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Emergence of Smooth Muscle Cell Endothelin B–Mediated Vasoconstriction in Lambs With Experimental Congenital Heart Disease and Increased Pulmonary Blood Flow

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**Background**—Endothelin-1 (ET-1) has been implicated in the pathophysiology of pulmonary hypertension. In 1-month-old lambs with increased pulmonary blood flow, we have demonstrated early alterations in the ET-1 cascade. The objective of this study was to investigate the role of potential later alterations of the ET cascade in the pathophysiology of pulmonary hypertension secondary to increased pulmonary blood flow.

**Methods and Results**—Eighteen fetal lambs underwent in utero placement of an aortopulmonary vascular graft (shunt) and were studied 8 weeks after spontaneous delivery. Compared with age-matched control lambs, lung tissue ET-1 levels were increased in shunt lambs (317.2 ± 113.8 versus 209.8 ± 61.8 pg/g, P < 0.05). In shunt lambs (n = 9), exogenous ET-1 induced potent pulmonary vasoconstriction, which was blocked by the ET_A receptor antagonist PD 156707 (n = 3). This pulmonary vasoconstriction was mimicked by exogenous Ala 1,3,11,15 ET-1 (4 Ala ET-1), the ET_B receptor agonist, and was blocked by the ET_B receptor antagonist BQ 788 (n = 3). However, in control lambs (n = 7), ET-1 and 4 Ala ET-1 did not change pulmonary vascular tone. In contrast to 4-week-old shunt lambs, immunohistochemistry revealed the emergence of ET_B receptors on smooth muscle cells in the vasculature of 8-week-old shunt lambs.

**Conclusions**—Over time, increased pulmonary blood flow and/or pressure results in the emergence of ET_B-mediated vasoconstriction, which coincides with the emergence of ET_B receptors on smooth muscle cells. These data suggest an important role for ET_B receptors in the pathophysiology of pulmonary hypertension in this animal model of increased pulmonary blood flow. (Circulation. 2003;108:1646-1654.)

**Key Words:** endothelin • pulmonary heart disease • heart defects, congenital

The development of pulmonary hypertension and its associated increased vascular reactivity is a common accompaniment of congenital heart disease with increased pulmonary blood flow. Recent evidence suggests that pulmonary vascular tone and vascular smooth muscle cell proliferation is regulated by a complex interaction of vasoactive substances that are produced locally by the vascular endothelium, such as nitric oxide (NO) and endothelin-1 (ET-1). Endothelial injury secondary to increased pulmonary blood flow and/or pressure disrupts these regulatory mechanisms and is a potential factor in the development of pulmonary hypertension. ET-1 is a 21-amino-acid polypeptide produced by vascular endothelial cells that has potent vasoactive properties and is mitogenic for vascular smooth muscle cells. Several studies demonstrate increased ET-1 concentrations in children with increased pulmonary blood flow and pulmonary hypertension, suggesting a role for ET-1 in the pathophysiology of pulmonary hypertension. To better define the role of ET-1 in the pathogenesis of pulmonary hypertension, we established a model of pulmonary hypertension with increased pulmonary blood flow in the lamb after in utero placement of an aorta-to-pulmonary vein graft. At 1 month of age, these lambs have a pulmonary-to-systemic blood flow ratio of 2.1, a mean pulmonary arterial pressure that is 50% to 75% of mean systemic arterial pressure, and pulmonary vascular remodeling characteristic of children with pulmonary hypertension and increased pulmonary blood flow. Previously, we demonstrated that these lambs display alterations in the ET-1 cascade at 4 weeks of age. These include
### Methods

#### Surgical Preparation and Care

**Ewes**

Eighteen mixed-breed Western pregnant ewes (135 to 141 days of gestation; term = 145 days) were operated on under sterile conditions as previously described. Through a left lateral fetal thoracotomy, an 8.0-mm Gore-tek vascular graft (~2 mm long) (W.L. Gore and Associates) was anastomosed between the ascending aorta and main pulmonary artery with care to preserve the integrity of the vascular endothelium. The heart and lungs were removed en bloc. The lungs were dissected for the surgical preparations.

**Lambs**

Eight weeks after spontaneous delivery, 35 lambs (18 shunted and 17 age-matched controls) were anesthetized with ketamine hydrochloride, diazepam, and fentanyl citrate, mechanically ventilated, and instrumented to measure vascular pressures and flow as previously described.

