Nitric oxide-endothelin-1 interactions after acute ductal constriction in fetal lambs

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Ovadia, Boaz, Janine M. Bekker, Robert K. Fitzgerald, Alexander Kon, Stephan Thelitz, Michael J. Johengen, Karen Hendricks-Munoz, Rene Gerrets, Stephen M. Black, and Jeffrey R. Fineman. Nitric oxide-endothelin-1 interactions after acute ductal constriction in fetal lambs. Am J Physiol Heart Circ Physiol 282: H862–H871, 2002. First published November 15, 2001; 10.1152/ajpheart.00417.2001.—Acute partial compression of the fetal ductus arteriosus (DA) results in an initial increase in pulmonary blood flow (PBF) that is followed by acute vasoconstriction. The objective of the present study was to determine the role of nitric oxide (NO)-endothelin-1 (ET-1) interactions in the acute changes in pulmonary vascular tone after in utero partial constriction of the DA. Twelve late-gestation fetal lambs (132–140 days) were instrumented to measure vascular pressures and left PBF. After a 24-h recovery period, acute constriction of the DA was performed by partially inflating a vascular occluder, and the hemodynamic variables were observed for 4 h. In control lambs (n = 7), acute ductal constriction initially increased PBF by 627% (P < 0.05). However, this was followed by active vasoconstriction, such that PBF was restored to preconstriction values by 4 h. This was associated with a 45% decrease in total NO synthase (NOS) activity (P < 0.05) and a 106% increase in plasma ET-1 levels (P < 0.05). Western blot analysis demonstrated no changes in lung tissue endothelial NOS, proET-1, endothelin-converting enzyme-1, or ET receptor protein levels. The infusion of PD-156707 (an ET receptor antagonist, n = 5) completely blocked the vasoconstriction and preserved NOS activity. These data suggest that the fetal pulmonary vasoconstriction after acute constriction of the DA is mediated by NO-ET-1 interactions. These include an increase in ET receptor-mediated vasoconstriction and an ET receptor-mediated decrease in NOS activity. The mechanisms of these NO-ET-1 interactions, and their role in mediating acute changes in PBF, warrant further studies.

In decreases in fetal pulmonary arterial pressure induced by mechanical constriction of the ductus arteriosus induce an acute increase in pulmonary blood flow that is followed by active vasoconstriction (1). This so-called “myogenic response,” which returns pulmonary blood flow to preconstriction values within 2–4 h, may represent an adaptive response of the fetal pulmonary vasculature to maintain the normal low flow (44). However, chronic ductal constriction results in pulmonary vascular remodeling and many of the pathophysiological features of persistent pulmonary hypertension of the newborn (3, 29, 49). In fact, fetal ductal constriction secondary to maternal indomethacin use has been associated with persistent pulmonary hypertension of the newborn (45).

Previous studies have demonstrated that vasoactive factors produced by the vascular endothelium, such as nitric oxide (NO) and endothelin-1 (ET-1), are important mediators of the fetal and transitional pulmonary circulations (11). NO is produced from its precursor, L-arginine, after the activation of endothelial NO synthase (eNOS) (31). Once released, NO diffuses into the smooth muscle cell and generates cGMP (the second messenger of NO-mediated relaxation) after the activation of soluble guanylate cyclase (18). ET-1 is produced from a 203-amino acid peptide precursor (preproET-1), which is then cleaved to form endothelin-1 (Big ET-1) (51). Big ET-1 is then cleaved by the metallopeptase endothelin-converting enzyme-1 (ECE-1) into its functional form (46). The complex pulmonary vasoactive effects of ET-1, which may include either pulmonary vasoconstriction and/or pulmonary vasodilation, are mediated by at least two different receptors: ET and ETB receptors, which are located on vascular smooth muscle cells, mediate vasoconstriction, whereas ETB receptors, which are located on vascular endothelial cells, mediate vasodilation (4, 37).

Evidence that the pulmonary vascular endothelium regulates vascular tone has led to the hypothesis that aberrations in endothelial function participate in the...
pathophysiology of pulmonary hypertensive disorders. For example, chronic ductal constriction is associated with decreased NO activity and increased ET-1-mediated vasoconstriction (5, 23, 39, 47). In addition, Abman and colleagues (21, 43) have recently demonstrated that acute ductal constriction impairs endothelium-dependent relaxation and that ET blockade attenuates the subsequent decrease in pulmonary blood flow. These data suggest a role for both NO and ET-1 in the acute myogenic response after acute ductal constriction. Increasing data also suggest that endogenous NO and ET-1 participate in the regulation of vascular smooth muscle cells and inhibits ET-1 secretion and gene expression in vascular endothelial cells (6, 24, 33). In addition, we have recently demonstrated in the postnatal pulmonary circulation that exogenous NO increases ET-1 release and decreases NOS activity via an ET<sub>A</sub> receptor-dependent mechanism (26, 48). The potential role of these NO-ET-1 interactions in mediating the acute changes in fetal pulmonary blood flow has not been investigated.

