, opening of the vascular graft resulted in a rapid increase in PBF (from 29.14 ± 7.7 to 79.98 ± 18.49 ml·min⁻¹·kg⁻¹ at 15 min; P < 0.05), which did not change further over the 4-h study period (Fig. 4A). Shunt opening resulted in a rapid decrease in LPVR (from 0.26 ± 0.09 to 0.14 ± 0.02 mmHg·ml⁻¹·min⁻¹·kg⁻¹ at 15 min; P < 0.05; Fig. 4B). However, unlike in control and tezosentan-treated lambs, LPVR increased from 15 min to 4 h after shunt opening, returning to baseline values by 2 h (ANOVA; P < 0.05; Fig. 4B). Furthermore, by 4 h, LPVR was higher than in control lambs (P < 0.05; Fig. 4B). Pulmonary artery pressure and left atrial pressure increased, and systolic, mean, and diastolic systemic arterial pressure decreased after shunt opening, whereas heart rate did not change (Table 3). Systemic arterial pH (from 7.41 ± 0.04 to 7.38 ± 0.07), PCO₂ (from 40.0 ± 3.5 to 44.5 ± 11.6 Torr), and PaO₂ (from 97.2 ± 8.7 to 88.1 ± 15.6 Torr) did not change. NOS activity was not detectable in l-NNA-treated lungs.
In sham-operated lambs, PBF, LPVR, mean pulmonary artery pressure, left and right atrial pressure, systemic arterial pressure, and heart rate did not change significantly during the 4-h study period (Table 4). Systemic arterial pH (from 7.41 ± 0.04 to 7.39 ± 0.07), \( \text{PCO}_2 \) (from 46.0 ± 3.2 to 45.5 ± 2.3 Torr), and \( \text{PaO}_2 \) (from 95.8 ± 11.3 to 99.1 ± 29.5 Torr) did not change. Plasma ET-1 levels (from 6.02 ± 1.1 to 5.78 ± 2.2 pg/ml) and tissue ECE-1 levels (0.140 ± 0.01 vs. 0.18 ± 0.03 pg/ml) did not change.

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**Fig. 3. Immunohistochemical detection of iNOS and nNOS protein in juvenile lamb lungs.** Lung tissue samples cryosectioned at 5 µm were blocked and probed for iNOS or nNOS as described in MATERIALS AND METHODS. Bound antibody was detected using a goat anti-mouse Alexafluor-546, and sections were imaged by fluorescence. Both iNOS (A) and nNOS (B) protein levels are detected by immunohistochemistry but are unchanged in acute shunt (top) or sham-operated (bottom) lambs. Representative vessels are shown. Scale bar = 50 µm.
pmol·min⁻¹·mg⁻¹) were also unchanged. In addition, both total (from 0.58 ± 0.20 to 0.48 ± 0.12 pmol·min⁻¹·mg protein⁻¹) and calcium-independent (from 0.08 ± 0.08 to 0.09 ± 0.04 pmol·min⁻¹·mg protein⁻¹) NOS activity was unchanged, and eNOS (from 0.38 ± 0.09 to 0.34 ± 0.09, densitometric values) protein levels were unchanged. iNOS protein levels were minimally detectable, and nNOS protein levels were not detectable by Western blot analysis. Immunohistochemistry did detect low levels of both iNOS and nNOS, which did not change during the 4-h study period (Fig. 3).

**DISCUSSION**

Vascular dysfunction is integral to the pathophysiology of a wide array of disease processes, including congenital heart disease (11, 13, 14). In these patients, pulmonary vascular dysfunction contributes to the dynamic alterations in PVR that complicate the postoperative period. Postoperative pulmonary vascular dysfunction may be secondary to a number of factors, including abnormal pulmonary mechanical forces, ischemia-reperfusion injury, and cardiopulmonary bypass (6, 34, 36, 37, 44). The current study suggests that the abnormal forces associated with acute increases in PBF alone are sufficient to produce altered ET-1 and NO signaling in the intact lamb. Specifically, we found that surgically induced increases in PBF are associated with increases in plasma ET-1 levels and ET-receptor-mediated decreases in lung tissue NOS activity. Together, these changes tend to increase PVR postoperatively.