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<table>
<thead>
<tr>
<th>General Hemodynamics</th>
<th>Control Lambs</th>
<th>Shunted Lambs</th>
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</thead>
<tbody>
<tr>
<td>Age, d</td>
<td>63.1±5.9</td>
<td>61.2±6.9</td>
</tr>
<tr>
<td>Weight, kg</td>
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<td>18.9±4.9*</td>
</tr>
<tr>
<td>PAP, mm Hg</td>
<td>17.6±4.6</td>
<td>26.5±10.6*</td>
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<tr>
<td>SAP, mm Hg</td>
<td>81.1±13.8</td>
<td>68.3±16.2*</td>
</tr>
<tr>
<td>PAP/SAP</td>
<td>0.23±0.09</td>
<td>0.41±0.19*</td>
</tr>
<tr>
<td>Left pulmonary vascular resistance, mm Hg · mL⁻¹ · min⁻¹ · kg⁻¹</td>
<td>0.283±0.09</td>
<td>0.141±0.05*</td>
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<tr>
<td>Left pulmonary blood flow, mL · kg⁻¹ · min⁻¹</td>
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<td>121.7±40.0*</td>
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<tr>
<td>Heart rate, bpm</td>
<td>136.9±22.5</td>
<td>153.9±22.3</td>
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<tr>
<td>Left atrial pressure, mm Hg</td>
<td>7.7±3.0</td>
<td>10.4±4.9*</td>
</tr>
<tr>
<td>Right atrial pressure, mm Hg</td>
<td>7.4±2.9</td>
<td>5.4±2.5</td>
</tr>
<tr>
<td>QO/Qs</td>
<td>1.00</td>
<td>3.0±0.9*</td>
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PAP indicates pulmonary arterial pressure; SAP, systemic arterial pressure. Values are mean ± SD. n=15 shunt lambs and 13 age-matched controls. Values in shunted lambs were obtained before closure of the vascular graft.

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Twenty-three lambs (12 shunted and 11 age-matched controls; Pozzi Livestock, Valley Ford, Calif) then underwent a hemodynamic study as described below. After the last protocol, the lambs were killed by an intravenous injection of pentobarbital sodium (Euthasol CII, Central City Medical), followed by bilateral thoracotomy. Twelve of the lambs (6 shunted and 6 twin controls) were killed without undergoing any hemodynamic study. These lungs were removed and prepared for tissue ET-1 levels and Western blot analysis, RNase protection assays, and immunohistochemistry.

All protocols and procedures were approved by the Committee on Animal Research of the University of California, San Francisco. All animals were euthanized by appropriate methods as described in the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

#### Experimental Protocols

**In Vivo ET-1–Dependent Responses**

The responses to ET-1 and 4 Ala ET-1 were determined in control lambs at rest and shunt lambs at rest with the vascular graft open. However, the responses to vasodilating agents may be dependent on the resting tone of the vascular bed studied. Therefore, to ensure that response differences were not tone dependent, the vasodilator responses in control lambs were also studied with increased tone during an intravenous infusion of U46619 (a thromboxane A₂ mimic), and shunted lambs were also studied after the vascular graft was closed, when pulmonary blood flow is similar to that of controls.

**Control lambs:** After a 45-minute recovery period from surgery, baseline measurements of the hemodynamic variables were measured. In 7 lambs, ET-1 (250 ng/kg) or 4 Ala ET-1 (1725 ng/kg) was then injected into the pulmonary artery in random order. To determine the selectivity of the agonists and the contribution of each receptor to basal tone, the responses to ET-1 and 4 Ala ET-1 were studied before and during the infusion of PD 156707 (1.0 mg · kg⁻¹ · h⁻¹; an ET₁ receptor antagonist) and BQ 788 (1.0 µg · kg⁻¹ · min⁻¹; an ET₆ receptor antagonist) in 4 additional lambs. After the lambs had recovered from the last agent, an infusion of U46619 (a thromboxane A₂ mimic) was then begun into the inferior vena cava. After 15 minutes of steady-state pulmonary hypertension, baseline measurements were again obtained, ET-1 and 4 Ala ET-1 were administered, and the hemodynamic variables were measured as described above.