The objective of this study was to determine the role of NO-ET-1 interactions in the dynamic changes in fetal pulmonary blood flow after acute mechanical constriction of the ductus arteriosus. To determine potential alterations in NO and ET-1 after acute ductal constriction, we determined lung tissue NOS activity, plasma ET-1 levels, and lung tissue protein levels of eNOS, preproET-1, ECE-1, and ETB receptors before and after acute ductal constriction. To determine potential NO-ET-1 interactions after ductal constriction, these alterations were compared in an additional group of fetal lambs that were pretreated with the ET<sub>A</sub> receptor antagonist PD-156707. Lastly, to isolate potential changes related to the experimental protocol, we determined potential alterations in additional fetal lambs without ductal constriction.

METHODS

Surgical preparation. Seventeen mixed-breed Western pregnant ewes (132–140 days gestation, term = 145 days) were operated on under sterile conditions with the use of local (2% lidocaine hydrochloride) and intravenous anesthesia (0.002 mg·kg<sup>-1</sup>·min<sup>-1</sup> diazepam and 0.3 mg·kg<sup>-1</sup>·min<sup>-1</sup> ketamine hydrochloride). Fetal anesthesia consisted of local anesthesia with 2% lidocaine hydrochloride and 15 mg/kg ketamine hydrochloride intramuscularly. Through a uterine incision, the fetal forelimb was exposed. Polyvinyl catheters were inserted into the fetal pedal artery and vein and were advanced to the aorta and inferior vena cava, respectively. A left lateral thoracotomy was performed in the fourth intercostal space. The pericardium was incised along the main pulmonary trunk. Teflon cannulas attached to polyvinyl catheters were inserted into the proximal main pulmonary trunk, left pulmonary artery, and left atrium. An ultrasonic flow transducer (Transonic Systems; Ithaca, NY) was placed around the left pulmonary artery. The ductus arteriosus was dissected free and infiltrated with 10% formalin to prevent ductal constriction during manipulation. A vascular occluder was then placed around the ductus arteriosus but left deflated. A side-biting vascular clamp was utilized to isolate peripheral lung tissue from the right upper lobe, and the incision was cauterized. Approximately 300 mg of peripheral lung were obtained for the biopsy to determine NOS activity and protein levels of eNOS, preproET-1, ECE-1, and ET<sub>B</sub> receptor. The thoracotomy incision was then closed in layers. Warm saline was instilled to replace the lost amniotic fluid, and the uterine incision was closed. A polyvinyl catheter was placed in the amniotic cavity. The catheters were filled with heparin sodium, plugged, and brought to the skin along with the transducer cables, where they were protected in a pouch secured to the ewe’s flank. After recovery from anesthesia, the ewe was returned to the cage. Antibiotics (1 million units of penicillin G procaine and 100 mg of gentamicin sulfate) were administered intravenously to the ewe and into the amniotic cavity during surgery and daily thereafter. Buprenorphine (0.01 mg/kg im) was administered for postoperative analgesia. All protocols were approved by the Committee of Animal Research at the University of California, San Francisco.

Experimental protocol. After a 24-h recovery period, the ewe was placed in a study cart with free access to food and water. The fetal catheters were connected to transducers, and 60 min were allowed for stabilization. An infusion of normal saline (n = 12, vehicle control) or PD-156707 (n = 5, a selective ET<sub>A</sub> receptor antagonist, 1.0 mg·kg<sup>-1</sup>·h<sup>-1</sup>) was then begun into the left pulmonary artery and continued throughout the study period. The dose of PD-156707 was chosen after several previous studies (19, 26, 32, 34, 35) showed that a 30-min infusion completely blocked the vasoconstricting effects of exogenous ET-1 and resulted in steady-state plasma concentrations that blocked ET<sub>A</sub> receptors in vivo. Thirty minutes after initiation of the infusion, baseline measurements of the hemodynamic variables (pulmonary and systemic arterial pressure, left pulmonary blood flow, left atrial pressure, and amniotic cavity pressure), systemic arterial blood gases, and pH were measured (preconstriction). Blood was collected from the femoral artery for plasma ET-1 determinations.

In 12 of the fetal lambs, the vascular occluder placed around the ductus arteriosus was then inflated with normal saline to increase mean pulmonary arterial pressure by 15–20 mmHg. The hemodynamic variables were monitored continuously, and systemic arterial blood gases were sampled intermittently. The occluder was occasionally adjusted to maintain the increase in mean pulmonary arterial pressure. After 4 h, blood was again obtained for plasma ET-1 concentrations. A repeat cesarean section was then performed, and a peripheral fetal lung biopsy was performed as described above.

To ensure that the potential changes demonstrated resulted from ductal constriction and not from other aspects of the protocol, five of the vehicle-treated fetal lambs underwent the exact protocol without inflation of the vascular occluder (occluder not inflated).

