eNOS and prostanoid enzymes in lungs of newborn piglets with chronic aortopulmonary shunts

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IT IS WELL KNOWN that infants with congenital heart lesions that are associated with increased pulmonary blood flow develop pulmonary hypertension (2, 7, 11, 15, 28). The mechanisms underlying the development of pulmonary hypertension in these infants remain uncertain but include the possibility that production of factors known to regulate pulmonary vascular pressure has been altered (2, 7, 11, 15, 28). For example, decreased production of vasodilators, such as nitric oxide (NO) and prostacyclin, or increased production of vasoconstrictors, such as thromboxane, could contrib-
ute to the development of pulmonary hypertension (2, 7, 11, 15, 28). In turn, a change in the abundance of the proteins responsible for their production could underlie altered production of these vasoactive factors. In other words, reduced amounts of endothelial nitric oxide synthase (eNOS), an enzyme responsible for basal pulmonary vascular NO production, could contribute to pulmonary hypertension. Similarly, altered amounts of prostanoid enzymes underlying either prostacyclin and/or thromboxane production could contribute to the pulmonary hypertension that develops in infants with increased pulmonary blood flow.

Experimental attempts to produce increased-flow pulmonary hypertension include placement of an aortopulmonary shunt in newborn piglets (10, 26). Previous investigators reported that piglets studied 4–12 wk after placement of an aortopulmonary shunt exhibited elevated pulmonary arterial pressure and thickened pulmonary arteries (10, 26). The effect of prolonged aortopulmonary shunts on amounts of eNOS and/or prostanoid enzymes in lungs of newborn piglets has not been reported. Our purpose was to test the hypothesis that abundance of either eNOS or prostanoid enzymes would be altered in lungs of newborn piglets 5–6 wk after placement of an aortopulmonary shunt. In addition, because both NO and prostanoids have been shown to contribute to pulmonary vascular dilation to ACh (4, 19, 31, 33), we tested the hypothesis that ACh-induced dilation would be altered in lungs of piglets with chronic aortopulmonary shunts.

METHODS

Animals. Newborn piglets arrived a few days before surgery at age 1–3 days and were placed in an environmental chamber so that they could acclimate and learn to ad libitum feed sow milk replacer from a feeding device attached to the chamber. At age 4–6 days, left thoracotomies were performed in all piglets. An aortopulmonary shunt was placed in five piglets, and six piglets underwent a sham operation. For the thoracotomy, each piglet was preanesthetized with ketamine (10 mg/kg im) and acepromazine (1 mg/kg im) and then intubated. The piglet was ventilated with a bellows-type anesthesia machine that deliv-
ered supplemental oxygen as needed, a tidal volume of 10–15 ml/kg, and a respiratory rate of 20–30 breaths/min. To maintain temperature at 39–40°C, the piglet was placed on a heating blanket. After percutaneous placement of an intravenous catheter in an ear vein, the piglet received prophylactic antibiotic therapy (cefazolin, 25 mg/kg iv) and was anesthetized with fentanyl (20 μg/kg iv). Throughout the operation, the piglet received a continuous intravenous infusion of normal saline (4–5 ml·kg⁻¹·h⁻¹) and additional fentanyl as needed. For the shunted piglets, a 5-mm Gortex graft was sewn end to side to the aorta and then to the pulmonary artery to create the aortopulmonary shunt. For the sham piglets, the aorta and pulmonary artery were dissected and transiently clamped for the same duration of time necessary to sew in a shunt. To minimize movement during shunt placement, vecuronium (0.1 mg/kg) was given during the time that the aorta and pulmonary artery were clamped. Postoperatively, each piglet received prophylactic antibiotics (cefazolin, 25 mg/kg im two times daily for 3 days) and analgesia (buprenorphine, 10 μg/kg im two times daily for 1 day). Piglets were maintained in the environmental chamber and fed ad libitum sow milk replacer until 3 wk of age. Piglets were then weaned, placed in a pen, and fed dry chow two times a day for an additional 2–3 wk.