**Shunted lambs:** After a 45-minute recovery period from surgery, baseline measurements of the hemodynamic variables were measured. In 9 shunt lambs, ET-1 and 4 Ala ET-1 were then administered as described above. In 3 additional lambs, the responses to ET-1 and 4 Ala ET-1 were studied before and during the infusion of PD 156707 and BQ 788 as described above. The vascular graft was then closed. After a 60-minute recovery, the responses to ET-1 and 4 Ala ET-1 were repeated.

#### ET-1 Determinations

Lung tissues were homogenized in 1 mol/L acetic acid containing 10 µg/mL pepstatin (Peptide International) and immediately boiled for 10 minutes. The homogenates were centrifuged at 25 000g for 30 minutes at 4°C, and the supernates were stored at −30°C before being assayed for immunoreactive endothelin as previously described.

**Tissue Preparation**

The heart and lungs were removed en bloc. The lungs were dissected with care to preserve the integrity of the vascular endothelium. Sections (2 to 3 g) from each lobe of the lung were removed. These tissues were snap-frozen in liquid N₂ and stored at −70°C until analysis.

For RNA and protein isolation, the snap-frozen lung tissue was prepared as previously described. For immunohistochemistry, the pulmonary vascular tree was rinsed with cold (4°C) PBS to remove blood and fixed by perfusion with cold (4°C) 4% paraformaldehyde. The pulmonary artery was then clamped. The airways were fixed at 20 cm H₂O pressure by filling the trachea with cold (4°C) 4%
paraformaldehyde. When the lungs were distended at this pressure, the trachea was clamped. The lungs were fixed for 24 hours at 4°C by immersion in 4% paraformaldehyde. Representative slices from each lobe were removed, placed in 30% sucrose until they sank, placed in OCT, frozen on dry ice, and stored at −70°C until sectioned. Sections (5 to 10 μm) were cut with a cryostat, transferred to aminoaalkylsilane-treated slides (Superfrost Plus, Fisher Scientific), and stored at −70°C.16

Western Blot Analysis
Western blot analysis was performed as previously described.14,17 The ET₄ receptor antiserum was generated as previously described.16 The ET₄ receptor antiserum was obtained from Maine Biotechnology Services. The prepro-ET-1 antibody was obtained from Affinity Bioreagents. The specificity of the prepro-ET-1 antibody was verified with those of 8-week-old lambs.

Immunohistochemistry was performed as previously described.14 In addition, immunohistochemistry was performed with a specific rabbit antiserum raised against ETB. This antiserum was prepared by injecting rabbits with a highly antigenic protein fragment based on rat ETB (sequence: NH₂-SCLKFKANDHGYDNF-COOH). Rabbits were bled at 6, 8, and 10 weeks, and the 8-week bleed was immunopurified (BioSynthesis Inc). Frozen tissue sections (7 μm) were allowed to come to room temperature. Samples were fixed for 10 minutes in cold acetone, then washed 3 times with PBS. To eliminate nonspecific binding of the primary antiserum to tissue proteins, tissue sections were incubated with 1% horse serum in PBS (blocking solution) for 1 hour. Then, tissue sections were incubated with 1% horse serum in PBS (blocking solution) for 1 hour. A minimum of 3 different sets of control and shunt lung tissue were prepared and examined.

RNA Probe Synthesis and RNase Protection Assay
The plasmid containing the cDNA fragment of interest was linearized with the appropriate restriction enzyme (Gibco-BRL). Antisense [32P]UTP radiolabeled cRNA probes (New England Nuclear) were synthesized by in vitro transcription using either T3 or T7 RNA polymerases (Boehringer-Mannheim) in the presence of cold rCTP, rGTP, and rATP.17

RNase protection assays were performed as previously described.17 Also included was a probe for 18S to serve as a control for the amount of input total RNA and the recovery of protected probe fragments.