In four additional preliminary studies, the fetal lamb was operated on as described above. However, 30 min after the initial lung biopsy, the chest remained open and the vascular occluder was not inflated. Additional lung biopsies were performed 30 min and 4 h after ductal constriction.

At the end of the protocol, the fetus and ewe were killed with a lethal injection of pentobarbital sodium followed by bilateral thoracotomy as described in the National Institutes
of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. Fetal weight was then obtained.

Measurements. Pressures were measured by Statham P23Db pressure transducers (Statham Instruments). Mean pressures were obtained by electrical integration. All pressures obtained in utero were zeroed against the amniotic cavity pressure. Left pulmonary blood flow was measured on an ultrasonic flowmeter (Transonic Systems). All hemodynamic variables were continuously recorded on a Gould multichannel electrostatic recorder (Gould; Cleveland, OH). Systemic arterial blood gases and pH were measured on a Corning 158 pH/blood gas analyzer (Corning Medical and Scientific; Medfield, MA). Pulmonary vascular resistance was calculated as follows: (mean pulmonary arterial pressure – left atrial pressure)/left pulmonary blood flow/kg fetal weight. The fetal weight before beginning the infusions was estimated using standardized fetal sheep growth charts established in our laboratory.

Plasma ET-1 determinations. Three milliliters of systemic arterial blood were collected and placed in iced polypropylene tubes with 100 μl apotinin and 0.1 mM EDTA. The tubes were immediately centrifuged at 4,000 g for 20 min. Collected plasma was treated with equal volumes of 0.1% trifluoracetic acid and stored at –70°C. The acidified supernatant was centrifuged at 1,000 g for 20 min and loaded on a 3 × 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°C or assayed immediately for immunoreactive ET-1. ET-1 standard, 125I-labeled ET-1 (1125I]-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 (26).

Assay for NOS activity. This was performed using the conversion of [3H]arginine to [3H]citrulline as a measure of NOS activity essentially as described by Bush et al. (7). Briefly, 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 tetrahydrobiopterin, 100 μM FAD, 1 mM MgCl2, 5 μM unlabeled l-arginine, 15 nM [3H]arginine, 25 units calmodulin, 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 Nω-nitro-l-arginine methyl ester (l-NAME). Assays were incubated for 30 min such that no more than 20% of the [3H]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] and then applied to columns containing 1 ml of Dowex AG50W-X8 resin, Na+ form, preequilibrated with 1 N NaOH. [3H]Citrulline was then quantitated by scintillation counting.

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 g × 10 min at 4°C). Supernatant fractions were then assayed for protein concentration using the Bradford reagent (Bio-Rad; Richmond, CA) and used for Western blot analysis. Western blot analysis was performed as previously described (5, 26). Briefly, protein extracts (25 μg) were separated on 7.5% denaturing polyacrylamide gels for eNOS and ECE-1α, 10% denaturing polyacrylamide gels for ETα receptor, and 4–20% gradient denaturing polyacrylamide gradient gels for preproET-1. A positive control was also included for the ECE-1α Western blot. This consisted of protein extracts (10 μg) prepared from COS-7 cells transiently transfected with a mammalian expression vector containing the full-length bovine ECE-1α cDNA (a generous gift from Dr. M. Yanagisawa, Howard Hughes Medical Institute, University of Texas Southwestern, Dallas, TX). All gels were electrophoretically transferred to Hybond-polyvinylidene difluoride membranes (Amersham; Arlington Heights, IL). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween. After being blocked, the membranes were incubated at room temperature with the appropriate dilution of the antisem of interest (1:2,500 for eNOS, 1:1,000 for ECE-1α and ETα receptor, and 1:500 for preproET-1), washed with TBS containing 0.1% Tween, and then incubated with either an anti-mouse IgG-horseradish peroxidase conjugate (1:1,000 dilution) for eNOS, a goat anti-rabbit IgG-horseradish peroxidase conjugate for ECE-1α and ETα receptor, or a goat anti-sheep IgG-horseradish peroxidase conjugate for preproET-1. After the membranes were washed, chemiluminescence was used to detect the protein bands.

The preproET-1 antibody 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 and 15 μl antisemum) protein. ECE-1α antisera were generated as previously described (26). The ETα receptor antisem was obtained from Maine Biotechnology Services (Portland, ME). The eNOS-specific monoclonal antiserum was purchased from Transduction Laboratories (Lexington, KY). The purified ET-1 was purchased from Sigma (St. Louis, MO).

Positive controls were run to demonstrate antibody specificity. The methodology and exposure times used were those that we have previously demonstrated to be within the linear range of the autoradiographic film and able to detect changes in lung protein expression (5, 26).

Statistical analysis. The means ± SD were calculated for the baseline hemodynamic variables, systemic arterial blood gases, pH, plasma ET-1 concentrations, and tissue NOS activity. The general hemodynamic variables, systemic arterial blood gases, and pH were compared over time within each group by ANOVA for repeated measures followed by Student-Newman-Keuls test for post hoc testing of multiple comparisons as appropriate. For NOS activity, results from preocclusion lungs were assigned a value of 1 (relative activity). Pre- and postdural constriction ET-1 concentrations and NOS activity were compared by the paired t-test. Comparisons between treatment groups (PD-156777 vs. control) were made by 2-way ANOVA for repeated measures followed by the unpaired t-test.