*Measurements in anesthetized animals.* After surgery (5–6 wk), all piglets were weighed, preanesthetized with ketamine (30 mg/kg im), and then anesthetized with pentobarbital sodium (10 mg/kg iv). Additional intravenous pentobarbital sodium was given as needed via an ear vein to maintain anesthesia during placement of the catheters. First, the trachea of the piglet was cannulated so that the animal could be ventilated if necessary. Next, a catheter was placed in the right femoral artery for monitoring systemic blood pressure and arterial blood gases. Then, another catheter was placed through the right external jugular vein into the pulmonary artery to monitor pulmonary arterial pressure. To obtain the pulmonary wedge pressure, the pulmonary arterial catheter was advanced into a distal pulmonary vessel. The zero reference for the vascular pressures was the midthorax. To measure cardiac output by the thermodilution technique (model 9520 thermodilution cardiac output computer; Edwards Laboratory), a thermistor was placed in the aortic arch via the left femoral artery, and a catheter that served as an injection port was placed in the left ventricle via the left carotid artery. Cardiac output was measured at end expiration as the mean of three injections of 3 ml of 0.9% saline (0°C). For one of the shunted piglets, equipment (Baxter Vigilance Monitor) became available to measure oxygen saturations both proximal to the shunt, in the right ventricle, and distal to the shunt in the pulmonary artery.

At the completion of the in vivo measurements, all animals were given heparin (1,000 IU/kg iv) and additional anesthesia (3–5 mg/kg pentobarbital sodium iv) and then were exsanguinated. Lungs were left in situ and used for perfusion as described below.

*Measurements in isolated perfused lungs.* For lung perfusion, the tracheal cannula was attached to a large-animal piston-type ventilator, and the lungs were ventilated with a normoxic gas mixture (17% O₂-6% CO₂-balance N₂) using a tidal volume of 15–20 ml/kg and a respiratory rate of 15–20 breaths/min (mean airway pressure of 4–6 cmH₂O). A midline sternotomy was performed, and, for the shunted piglets, a clamp was placed across the shunt. Saline-filled cannulas were placed in the pulmonary artery and left atrium through incisions in the right and left ventricles. The diaphragm and all abdominal contents were removed. The vascular cannulas were connected to the perfusion circuit described previously (13, 14). The perfusion circuit was filled with 200 ml of the animal’s own blood collected during exsanguination (as described in *Measurements in anesthetized animals*), mixed with 100 ml of 3% albumin-saline. The perfusion circuit included a rotary pump (model 7523-00; Cole-Parmer Masterflex) that continuously circulated the perfusate from a reservoir through a bubble trap into the pulmonary arterial cannula, through the lungs to the left atrial cannula, and back to the reservoir. Pulmonary arterial, left atrial, and airway pressures were continuously monitored. The most dependent edge of the lung was used as the zero reference for vascular pressures.

After connection to the perfusion circuit, the lungs were perfused for 30–60 min until a stable pulmonary arterial pressure was achieved. The perfusate flow and left atrial pressure were adjusted to respective levels of 50 ml·min⁻¹·kg⁻¹ and 0 cmH₂O and were maintained constant for the remainder of the study.

Changes in pulmonary arterial pressure in response to ACh were then measured. Because we knew from our first few studies (see RESULTS) that baseline pulmonary arterial pressure is greater in shunted than in sham animals, the pulmonary arterial pressure of the sham animals was elevated to a level comparable to that of the shunted animals by adding 1 M KCl to the perfusate of the sham lungs. ACh was added cumulatively to the reservoir to achieve concentrations of 10⁻⁹ to 10⁻⁶ M. The ACh responses were transient so that the pulmonary arterial pressure was allowed to return to baseline between doses. After the response to ACh was measured, the endothelium-independent agent papaverine was added to the reservoir (300 μg/ml of perfusate) and allowed to circulate for 20 min to determine the contribution of vasomotor tone to pulmonary arterial pressure.