Immunohistochemistry
Immunohistochemistry was performed as previously described.16 Studies were done on serial sections of control and shunt ovine lung with our rabbit anti-ET₄ antibody raised as described previously.14 In addition, immunohistochemistry was performed with a specific rabbit antiserum raised against ETB. This antiserum was prepared by injecting rabbits with a highly antigenic protein fragment based on rat ETB (sequence: NH₂-SCLKFKANDHGYDNF-COOH). Rabbits were bled at 6, 8, and 10 weeks, and the 8-week bleed was immunopurified (BioSynthesis Inc). Frozen tissue sections (7 μm) were allowed to come to room temperature. Samples were fixed for 10 minutes in cold acetone, then washed 3 times with PBS. To eliminate nonspecific binding of the primary antiserum to tissue proteins, tissue sections were incubated with 1% horse serum in PBS (blocking solution) for 1 hour. Then, tissue sections were incubated with 1% horse serum in PBS (blocking solution) for 1 hour. A minimum of 3 different sets of control and shunt lung tissue were prepared and examined.

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Lung sections from four 4-week-old shunt and control lambs were also analyzed to localize ET₄ and ET₃ receptors.

Statistical Analysis
The mean±SD was calculated for the hemodynamic variables, systemic arterial blood gases, and pH and tissue ET-1 levels. Comparisons were made by the paired t test using the Bonferroni correction, the unpaired t test, or ANOVA for repeated measures with multiple-comparison testing.

Quantification of autoradiographic results was performed by scanning the bands of interest into an image editing software program (Adobe Photoshop, Adobe Systems). For RNAse protection assays, to control for the amount of input RNA and the recovery of protected probe fragments, the mRNA signal of interest was normalized to the corresponding 18S signal for each lane. The mean±SD was calculated for the relative RNA and protein. The unpaired t test or ANOVA was used for comparisons between 4- and 8-week-old, control and shunt lambs for repeated measures with multiple-comparison testing. A value of P<0.05 was considered statistically significant.

Results
At 2 months of age, shunted lambs weighed significantly less than controls (18.9±4.9 versus 22.6±4.2 kg, P<0.05). The ratio of pulmonary to systemic blood flow (Qp/Qs) was 3.0±0.9. Mean pulmonary arterial pressure was increased to 41% of systemic values. This was associated with an increase in pulmonary blood flow and left atrial pressure (P<0.05). Mean systemic arterial pressure and the calculated left pulmonary vascular resistance were decreased (P<0.05) (Table). Peripheral lung tissue immunoreactive ET-1 concentrations were greater in shunted lambs than control lambs (317.2±113.8 versus 209.8±61.8 pg/g, P<0.05).

The baseline hemodynamics and protein concentrations of the 4-week-old lambs used in this study were similar to those previously published (data not shown).13,14 In addition, the
baseline hemodynamics of the 4- and 8-week-old shunt lambs used in this study were similar.

**Hemodynamic Study**

**Control Lambs**

In control lambs, the intrapulmonary injection of ET-1 and 4 Ala ET-1 did not change mean pulmonary arterial pressure, left pulmonary blood flow, or left pulmonary vascular resistance (Figure 1). ET-1 increased mean systemic arterial pressure from 67.5 ± 7.7 to 90.6 ± 7.8 mm Hg (P < 0.05).

Similarly, during steady-state pulmonary hypertension induced by the infusion of U46619, intrapulmonary injections of ET-1 or 4 Ala ET-1 did not change mean pulmonary arterial pressure or pulmonary vascular resistance (data not shown).

At rest, the infusion of PD 156707 induced a modest decrease in mean pulmonary arterial pressure (−8.0 ± 0.6%, P < 0.05) and left pulmonary vascular resistance (−9.9 ± 2.6%, P < 0.05) (n = 4). Pulmonary blood flow was unchanged. During the infusion of PD 156707, the increase in systemic arterial pressure induced by ET-1 was blocked, and the response to 4 Ala ET-1 was unchanged (data not shown). The infusion of BQ 788 did not change the baseline hemodynamic variables (n = 4). In addition, during the infusion of BQ 788, the response to ET-1 and 4 Ala ET-1 was unchanged (data not shown).