Quantitation of autoradiographic results was performed by scanning (Hewlett-Packard SCA Jet IICX, Hewlett-Packard; Palo Alto, CA) the bands of interest into an image-editing software program (Adobe Photoshop, Adobe Systems; Mt. View, CA). Band intensities from Western blot analyses were analyzed densitometrically on a Macintosh computer (model 9500, Apple Computer; Cupertino, CA) using the public domain NIH Image program (developed at NIH and available on the Internet at http://rsb.info.nih.gov/nih-image). For Western blot analysis, to ensure equal protein loading, duplicate polyacrylamide gels were run. One
was stained with Coomassie blue. In the expected molecular weight range of each protein of interest, the density of the Coomassie blue bands was determined and utilized to normalize the Western blot band intensities. Results from pre-occlusion lungs were assigned a value of 1 (relative protein). The means ± SD were calculated for the relative protein at pre- and postductal constriction. Comparisons were made by the paired t-test. P < 0.05 was considered statistically significant.

RESULTS

There were no differences in gestational age, weight, sex distribution, or baseline hemodynamic variables between control, PD-156707-treated, and nonconstricted fetal lambs (data not shown).

In control lambs, acute ductal constriction rapidly increased mean pulmonary arterial pressure and left pulmonary blood flow (P < 0.05). Left pulmonary vascular resistance decreased (P < 0.05). Mean systemic arterial pressure, left atrial pressure, systemic arterial blood gases, and pH were all unchanged (Table 1). During the 4-h study period, pulmonary arterial pressure remained increased, but left pulmonary blood flow and pulmonary vascular resistance returned to pre-constriction values (Fig. 1). In fact, compared with 30-min postconstriction, pulmonary blood flow was decreased after 4 h and pulmonary vascular resistance was increased (P < 0.05; Fig. 1).

To determine the effects of ductal constriction on endogenous NO activity, we determined tissue NOS activity and eNOS protein levels before and 4 h after ductal constriction. We found that NOS activity decreased by 43% (P < 0.05; Fig. 2 and Table 2). However, eNOS protein levels were unchanged (Fig. 3 and Table 2). In the acute open-chest protocol, we found that total NOS activity was already decreased after 30 min of ductal constriction (~49.0%, P < 0.05, n = 4).

To determine the effects of ductal constriction on endogenous ET-1 production, we determined plasma ET-1 concentrations and lung preproET-1, ECE-1, and ET$_B$ receptor protein levels before and 4 h after ductal constriction. We found that plasma ET-1 concentrations were increased by 106% (P < 0.05; Fig. 4). In addition, Western blot analysis demonstrated no change in preproET-1, ECE-1a, or ET$_B$ receptor protein levels during the study period (Figs. 5–7 and Table 2). To determine potential NO-ET-1 interactions after ductal constriction, an additional group of fetal lambs were pretreated with the ET$_A$ receptor antagonist PD-156707. The infusion of PD-156707 decreased mean pulmonary arterial pressure (from 57.6 ± 10.1 to 52.0 ± 7.3 mmHg, P < 0.05). Mean systemic arterial

Table 1. Hemodynamic changes associated with acute ductal constriction in vehicle-treated fetal lambs