For the shunted piglets, patency of the shunt was confirmed by visual examination at the end of perfusion. For all piglets, a piece of lung was immediately frozen in liquid nitrogen and stored at ~80°C for later analysis of eNOS and the prostanoid enzymes cyclooxygenase (COX)-1, COX-2, prostacyclin synthase, and thromboxane synthase in whole lung homogenate samples as described below. For lungs of some piglets (n = 4 shunted, n = 4 sham), the remainder of the lungs was then submersed in saline and stored overnight at 4°C. The next morning, two size groups of pulmonary arteries (small pulmonary arteries = 100–600 μm in diameter and larger pulmonary arteries = 700–1,200 μm in diameter) were dissected from the stored lungs and then immediately frozen in liquid nitrogen for later analysis of eNOS and prostanoid enzymes (COX-1, COX-2, prostacyclin synthase, and thromboxane synthase) in pulmonary artery homogenate samples as described below.

**Immunoblot analysis.** We performed preliminary studies with different amounts of total protein to determine the dynamic range of the immunoblot analysis for each protein and tissue homogenate. An amount of protein that was within the dynamic range of the immunoblot analysis was then used to compare protein abundance between homogenates from shunted and sham-operated piglets as described below. For example, Fig. 1 shows an immunoblot for eNOS and tissue homogenate. An amount of protein that was greater in shunted than in sham animals, the pulmonary arterial pressure of the sham animals was elevated to a level comparable to that of the shunted animals by adding 1 M KCl to the perfusate of the sham lungs. ACh was added cumulatively to the reservoir to achieve concentrations of 10⁻⁹ to 10⁻⁶ M. The ACh responses were transient so that the pulmonary arterial pressure was allowed to return to baseline between doses. After the response to ACh was measured, the endothelium-independent agent papaverine was added to the reservoir (300 μg/ml of perfusate) and allowed to circulate for 20 min to determine the contribution of vasomotor tone to pulmonary arterial pressure.

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![Fig. 1. Immunoblot for endothelial nitric oxide synthase (eNOS) using small pulmonary artery (Pa) homogenate samples from sham-operated and shunted piglets containing total protein amounts of 1, 2.5, 5, 7.5, and 10 μg. Note that for 1, 2.5, 5, and 7.5 μg of total protein, the intensity of staining for eNOS increases with increasing amounts of protein.](http://ajplung.physiology.org/)

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using small pulmonary artery homogenate samples containing total protein amounts of 1, 2.5, 5, 7.5, and 10 \( \mu \text{g} \). On the basis of these findings, to compare eNOS abundance in small pulmonary artery homogenates between shunted and sham-operated piglets, we followed methods as described below using 2.5-\( \mu \text{g} \) total protein samples.