Acute increases in PBF have been examined in the fetal lamb after mechanical constriction of the ductus arteriosus. In this model, acute increases in PBF and decreases in PVR are reversed within 2–4 h by intense pulmonary vasoconstriction (1, 31). Recent data demonstrate that increased plasma ET-1 levels and ETA-receptor-mediated decreases in lung tissue NOS activity are associated with these changes (31). In the current study, acute increases in PBF were induced by the surgical placement of a large (8 mm) aortopulmonary shunt in intact juvenile (4–6 wk) lambs. Shunt placement resulted in a rapid sharp increase in PBF and decrease in PVR. However, very rapidly, PBF and PVR reached a plateau such that neither was significantly different at 4 h compared with 15 min after shunt opening. In isolation, it is unclear whether this plateau represents maximal flow through the pulmonary circulation or a limitation in response to an increase in pulmonary vascular tone. However, the fact that plasma ET-1 levels were increased and lung tissue NOS activity was decreased at 4 h after shunt opening suggests that pulmonary vascular tone may be increased.

To elucidate the role of ET-1 signaling in these animals, a group of lambs was treated with tezosentan, a combined ETA- and ETB-receptor antagonist, before and after shunt placement. Treatment with tezosentan augmented the increase in PBF and decrease in PVR, preventing a sustained plateau, such that PBF was higher and PVR was lower than...
controls at 3 and 4 h. Furthermore, lung tissue NOS activity was increased in tezosentan-treated lambs as opposed to the decrease observed in control lambs. These data indicate that ET-receptor activation may modulate a flow- and/or pressure-induced decrease in eNOS activity that occurs with an acute increase in PBF. Moreover, these data suggest that PBF after shunt placement is limited, at least in part, by active vasoconstriction because treatment with tezosentan allowed for a continued increase in PBF and decrease in PVR over time.

To elucidate the role of NO signaling in these animals, another group of lambs was treated with L-NNA, an inhibitor of NOS. Treatment with L-NNA did not affect the rapid increase in PBF or decrease in PVR observed after shunt opening. However, unlike vehicle or tezosentan-treated lambs, L-NNA-treated lambs demonstrated an increase in PVR over time, reaching baseline values by 2 h. These data suggest that whereas mechanical forces (i.e., vessel recruitment and vascular elasticity) may account for much of the decrease in PVR observed immediately after shunt placement, NO may play an important role in maintaining a low PVR once PBF is increased. Unlike PVR, PBF did not differ between L-NNA-treated lambs and vehicle-treated lambs, despite the fact that NOS activity was completely blocked. The reasons for PBF not further decreasing in L-NNA-treated lambs are unclear. However, in the presence of an aortopulmonary communication, PBF is dependent on several factors, which include the ratio of pulmonary to systemic vascular resistance. Indeed, recently published studies (15, 24) on the postoperative management of patients with single-ventricle physiology advocate a pharmacological decrease in SVR, as opposed to increased PVR, to decrease pulmonary overcirculation and maintain adequate systemic oxygen delivery. In this study, infusion of L-NNA was associated with an increase in systemic blood pressure, suggesting an increase in SVR, which may have affected the ratio of pulmonary to systemic vascular resistance to favor the maintenance of PBF in the face of an increasing PVR. The instrumentation utilized in the current study did not allow for direct measurements of SVR without significant assumptions. However, L-NNA has been shown to increase SVR in other studies at the dose utilized in this study (16).

The acute increase in PBF was associated with a decrease in NOS activity under normal conditions and an increase in NOS activity during ET-receptor blockade. In the vasculature, NO may be produced by three major NOS isoforms: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). Both nNOS and eNOS are constitutive and calcium dependent, whereas iNOS is inducible and calcium independent. All three isoforms have been identified in the developing sheep lung (42). To determine the potential contributions of these isoforms in our studies, NOS activity assays were performed with and without calcium. We found that the majority of total NOS activity was calcium dependent and the protein expression of nNOS was minimal, suggesting that changes in eNOS activity were dominant. In fact, Western blot analysis could not detect nNOS protein levels, so we utilized immunohistochemistry to confirm the presence of nNOS in our lambs, as previously described (42). A large body of evidence indicates that eNOS can be regulated at the transcriptional and posttranslational levels (23, 33). For example, laminar shear stress has been shown to increase eNOS transcription, and a number of factors, including intracellular location, protein-protein interactions, phosphorylation, and substrate and cofactor availability, have all been shown to regulate eNOS activity (23, 33, 35, 40). In the current study, the change in NOS activity was not associated with changes in protein levels, suggesting a posttransla-