<table>
<thead>
<tr>
<th></th>
<th>Preconstriction</th>
<th>15 min</th>
<th>30 min</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP, mmHg</td>
<td>61.7 ± 6.5</td>
<td>78.3 ± 7.8*</td>
<td>80.0 ± 5.8*</td>
<td>80.3 ± 6.1*</td>
<td>79.7 ± 7.2*</td>
<td>76.0 ± 7.5*</td>
<td>71.7 ± 7.6*</td>
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<tr>
<td>Left pulmonary vascular resistance, mmHg·ml⁻¹·min⁻¹·kg⁻¹</td>
<td>14.1 ± 7.5</td>
<td>3.2 ± 1.1*</td>
<td>5.3 ± 4.2</td>
<td>14.1 ± 22.0</td>
<td>12.1 ± 16.4</td>
<td>10.7 ± 8.0</td>
<td>18.9 ± 14.5</td>
</tr>
<tr>
<td>Left pulmonary blood flow, ml·kg⁻¹·min⁻¹</td>
<td>4.9 ± 3.2</td>
<td>20.8 ± 10.1*</td>
<td>26.5 ± 27.3*</td>
<td>24.1 ± 26.2*</td>
<td>14.5 ± 10.5</td>
<td>10.3 ± 6.2</td>
<td>6.7 ± 6.5</td>
</tr>
<tr>
<td>SAP, mmHg</td>
<td>57.4 ± 6.5</td>
<td>58.0 ± 6.4</td>
<td>58.9 ± 6.3</td>
<td>57.1 ± 4.9</td>
<td>59.1 ± 4.6</td>
<td>58.3 ± 3.4</td>
<td>54.0 ± 6.3</td>
</tr>
<tr>
<td>PAP-SAP, mmHg</td>
<td>4.3 ± 4.7</td>
<td>20.6 ± 7.3*</td>
<td>23.1 ± 5.9*</td>
<td>21.7 ± 6.1*</td>
<td>19.7 ± 4.4*</td>
<td>17.7 ± 6.0*</td>
<td>17.7 ± 3.9*</td>
</tr>
<tr>
<td>pH, units</td>
<td>7.32 ± 0.08</td>
<td>7.30 ± 0.15</td>
<td>7.31 ± 0.08</td>
<td>7.32 ± 0.08</td>
<td>7.31 ± 0.07</td>
<td>7.29 ± 0.08</td>
<td>7.29 ± 0.08</td>
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<td>PaCO$_2$, Torr</td>
<td>54.5 ± 5.5</td>
<td>50.2 ± 6.1</td>
<td>51.3 ± 7.4</td>
<td>50.8 ± 4.1</td>
<td>48.5 ± 5.5*</td>
<td>50.5 ± 10.5</td>
<td>50.2 ± 5.7</td>
</tr>
<tr>
<td>PaO$_2$, Torr</td>
<td>14.7 ± 2.3</td>
<td>14.0 ± 3.1</td>
<td>13.5 ± 3.3</td>
<td>13.8 ± 4.1</td>
<td>14.3 ± 3.7</td>
<td>14.2 ± 4.3</td>
<td>14.0 ± 2.9</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 lambs. PAP, pulmonary arterial pressure; SAP, systemic arterial pressure; PaCO$_2$ and PaO$_2$, arterial PCO$_2$ and PO$_2$, respectively. *P < 0.05 vs. preconstriction values (ANOVA).
pressure, left pulmonary blood flow, left pulmonary vascular resistance, left atrial pressure, systemic arterial blood gases, and pH were unchanged.

In PD-156707-treated lambs, acute ductal constriction rapidly increased mean pulmonary arterial pressure and left pulmonary blood flow ($P < 0.05$). Left pulmonary vascular resistance decreased ($P < 0.05$). Mean systemic arterial pressure, left atrial pressure, systemic arterial blood gases, and pH were all unchanged (Table 3). During the 4-h study period, pulmonary arterial pressure remained increased, but left pulmonary blood flow and pulmonary vascular resistance remained unchanged (Table 3 and Fig. 8). In fact, in PD-156707-treated lambs, pulmonary blood flow was significantly increased compared with control lambs, and pulmonary vascular resistance was significantly decreased, after 4 h of ductal constriction ($P < 0.05$; Fig. 8).

### Table 2. Changes in NOS activity and protein levels associated with acute ductal constriction

<table>
<thead>
<tr>
<th>Groups</th>
<th>NOS Activity</th>
<th>eNOS Protein</th>
<th>Prepro-ET-1 Protein</th>
<th>ECE-1 Protein</th>
<th>ETb Receptor Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>Control</td>
<td>0.347 ± 0.19</td>
<td>0.242 ± 0.25</td>
<td>32.002 ± 6.577</td>
<td>29.834 ± 15.147</td>
<td>1,052,226 ± 625,596</td>
</tr>
<tr>
<td>PD-156707-</td>
<td>0.512 ± 0.21</td>
<td>1.05 ± 0.21</td>
<td>1,896,602 ± 718,951</td>
<td>3,479,167 ± 861,104</td>
<td>1,072,624 ± 587,837</td>
</tr>
<tr>
<td>treated</td>
<td>0.244 ± 0.17</td>
<td>0.538 ± 0.24</td>
<td>68,612 ± 37,613</td>
<td>143,928 ± 115,341</td>
<td>140,765 ± 140,765</td>
</tr>
<tr>
<td>Nonoccluded</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. Nitric oxide synthase (NOS) activity was measured in pmol·min$^{-1}$·mg$^{-1}$; protein levels are densitometric values. eNOS, endothelial NOS; ET-1, endothelin-1; ECE, endothelin-converting enzyme; Pre, preconstriction; Post, postconstriction. *$P < 0.05$ vs. preconstriction values.
In PD-156707-treated lambs, tissue NOS activity did not decrease after ductal constriction but significantly increased by 104% \((P < 0.05; \text{Fig. 2 and Table 2). This was associated with an increase in eNOS protein levels of 94\% (P < 0.05; \text{Fig. 3 and Table 2). Plasma ET-1 concentrations were increased to values that were similar to control lambs (Fig. 4). Similar to control lambs,}}\)

protein levels of proproET-1, ECE-1, and ET\(_B\) receptors were unchanged after ductal constriction (Figs. 5–7 and Table 2).