**Immunoblot analysis for whole lung homogenate samples.** Tissue pieces that did not contain large airways or large vessels were selected from frozen perfused lungs of sham and shunted piglets. The tissue pieces were homogenized in 10 mM HEPES buffer containing 250 mM sucrose, 3 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4, on ice using three 15-s pulses of a Polytron blender, taking care to avoid foaming of the homogenate. Protein concentration of the lung homogenate was determined by the Bio-Rad protein assay. Each lung homogenate was diluted with PBS to obtain a protein concentration of 1 mg/ml. Forty microliters of each sample were solubilized in 40 \( \mu \text{l} \) of 4% (wt/vol) SDS, 2.5% (vol/vol) β-mercaptoethanol, 10% glycerol, and 0.05% bromphenol blue, pH 6.8; Novex), heated to 80°C for 15 min, and centrifuged for 3 min at 5,600 \( \times \) g to remove denatured protein. Equal volumes of these supernatants were then applied to Tris-glycine precast 5% polyacrylamide gels (Novex) so that 15-\( \mu \text{g} \) protein whole lung homogenate samples were loaded on both gels. We used 15-\( \mu \text{g} \) protein samples for eNOS. Electrophoresis was carried out in 25 mM Tris, 192 mM glycine, and 0.1% SDS (pH = 8.3) at 125 volts for 1.7 h. The proteins were transferred from the gel to a nitrocellulose membrane (Novex) at 100 volts for 1 h in 25 mM Tris, 192 mM glycine, and 20% methanol (pH = 8.3). The membrane was incubated overnight at 4°C in PBS containing 10% nonfat dried milk and 0.1% Tween 20 to block nonspecific protein binding. To detect eNOS, the nitrocellulose membrane was incubated for 1 h at room temperature with the primary antibody (Transduction Laboratories) diluted 1:500 in PBS containing 0.1% Tween 20 and 1% nonfat dried milk (carrier buffer) followed by incubation for 30 min at room temperature with a biotinylated secondary antibody (Vector Elite, ABC Kit; Vector Laboratories) diluted 1:5,000 in the carrier buffer, followed by incubation for 30 min at room temperature with streptavidin-horseradish peroxidase conjugate (Amersham) diluted 1:1,500 in PBS containing 0.1% Tween 20. The nitrocellulose membrane was washed three times between the first two incubations with the carrier buffer, three times with the carrier buffer, and one time with PBS containing 0.1% Tween 20 after the final incubation. To visualize the biotinylated antibody, the membranes were developed using enhanced chemiluminescence (ECL) reagents (Amersham), and the chemiluminescent signal was captured on X-ray film (ECL Hyperfilm; Kodak). The bands for eNOS were quantified using densitometry.

Similar procedures were followed using primary antibodies for COX-1 (Cayman), COX-2 (Oxford), prostacyclin synthase (Cayman), thromboxane synthase (from Dr. W. B. Campbell, Medical College of Wisconsin), and appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma or Zymed). For determinations of COX-1, prostacyclin synthase, and thromboxane synthase we used 15-\( \mu \text{g} \) protein samples. For COX-2 analysis, we used 40-\( \mu \text{g} \) protein samples.

After analyses for eNOS or the prostanoid enzymes, the membranes were reprobed for the smooth muscle cell marker actin (Sigma). All of the reprobed membranes contained whole lung homogenate total protein amounts outside the dynamic range of the immunoblot analysis for actin. Therefore, we performed additional studies to compare actin abundance between whole lung homogenates from shunted and sham-operated piglets. For these studies, we used samples containing total protein amounts within the dynamic range for actin analysis, i.e., 2.5-\( \mu \text{g} \) protein samples. The immunoblot analysis for pulmonary artery homogenate samples. For both size groups of pulmonary artery homogenate samples, procedures similar to those described above for whole lung homogenate samples were applied to frozen samples of 100- to 600-\( \mu \text{m} \)-diameter (small) or 700- to 1,200-\( \mu \text{m} \)-diameter (larger) pulmonary arteries. For eNOS analysis, 2.5-\( \mu \text{g} \) total protein samples were used for both size groups of pulmonary artery homogenates. For all of the prostanoid enzymes, 7.5-\( \mu \text{g} \) total protein samples were used for the pulmonary artery homogenates. After analyses for eNOS or the prostanoid enzymes, the membranes were reprobed for the smooth muscle cell marker actin (Sigma). All of the reprobed membranes contained pulmonary artery homogenate total protein amounts outside the dynamic range of the immunoblot analysis for actin. Therefore, we performed ad