**Fig. 5.** Lung tissue NOS activity and eNOS protein levels. A: relative lung tissue NOS activity (normalized to baseline) increases at 4 h in Tezo-treated lambs as opposed to the decrease seen in control animals. B: eNOS protein levels in lung tissue do not change before and 4 h after opening of aortopulmonary shunt in Tezo-treated lambs. B, top: representative Western blots are shown for protein extracts prepared from lung tissue separated on a 7.5% SDS-polyacrylamide gel, electrophoretically transferred to Hybond membranes, and analyzed using a specific antiserum raised against eNOS (equal protein loading is shown by signal obtained from β-actin). B, bottom: densitometric values for eNOS protein (normalized to β-actin) from Tezo-treated lambs. eNOS protein levels do not change in Tezo-treated lambs by 4 h after aortopulmonary shunt placement. Control, n = 9; Tezo-treated, n = 6. Values are means ± SE. *P < 0.05 vs. baseline.

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Table 3. Hemodynamic changes after aortopulmonary shunt opening in L-NNA-treated lambs

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Post-L-NNA</th>
<th>15</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
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<tbody>
<tr>
<td><strong>MPAP, mmHg</strong></td>
<td>12.69±2.8</td>
<td>21.91±5.2*</td>
<td>29.58±6.6*</td>
<td>34.95±5.7*</td>
<td>33.72±4.8*</td>
<td>34.27±6.2*</td>
<td>34.95±5.7*</td>
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<tr>
<td><strong>LA, mmHg</strong></td>
<td>5.53±2.2</td>
<td>10.97±4.8*</td>
<td>10.73±6.5*</td>
<td>11.75±5.1*</td>
<td>10.62±4.0*</td>
<td>8.94±2.0*</td>
<td>8.94±2.0*</td>
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<tr>
<td><strong>RA, mmHg</strong></td>
<td>4.80±1.4</td>
<td>4.44±2.7</td>
<td>5.54±1.8</td>
<td>5.95±1.7</td>
<td>5.75±1.5</td>
<td>6.13±1.8</td>
<td>6.13±1.8</td>
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<td><strong>Systolic SAP, mmHg</strong></td>
<td>8.49±1.1</td>
<td>106.24±14.3*</td>
<td>115.80±24.5*</td>
<td>115.02±30.4*</td>
<td>114.03±28.2*</td>
<td>119.07±26.0*</td>
<td>124.13±34.7*</td>
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<td><strong>MSAP, mmHg</strong></td>
<td>7.40±0.2</td>
<td>79.95±7.5*</td>
<td>40.54±6.7*</td>
<td>49.13±5.5*</td>
<td>42.59±12.1*</td>
<td>38.08±5.7*</td>
<td>37.92±9.6*</td>
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<td><strong>HR, beats/min</strong></td>
<td>143.60±7.7</td>
<td>143.40±32.6</td>
<td>150.40±41.2</td>
<td>154.20±33.4</td>
<td>148.60±32.4</td>
<td>140.60±30.4</td>
<td>145.40±30.5</td>
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</table>

Values are means ± SD; *P < 0.05 vs. baseline; †P < 0.05 vs. previous column; ‡P < 0.05 vs. post-L-NNA (ANOVA).