In fetal lambs without ductal constriction, hemodynamic variables, systemic arterial blood gases, and pH were unchanged during the 4-h study period (Table 4). Similar to the PD-156707-treated lambs, tissue NOS activity (114\%) and eNOS protein levels (107\%) increased \((P < 0.05; \text{Table 2 and Figs. 2 and 3). Plasma ET-1 levels (from 22.5 ± 2.1 to 21.2 ± 3.0 pg/ml) were unchanged.}}\)

**DISCUSSION**

In the present study, we tested the hypothesis that NO-ET-1 interactions mediate the dynamic changes in pulmonary blood flow after acute partial constriction of the ductus arteriosus in the fetal lamb. As previously demonstrated, we found that acute constriction of the ductus arteriosus induced an initial pulmonary vasodilation that was followed by active pulmonary vasoconstriction, returning pulmonary blood flow to preconstriction values within 2–4 h. Associated with these changes, total lung NOS activity decreased by 43\% and plasma ET-1 levels increased by 106\%. The infusion of PD-156707, an ETA receptor antagonist, blocked the active vasoconstriction while preserving NOS activity. These data suggest that the active vasoconstriction after ductal constriction is mediated, at least in part, by the ET\(_A\) receptor. Its effects include an increase in ET\(_A\) receptor-mediated vasoconstriction and/or an ET\(_A\) receptor-mediated decrease in NOS activity. The latter represents a novel NO-ET-1 interaction of the fetal
pulmonary vasculature with important physiological and clinical implications.

Increasing data suggest that both NO and ET-1 are important mediators of the normal perinatal circulation. For example, studies (30, 38, 42) have revealed maturational increases in NO-mediated relaxation and eNOS gene expression during the late fetal and early postnatal period. This maturational increase in NO production parallels the dramatic decrease in pulmonary vascular resistance that occurs at birth. In addition, in the late-gestation fetal lamb, an infusion of the NOS inhibitor \( \text{N}^{\text{H}}\text{G}-\text{L-arginine} \) markedly increases resting tone and attenuates the increase in pulmonary blood flow associated with ventilation at birth (2, 10, 12). Conversely, selective ET\(_A\) receptor blockade produces only small decreases in resting fetal pulmonary resistance and does not attenuate the increase in pulmonary blood flow associated with ventilation at birth (2, 10, 12).

Table 3. Hemodynamic changes associated with acute ductal constriction in PD-156707-treated fetal lambs

<table>
<thead>
<tr>
<th>Hemodynamic Variable</th>
<th>Preconstriction</th>
<th>15 min</th>
<th>30 min</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP, mmHg</td>
<td>52.0 ± 3.7</td>
<td>66.0 ± 7.2*</td>
<td>67.6 ± 6.8*</td>
<td>68.4 ± 5.9*</td>
<td>66.0 ± 5.5*</td>
<td>63.2 ± 2.7*</td>
<td>60.0 ± 6.0*</td>
</tr>
<tr>
<td>Left pulmonary vascular resistance, mmHg·ml⁻¹·min⁻¹·kg⁻¹</td>
<td>6.4 ± 4.6</td>
<td>2.5 ± 1.8*</td>
<td>1.62 ± 0.7*</td>
<td>2.3 ± 1.1*</td>
<td>3.17 ± 2.5*</td>
<td>3.4 ± 3.1*</td>
<td>3.4 ± 2.9*</td>
</tr>
<tr>
<td>Left pulmonary blood flow, ml·kg⁻¹·min⁻¹</td>
<td>11.1 ± 7.3</td>
<td>33.6 ± 17.5*</td>
<td>41.1 ± 16.7*</td>
<td>33.3 ± 18.3*</td>
<td>30.2 ± 21.9*</td>
<td>27.3 ± 18.9*</td>
<td>24.6 ± 16.0*</td>
</tr>
<tr>
<td>SAP, mmHg</td>
<td>49.4 ± 6.1</td>
<td>48.8 ± 6.1</td>
<td>50.8 ± 7.6</td>
<td>51.6 ± 7.9</td>
<td>50.0 ± 7.1</td>
<td>48.8 ± 5.9</td>
<td>44.8 ± 6.1*</td>
</tr>
<tr>
<td>PAP-SAP, mmHg</td>
<td>2.6 ± 2.4</td>
<td>17.2 ± 7.8*</td>
<td>16.8 ± 3.9*</td>
<td>16.8 ± 3.6*</td>
<td>16.0 ± 3.2*</td>
<td>14.4 ± 4.6*</td>
<td>15.0 ± 5.1*</td>
</tr>
<tr>
<td>pH, units</td>
<td>7.30 ± 0.05</td>
<td>7.30 ± 0.05</td>
<td>7.27 ± 0.05</td>
<td>7.25 ± 0.06*</td>
<td>7.21 ± 0.07*</td>
<td>7.20 ± 0.07*</td>
<td>7.20 ± 0.09*</td>
</tr>
<tr>
<td>Paco(_2), Torr</td>
<td>46.2 ± 3.9</td>
<td>47.2 ± 4.2</td>
<td>48.6 ± 7.3</td>
<td>51.8 ± 4.6</td>
<td>51.4 ± 5.7</td>
<td>55.4 ± 6.1*</td>
<td>56.2 ± 7.4*</td>
</tr>
<tr>
<td>Paco(_2), Torr</td>
<td>15.2 ± 2.0</td>
<td>16.6 ± 4.0</td>
<td>14.8 ± 1.9</td>
<td>14.4 ± 2.6</td>
<td>14.4 ± 2.8</td>
<td>13.8 ± 1.8*</td>
<td>14.0 ± 1.2*</td>
</tr>
</tbody>
</table>