**Shunted Lambs**

In shunted lambs with the vascular graft open, the intrapulmonary injection of ET-1 increased mean pulmonary (25.1 ± 8.3 to 35.2 ± 11.8 mm Hg) and systemic (63.7 ± 12.2 to 73.3 ± 14.2) arterial pressure and left pulmonary vascular resistance (0.13 ± 0.04 to 0.23 ± 0.011 mm Hg · L⁻¹ · min⁻¹) (P < 0.05). Left pulmonary blood flow was unchanged. Similarly, the intrapulmonary injection of 4 Ala ET-1 increased mean pulmonary (25.0 ± 9.7 to 32.3 ± 12.0 mm Hg) and systemic (66.8 ± 12.4 to 73.6 ± 13.1 mm Hg) arterial pressure and left pulmonary vascular resistance (0.13 ± 0.07 to 0.22 ± 0.16 mm Hg · L⁻¹ · min⁻¹) (P < 0.05). Left pulmonary blood flow decreased (132.7 ± 41.3 to 122.1 ± 43.7 mL/min) (P < 0.05).

Similarly, after shunt closure, the intrapulmonary injections of ET-1 and 4 Ala ET-1 induced similar increases in mean pulmonary arterial pressure and left pulmonary vascular resistance (data not shown) (P < 0.05).

The percent increase in both left pulmonary vascular resistance and mean pulmonary arterial pressure induced by the injection of ET-1 was greater in shunted lambs than in control lambs (P < 0.05) (Figure 1). Similarly, the percent increase in both left pulmonary vascular resistance and mean pulmonary arterial pressure induced by the injection of 4 Ala ET-1 was greater in shunted lambs than in control lambs (P < 0.05) (Figure 1).

The infusion of PD 156707 decreased mean pulmonary arterial pressure (−15.4 ± 3.7%, P < 0.05) and left pulmonary vascular resistance (−25.0 ± 3.9%, P < 0.05) (n = 3). Pulmonary blood flow was unchanged. During the infusion of PD 156707, the increase in left pulmonary vascular resistance induced by ET-1 was blocked (90.1 ± 35.2% versus
but the response to 4 Ala ET-1 was unchanged. The infusion of BQ 788 did not change the baseline hemodynamic variables. However, during the infusion of BQ 788, the increase in left pulmonary vascular resistance in response to ET-1 was partially attenuated (90.1±35.2% versus 19.2±16.5%), whereas the response to 4 Ala ET-1 was completely blocked (107.5±22.0% versus 1.9±5.7%) (n=3).

To determine whether the alterations in ET-1 levels and physiological responses were associated with changes in gene expression, we performed Western blot analysis and RNAse protection assays. Compared with age-matched control lambs, the levels of both prepro-ET-1 and ECE-1 protein and mRNA were unchanged (Figures 2 and 5). Protein levels of ET<sub>A</sub> receptors were increased in shunt lambs (P<0.05), whereas the changes in ET<sub>A</sub> receptor mRNA did not reach statistical significance (Figures 3 and 5). Protein and mRNA levels of ET<sub>B</sub> receptors were also unchanged in shunt lambs compared with age-matched controls (Figures 3 and 5). However, compared with 4-week-old shunt lambs, protein levels of ET<sub>B</sub> receptor were significantly increased in 8-week-old shunt lambs (Figure 4).

In both 4- and 8-week-old control lambs, localization by immunohistochemistry demonstrated ET<sub>A</sub> receptors solely on vascular endothelial cells (Figure 6C and 6G) and ET<sub>A</sub> receptors solely in the vascular smooth muscle cell layer (Figure 6A and 6E). In 4- and 8-week-old shunt lambs ET<sub>A</sub> receptors were again found solely in the vascular smooth muscle cell layer (Figure 6B and 6F). In 4-week-old shunt lambs, ET<sub>B</sub> receptors were found predominantly on endothelial cells (>90%) and occasionally on smooth muscle cells. However, in 8-week-old shunt lambs, a significant subpopulation of ET<sub>B</sub> receptors were localized to the vascular smooth muscle cell layer (>50%) (Figure 6D and 6H).