Table 4. Hemodynamics in sham-operated lambs

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>60</th>
<th>120</th>
<th>180</th>
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</thead>
<tbody>
<tr>
<td><strong>MPAP, mmHg</strong></td>
<td>17.82±1.4</td>
<td>18.67±1.9</td>
<td>18.59±1.3</td>
<td>18.32±1.8</td>
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<tr>
<td><strong>LA, mmHg</strong></td>
<td>7.68±2.7</td>
<td>8.14±2.5</td>
<td>8.30±2.7</td>
<td>9.07±2.2*</td>
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<td><strong>RA, mmHg</strong></td>
<td>8.49±3.7</td>
<td>8.45±3.7</td>
<td>7.23±2.0</td>
<td>6.93±1.8</td>
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<td><strong>Systolic SAP, mmHg</strong></td>
<td>85.25±14.9</td>
<td>100.25±19.2</td>
<td>99.75±13.8</td>
<td>99.50±16.1</td>
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<tr>
<td><strong>MSAP, mmHg</strong></td>
<td>77.29±10.4</td>
<td>77.03±14.2</td>
<td>79.79±11.0</td>
<td>76.78±14.6</td>
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<td><strong>Diastolic SAP, mmHg</strong></td>
<td>69.75±2.1</td>
<td>66.50±11.2*</td>
<td>70.35±12.2</td>
<td>69.80±13.9</td>
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<tr>
<td><strong>HR, beats/min</strong></td>
<td>141.75±40.3</td>
<td>123.25±32.2</td>
<td>126.00±19.3</td>
<td>136.00±19.5</td>
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<td><strong>Flow, kg·min⁻¹·min⁻¹</strong></td>
<td>27.43±10.1</td>
<td>34.64±11.4</td>
<td>32.35±12.7</td>
<td>31.81±10.3</td>
</tr>
<tr>
<td><strong>Left PVR, mmHg·ml⁻¹·min⁻¹·kg⁻¹</strong></td>
<td>0.419±0.2</td>
<td>0.348±0.2</td>
<td>0.386±0.2</td>
<td>0.342±0.2</td>
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</table>

Values are means ± SD; n = 4 lambs. PVR, pulmonary vascular resistance. *P < 0.05 vs. baseline (ANOVA).
a sustained decrease in PVR after shunt opening but did not alter the initial reduction in PVR and increase in PBF. In addition, PBF did not decrease as PBF increased from 15 min to 4 h in L-NNA-treated lambs. These data suggest that NO is critical in maintaining the reduction in PVR that follows acutely increased PBF but that it does not account for all of the hemodynamic manifestations that follow shunt placement. Mechanical forces and the balance between PVR and SVR are likely also critical.

Several important limitations of the present study are noteworthy. First, only one age group was studied. The pulmonary circulation undergoes significant developmental changes. Thus we cannot assume that the NO-ET-1 interactions described in the juvenile lamb would be the same in other age groups. Second, the model utilized in this study employs a very large (8 mm) aortopulmonary vascular graft. In practice, systemic to pulmonary shunts placed in patients with congenital heart disease are generally <4.5 mm. Furthermore, such a large shunt may obfuscate important changes in PBF that would otherwise accompany changes in PVR. Third, our model involved the placement of a shunt in an intact lamb with previously normal PBF. Clinically, systemic to pulmonary shunts are usually placed in the setting of significant abnormalities in PBF. Whereas more complex models are warranted to examine the effects of acute alterations in PBF in the setting of abnormal pulmonary circulations, it is critical to first establish the effects of increased PBF alone. The present study contributes to this effort. Fourth, a combined ET-receptor antagonist was utilized, which prevents insight into potential receptor-specific mechanisms. Future studies are needed to delineate the role of ET<sub>A</sub> and ET<sub>B</sub> receptors in the interactions described in the present study. Fifth, we did not directly measure systemic blood flow in this preparation, which would have provided important additional information, because these determinations would have necessitated the placement of two additional flow probes and the risk of vascular compression. However, by determining the pulmonary-to-systemic blood flow ratio (Q<sub>P</sub>/Q<sub>S</sub>) utilizing the Fick equation and assuming that left PBF is 40% of total flow, we can estimate systemic blood flow by dividing the total PBF by the Q<sub>P</sub>/Q<sub>S</sub>. With the use of these parameters, estimated systemic blood flow did not change over the study period in any of the study groups (data not shown). Finally, our study describes alterations over a 4-h period. Important vascular alterations likely manifest later after acute changes in PBF, and the changes we describe may lose relevance over time.

In summary, we found that surgically induced acute increases in PBF in the intact juvenile lamb result in increased plasma ET-1 levels and ET-receptor-mediated decreased NOS activity. These endothelial alterations favor active vasoconstriction, which limits PBF postoperatively. Interestingly, decreases in NO signaling and increases in ET-1 signaling are associated with pulmonary hypertension in a number of clinical settings as well as experimental models (2, 9, 10, 19, 21, 22, 46). Furthermore, the NO-ET-1 interactions described in the present study recapitulate alterations demonstrated with acute mechanical constriction of the ductus arteriosus in fetal lambs and chronic inhaled NO therapy in juvenile lambs (8, 29, 31). A better understanding of these novel endothelial interactions may result in postoperative strategies that improve the care of neonates, infants, and children with congenital heart disease and may have important implications for a number of pulmonary and systemic vascular disorders.

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