Values are means ± SD; \( n = 5 \) lambs. *\( P < 0.05 \) vs. preconstriction values; †\( P < 0.05 \) vs. previous column (ANOVA).
monary blood flow at birth (20, 50). Together, these data suggest a major role for NO in mediating resting fetal pulmonary vascular tone and the fall in pulmonary vascular resistance during the transitional pulmonary circulation and a minor role for basal ET-1-induced vasoconstriction in maintaining the high fetal pulmonary vascular resistance.

In the present study, acute ductal constriction increased plasma ET-1 concentrations by 106%. Increases in plasma ET-1 concentrations may result from increases in ET-1 production, ET-1 release, and/or decreased ET-1 clearance. The production of ET-1 begins with the cleavage of the translational product preproET-1 into a nonactive 38-amino acid residue known as Big ET-1. Big ET-1 is then cleaved into its functional form, ET-1, by the endopeptidase ECE-1 (46). ECE-1 exists in two isoforms, ECE-1α and ECE-1β, with ECE-1α considered to be the most biologically important (41). Because many studies have suggested that ET-1 production is regulated at the transcriptional level of preproET-1 and/or ECE-1, we determined potential changes in preproET-1 and ECE-1α protein levels. We found that both preproET-1 and ECE-1α protein levels were unchanged after acute ductal constriction, suggesting that the increased plasma concentrations are independent of changes in gene expression. In addition, the ETB receptor has been implicated in the clearance of ET-1 from the circulation, but we found no changes in ETB receptor protein levels (13). Rapid ET-1 release from intracellular secretory granules has been demonstrated after such stimuli as cytokines and stretch (25, 28). Therefore, the increase in plasma ET-1 induced by ductal constriction may represent an increase in ET-1 release. However, potential changes in ECE-1 activity, NO-induced displacement of ET-1 from its receptors, and/or potential changes in ET-1 clearance represent additional potential mechanisms that were not completely studied but warrant investigation.

To better determine the potential role of ET-1 during acute ductal constriction, we pretreated five additional fetal lambs with an ETa receptor antagonist. To selectively block ETa receptor activity, we utilized PD-156707, a nonpeptide ETa receptor antagonist. PD-156707 is highly selective for the ETa receptor and inhibits the binding of [125I]ET-1 to cloned human ETA receptor and ETB receptor with inhibitory constant values of 0.17 and 133.8 nM, respectively (34). In rabbits, PD-156707 infusion rates of 0.03 mg·kg⁻¹·h⁻¹ completely blocked the vasoconstricting effects of exogenous ET-1, with corresponding plasma concentrations that were <0.05 µg/ml (10⁻⁷ M) (19, 35). We also performed several preliminary studies in lambs that demonstrated that PD-156707 infusion rates of 1.0 mg·kg⁻¹·h⁻¹ completely and selectively block the vasoconstricting effects of exogenous ET-1 (250 ng/kg) and produced stable plasma concentrations of >500 ng/ml within 30 min of initiating the infusion (32). Therefore, in the present study, we utilized an infusion rate of 1.0 mg·kg⁻¹·h⁻¹, which was initiated 30 min before ductal constriction. In PD-156707-treated lambs, we found that ductal constriction resulted in a similar increase in plasma ET-1 levels as demonstrated in the control lambs. However, the acute pulmonary vasoconstriction after ductal constriction was completely blocked, suggesting an important role for the ETa receptor in mediating this constriction.

A second component that may mediate the pulmonary vasoconstriction after ductal constriction is a decrease in NOS activity. In control lambs, we found that total NOS activity decreased by 43% after 4 h. Because eNOS protein levels were unchanged and the decrease was demonstrated as early as 30 min after ductal constriction in our open-chest protocol, the change in activity is likely independent of changes in gene expression. However, the exact mechanisms for the decrease in NOS activity are currently unclear. Our previous in vitro studies (40, 48) utilizing cells cultured from postnatal lambs suggested that vascular smooth muscle cells produce superoxide via an ETa receptor-dependent mechanism. Superoxide may then decrease NOS activity either directly, via the conversion to hydrogen peroxide, or via the production of peroxynitrite. In the intact postnatal lamb, we recently demonstrated that exogenous NO increases ET-1 secretion and decreases endogenous lung NOS activity via ETa receptor signaling (26, 48). In these studies, the dominant mechanism is the formation of peroxynitrite from the binding of superoxide and NO, which nitrates and