**Discussion**

In a follow-up investigation to our previous studies, we found that exposure to 8 weeks of increased pulmonary blood flow and pressure results in progressive alterations in ET-1 signaling. Similar to alterations displayed at 4 weeks, shunted lambs had increased lung tissue concentrations of ET-1 and increased protein levels of ET<sub>A</sub> receptors. However, in contrast to decreased ET<sub>B</sub> protein levels and a loss of ET<sub>B</sub> receptor–mediated pulmonary vasodilation in 4-week-old shunt lambs, 8-week-old shunt lambs had an upregulation of ET<sub>B</sub> receptor protein and the emergence of ET<sub>B</sub> receptor–mediated pulmonary vasoconstriction. Immunohistochemistry revealed that both 4- and 8-week-old control lambs had ET<sub>B</sub> receptors solely on endothelial cells, whereas 8-week-old shunt lambs displayed a subpopulation of ET<sub>B</sub> receptors present in the vascular smooth muscle cell layer. ET<sub>A</sub> receptors were found on vascular smooth muscle cells in all groups. These data suggest a potentially important role for...
addition, the infusion of BQ 788 (an ET\textsubscript{A} receptor antagonist) blocked the vasoconstriction induced by 4 Ala ET-1, whereas PD 156707 (an ET\textsubscript{A} receptor antagonist) did not, further suggesting that 4 Ala ET-1–induced pulmonary constriction was secondary to ET\textsubscript{A} receptor activation. Interestingly, the infusion of BQ 788 did not change resting pulmonary vascular tone in either control or shunt lambs, suggesting that ET\textsubscript{A} receptor activation participates in agonist-induced responses but makes a minimal contribution to basal tone. Conversely, the increase in ET\textsubscript{A} receptor protein noted in shunt lambs was associated with an augmented pulmonary vasodilating response to PD 156707, the ET\textsubscript{A} receptor antagonist, suggesting that ET\textsubscript{A} receptor activation contributes to basal tone in shunt lambs.

In association with the emergence of ET\textsubscript{B} receptor–mediated pulmonary vasconstriction, there were changes in protein levels of ET\textsubscript{B} receptors. Previously, in 4-week-old shunt lambs, we demonstrated a decrease in ET\textsubscript{B} receptor lung protein levels compared with age-matched control lambs.

This was associated with loss of the pulmonary vasodilating response to the ET\textsubscript{B} receptor agonist 4 Ala ET-1. In the present study, we demonstrate no difference in lung ET\textsubscript{B} receptor protein between 8-week-old shunt lambs and age-matched control lambs. However, compared with 4-week-old shunt lambs, there was a significant increase in ET\textsubscript{B} receptor protein levels. In addition, immunohistochemistry reveals that a subset of ET\textsubscript{B} receptor expression predominately in 8-week-old shunt lambs now localizes to the smooth muscle cell layer. Taken together, these data suggest that increased pulmonary blood flow and/or pressure causes a decrease in endothelial ET\textsubscript{B} receptors and the emergence of smooth muscle cell ET\textsubscript{B} receptors. Because 4 Ala ET-1–induced pulmonary vasoconstriction is now present in 8-week-old shunt lambs, we speculate that the increased protein levels of ET\textsubscript{B} receptor at 8 weeks mediate the demonstrated pulmonary vasconstricting response. Although increasing in vitro data suggest that changes in mechanical forces alter ET-1 signaling, few data are available on the regulation of ET receptor signaling. Therefore, the potential mechanisms for the current receptor changes are unknown and warrant further investigation.

Changes in ET\textsubscript{B} receptors have previously been demonstrated in other animal models of pulmonary hypertension. For example, in contrast to the present study, rats exposed to monocrotaline displayed an increase in ET\textsubscript{B} receptor–mediated vasodilation during the development of pulmonary hypertension. In addition, in piglets exposed to hypoxia, there was loss of the normal postnatal increase in endothelial ET\textsubscript{B} receptors during the first 3 days of life, and in sheep with persistent pulmonary hypertension of the newborn, ET\textsubscript{B} receptor expression was decreased. However, in humans with advanced pulmonary vascular disease secondary to thromboembolic pulmonary hypertension, a recent report demonstrates a selective upregulation of ET\textsubscript{B} mRNA in the media of pulmonary arteries. The present study represents the first investigation of changes in ET receptors during the development of pulmonary hypertension secondary to congenital heart disease with increased pulmonary blood flow. Our findings are consistent with the recent findings in adults and further suggest an important role.

smooth muscle cell ET\textsubscript{B} receptors in the pathophysiology of pulmonary hypertension secondary to congenital heart disease.