### Table 1. Hemodynamic measurements in living anesthetized piglets

<table>
<thead>
<tr>
<th>Group</th>
<th>Age, days</th>
<th>Weight, kg</th>
<th>Pulmonary Arterial Pressure, cmH_2O</th>
<th>Wedge Pressure, cmH_2O</th>
<th>Aortic Pressure, cmH_2O</th>
<th>Cardiac Output, ml·kg⁻¹·min⁻¹</th>
<th>pH</th>
<th>Po_2, Torr</th>
<th>PcO_2, Torr</th>
<th>Hct, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n = 6)</td>
<td>43 ± 2</td>
<td>7.5 ± 0.6</td>
<td>14.8 ± 0.5</td>
<td>5.2 ± 0.8</td>
<td>107 ± 8</td>
<td>205 ± 19</td>
<td>7.43 ± 0.01</td>
<td>83 ± 2</td>
<td>36 ± 2</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>Shunt (n = 5)</td>
<td>43 ± 2</td>
<td>8.0 ± 0.6</td>
<td>31.1 ± 3.5*</td>
<td>4.7 ± 0.9</td>
<td>98 ± 7</td>
<td>385 ± 48*</td>
<td>7.43 ± 0.01</td>
<td>77 ± 2*</td>
<td>39 ± 2</td>
<td>30 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. *Significantly different from sham, P < 0.05, unpaired t-test.

### Table 2. Perfusion parameters and pulmonary arterial pressures

<table>
<thead>
<tr>
<th>Group</th>
<th>Perfusion Flow Rate, ml/min</th>
<th>Baseline Pulmonary Arterial Pressure, cmH_2O</th>
<th>Elevated Pulmonary Arterial Pressure, cmH_2O</th>
<th>Papaverine Pulmonary Arterial Pressure, cmH_2O</th>
<th>pH</th>
<th>Po_2, Torr</th>
<th>PcO_2, Torr</th>
<th>Hct, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n = 6)</td>
<td>377 ± 34</td>
<td>23 ± 3</td>
<td>41 ± 5</td>
<td>17 ± 1</td>
<td>7.40 ± 0.02</td>
<td>140 ± 6</td>
<td>42 ± 0.2</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>Shunt (n = 5)</td>
<td>404 ± 27</td>
<td>36 ± 5a</td>
<td>36b</td>
<td>22 ± 3a</td>
<td>7.39 ± 0.02</td>
<td>141 ± 9</td>
<td>44 ± 0.2</td>
<td>19 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. *Significantly different from sham, P < 0.05, unpaired t-test. †Elevated and baseline pulmonary arterial pressures are the same for the shunted lungs; see text for details.
ditional studies to compare actin abundance between pulmonary artery homogenates from shunted and sham-operated piglets using samples containing total protein amounts within the dynamic range for actin analysis, i.e., 0.5-µg protein samples.

Statistics. Data are presented as means ± SE. An unpaired t-test was used to compare the data between shunted and sham piglets. P ≤ 0.05 was indicative of statistical significance.

RESULTS

On the day of surgery, the ages and weights of the animals did not differ between piglets used for the sham (age = 5 ± 0.4 days, wt = 1,926 ± 118 g) and shunt (age = 5 ± 0.3 days, wt = 2,302 ± 117 g) operations. Likewise, on the day of the in vivo hemodynamic measurements, there was no difference between ages, weights, or hematocrits of the sham compared with the shunt-operated piglets (Table 1). The measured values of blood pH, PO2, and PCO2 obtained during in vivo hemodynamic measurements in anesthetized animals were similar between the sham and shunted piglets except for a slightly higher PO2 in the sham piglets (Table 1). Measurements of pulmonary arterial pressure, pulmonary wedge pressure, cardiac output, and aortic pressure are summarized in Table 1. Pulmonary arterial pressure was doubled, and cardiac output was 75% greater in shunted than in sham piglets. For the one shunted piglet in which we have measurements, the right ventricle oxygen saturation was 60%, and the distal pulmonary arterial pressure saturation was 86%, such that the pulmonary-to-systemic blood flow was 2.8 to 1 in this piglet.