### Table 4. Hemodynamic changes in nonoccluded fetal lambs

<table>
<thead>
<tr>
<th></th>
<th>Preconstriction</th>
<th>15 min</th>
<th>30 min</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP, mmHg</td>
<td>58.8 ± 8.3</td>
<td>58.0 ± 7.1</td>
<td>58.4 ± 12.1</td>
<td>60.2 ± 10.7</td>
<td>59.6 ± 10.0</td>
<td>60.0 ± 11.0</td>
<td>56.2 ± 8.0</td>
</tr>
<tr>
<td>Left pulmonary vascular resistance, mmHg·ml⁻¹·min⁻¹·kg⁻¹</td>
<td>9.7 ± 7.2</td>
<td>14.0 ± 10.2</td>
<td>13.6 ± 7.2</td>
<td>11.4 ± 8.9</td>
<td>9.0 ± 7.0</td>
<td>12.4 ± 8.8</td>
<td>9.1 ± 4.9</td>
</tr>
<tr>
<td>Left pulmonary blood flow, ml·kg⁻¹·min⁻¹</td>
<td>7.6 ± 5.3</td>
<td>6.2 ± 5.8</td>
<td>4.6 ± 3.5</td>
<td>7.1 ± 5.6</td>
<td>7.4 ± 3.9</td>
<td>5.0 ± 2.5</td>
<td>6.6 ± 3.8</td>
</tr>
<tr>
<td>SAP, mmHg</td>
<td>57.5 ± 7.7</td>
<td>56.8 ± 5.9</td>
<td>60.0 ± 11.0</td>
<td>60.5 ± 9.0</td>
<td>60.3 ± 9.8</td>
<td>60.0 ± 11.4</td>
<td>55.5 ± 7.2</td>
</tr>
<tr>
<td>PAP-SAP, mmHg</td>
<td>2.0 ± 2.8</td>
<td>2.3 ± 2.6</td>
<td>1.5 ± 1.9</td>
<td>2.3 ± 2.9</td>
<td>1.3 ± 1.5</td>
<td>2.0 ± 2.3</td>
<td>2.3 ± 2.6</td>
</tr>
<tr>
<td>pH units</td>
<td>7.35 ± 0.05</td>
<td>7.34 ± 0.06</td>
<td>7.34 ± 0.06</td>
<td>7.34 ± 0.06</td>
<td>7.33 ± 0.07</td>
<td>7.33 ± 0.07</td>
<td>7.33 ± 0.05</td>
</tr>
<tr>
<td>Paco₂, Torr</td>
<td>52.0 ± 3.9</td>
<td>49.8 ± 4.3</td>
<td>49.2 ± 4.0</td>
<td>50.2 ± 3.9</td>
<td>52.0 ± 4.5</td>
<td>51.0 ± 4.7</td>
<td>51.6 ± 2.1</td>
</tr>
<tr>
<td>Pao₂, Torr</td>
<td>14.6 ± 6.5</td>
<td>14.0 ± 7.1</td>
<td>15.2 ± 6.9</td>
<td>16.0 ± 7.6</td>
<td>15.4 ± 6.3</td>
<td>15.8 ± 5.5</td>
<td>15.0 ± 4.5</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 5 lambs.
irreversibly inhibits eNOS (26, 40, 48). In the present study, we demonstrated that the decrease in lung NOS activity after acute ductal constriction can be preserved by the infusion of PD-156707. Therefore, these data support a similar ETA receptor-dependent decrease in NOS activity in the fetal pulmonary circulation after ductal constriction. However, several other posttranslational modifications of NOS, such as protein kinase C-dependent phosphorylation and translocation of NOS within the cytosol, are also known to quickly decrease NOS activity (17, 27). These and other possible mechanisms are currently speculative and warrant further study.

In nonoccluded lambs, we found that total NOS activity increased. Because this was associated with a similar increase in eNOS protein levels, these data suggest that the experimental protocol, independent of ductal manipulation, upregulates eNOS gene expression. The etiology for this upregulation of NOS is unclear and warrants further studies. Potential etiologies include surgically induced increases in catecholamine and prostanoid production, both of which have been shown to upregulate eNOS in different cellular systems, and the fact that the second biopsy was taken ~36 h later in gestation, during a time when eNOS gene expression is increasing (8, 9, 14, 38). Because the experimental protocol alone increased NOS activity, the decrease in NOS activity after ductal constriction in control lambs would tend to be minimized by the protocol, further accentuating the fact that acute ductal constriction decreases NOS activity. Interestingly, eNOS protein levels were not increased in control lambs but remained increased in PD-156707-treated lambs. Because chronic ductal constriction decreases eNOS gene expression, perhaps an early decrease in NOS activity after ductal constriction can be prevented by the infusion of PD-156707. Therefore, these novel NO-ET-1 interactions associated with acute changes in pulmonary blood flow may have important physiological and pathophysiological implications. Additional studies of NO-ET-1 interactions in the normal and abnormal pulmonary circulation, and their mechanisms, are warranted.

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REFERENCES