The hemodynamic effects of ET-1 are mediated by at least 2 populations of receptors, ET\textsubscript{A} and ET\textsubscript{B} receptors. In the systemic vasculature, the response to exogenous ET-1 is consistently vasoconstricting, which is mediated via ET\textsubscript{A} receptor activation. However, in the pulmonary vasculature, the response to exogenous ET-1 is dependent on a variety of factors, including age, species, and the underlying disease state. In the fetal and young sheep, ET-1 induces pulmonary vasodilation, which is mediated via ET\textsubscript{B} receptor activation and the release of NO. In the adult sheep, ET-1 induces pulmonary vasoconstriction, which is associated with prostaglandin release and presumed ET\textsubscript{A} receptor activation. In the rabbit, rat, and human adult pulmonary circulation, pulmonary vasoconstricting ET\textsubscript{B} receptors have been identified in the medial layer of pulmonary arteries. However, in response to modest doses of ET-1, ET\textsubscript{B} receptor–induced pulmonary vasoconstriction has not been reported previously in the young sheep or human. With the use of 4 Ala ET-1, a selective ET\textsubscript{B} receptor agonist, we demonstrate significant pulmonary vasoconstriction in 8-week-old shunt lambs and no response in age-matched control lambs. In

Figure 4. Western blot analysis for ET\textsubscript{B} receptor in lung tissue from 4- and 8-week-old lambs. Top, Protein extracts (50 µg) prepared from lung tissue from 4 lambs (2 control and 2 shunt). Bottom, Densitometric values for relative ET\textsubscript{B} receptor protein (normalized to H11006) from 4 lambs (2 control and 2 shunt). Four-week-old values were assigned a value of 1. In control lambs, ET\textsubscript{B} receptor protein expression was decreased at 8 weeks compared with 4 weeks. However, in shunt lambs, ET\textsubscript{B} receptor protein expression was increased at 8 weeks compared with 4 weeks. Values are mean±SEM. *P<0.05 vs corresponding 4-week values.
for both ET\textsubscript{A} and ET\textsubscript{B} receptors in the pathophysiology of pulmonary hypertensive disorders.

In summary, in a model of increased pulmonary blood flow and pulmonary hypertension secondary to in utero aortopulmonary vascular graft placement, the present study describes the emergence of smooth muscle cell, ET\textsubscript{B} receptor–mediated pulmonary vasoconstriction over the second month of postnatal life. In addition, there are persistent increases in lung tissue ET-1...
levels and ET\textsubscript{A} receptor protein levels. These data suggest an increasingly important role for both ET\textsubscript{A} and ET\textsubscript{B} receptors in the pathophysiology of pulmonary hypertension secondary to congenital heart defects. With the recent development of ET receptor antagonists as potential therapeutic agents, these data have important clinical implications. Similar to the recent report in adults with advanced disease, these data suggest that combined ET\textsubscript{A} and ET\textsubscript{B} receptor antagonists may be more beneficial than selective ET\textsubscript{A} receptor antagonists in the treatment of pulmonary hypertension.\textsuperscript{28} In fact, bosentan, a combined receptor antagonist, has recently shown beneficial therapeutic effect in adults with primary pulmonary hypertension.\textsuperscript{29} Further investigations into the mechanisms of these alterations are needed and may have important clinical implications.

Figure 6. ET\textsubscript{A} and ET\textsubscript{B} protein expression in lung in vivo from tissue from 4- and 8-week-old lambs. Immunohistochemical localization of ET\textsubscript{A} and ET\textsubscript{B} protein expression in lung in vivo from 4- (A–D) and 8-week-old (E–H) lambs. Polyclonal rabbit ET\textsubscript{A} and ET\textsubscript{B} receptor antibodies and monoclonal mouse anti–smooth muscle cell (SMC)–actin antibodies were used to localize expression. ET\textsubscript{A} (A, B, E, F) and ET\textsubscript{B} (C, D, G, H) protein expression is shown in red and SMC-actin expression in green. Colocalization is shown in yellow. Magnification ×800. In both shunt and control lambs, ET\textsubscript{A} receptors localize to smooth muscle cells at both ages. In 4-week-old control lambs (A, C, E, G), ET\textsubscript{B} receptors localize to endothelial cells. In 4-week-old shunt lambs, ET\textsubscript{B} receptors were found predominantly on endothelial cells (>90%) (D). However, in the majority of 8-week-old shunt lamb vessels (>50%), subpopulations of ET\textsubscript{B} receptors localize to smooth muscle cells (H). Results are representative of 3 different sets of twin matches (control and shunt).
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