For the perfused lungs, there was no difference in perfusate hematocrit nor in values of pH, PO2, or PCO2 measured in the perfusate of isolated lungs from sham and shunted piglets (Table 2). In the perfused lungs, left atrial pressure was zero, and perfusate flow was the same for both groups (Table 2) and was maintained constant throughout the study so that changes and differences in pulmonary arterial pressure, respectively, represent changes and differences in pulmonary vascular resistance. The baseline pulmonary arterial pressure was greater in isolated perfused lungs of the shunted piglets compared with the sham piglets (Table 2). After the addition of KCl, the pulmonary arterial pressure in the sham group was elevated to a level

Fig. 2. ACh-induced changes in pulmonary arterial pressure in perfused lungs of sham-operated and shunted piglets. Data are means ± SE. *Different from sham, P < 0.05.

Fig. 3. Papaverine-induced changes in pulmonary arterial pressure in perfused lungs of sham-operated and shunted piglets. Data are means ± SE. *Different from sham, P < 0.05.

Fig. 4. ACh-induced changes in pulmonary arterial pressure expressed relative to dilation to the non-endothelium-dependent agent papaverine. Data are means ± SE. *Different from sham, P < 0.05.

Fig. 5. eNOS immunoblots in whole lung homogenates (A) and the following homogenates of two size groups of small pulmonary arteries: smaller pulmonary arteries = 100–600 µm in diameter (B) and larger pulmonary arteries = 700–1,200 µm in diameter (C). +, Positive control for eNOS (obtained from Transduction Laboratories). Note that there is no apparent difference in the intensity of the eNOS bands between sham-operated and shunted piglets for any of the lung tissue homogenates.
homogenates at an apparent molecular mass of 60 kDa and thromboxane synthase detected thromboxane synthase in all homogenates at an apparent molecular mass of 52 kDa (Fig. 7); and the antibody to thromboxane synthase detected prostacyclin synthase in all homogenates at an apparent molecular mass of 72 kDa (Fig. 6); the antibody to thromboxane synthase detected COX-2 in all homogenates at an apparent molecular mass of 70 kDa (Fig. 6); the antibody to thromboxane synthase detected COX-1 in all homogenates at an apparent molecular mass of 60 kDa (Fig. 7). Similar to findings for eNOS, as determined by densitometry, there was no difference in the absorbance of bands for any of the prostanoid enzymes for whole lung homogenates or for homogenates of either size group of small pulmonary arteries from shunted compared with sham piglets (Table 3).

Figure 8, A–C, shows representative immunoblot analyses for actin in reprobed whole lung and reprobed pulmonary artery homogenates. Figure 8, D–F, shows immunoblot analyses for actin abundance in the whole pulmonary arterial pressure in the lungs of sham piglets was greater than the pulmonary arterial pressure in the lungs of shunted piglets (Table 2). Of note, after the addition of papaverine and removal of vasomotor tone, the pulmonary arterial pressure in the lungs of shunted piglets was greater than the pulmonary arterial pressure in the lungs of sham piglets (Table 2).

Immunoblot analyses for eNOS in the whole lung homogenates and both size groups of small pulmonary artery homogenates (small pulmonary arteries 100–600 μm in diameter and larger pulmonary arteries 700–1,200 μm in diameter) are shown in Fig. 5. The antibody to eNOS detected eNOS in all homogenates at an apparent molecular mass of 135 kDa as determined from an exponential fit of molecular mass standards. The absorbance of the eNOS bands as determined by densitometry did not differ between shunted and sham piglets for either whole lung homogenates or for homogenates of either size group of small pulmonary arteries (Table 3).

Immunoblot analyses for the prostanoid enzymes COX-1, COX-2, prostacyclin synthase, and thromboxane synthase in the whole lung homogenates and both size groups of small pulmonary artery homogenates (small pulmonary arteries 100–600 μm in diameter and larger pulmonary arteries 700–1,200 μm in diameter) are shown in Figs. 6 and 7. The antibody to COX-1 detected COX-1 in all homogenates at an apparent molecular mass of 70 kDa (Fig. 6); the antibody to COX-2 detected COX-2 in all homogenates at an apparent molecular mass of 72 kDa (Fig. 6); the antibody to prostacyclin synthase detected prostacyclin synthase in all homogenates at an apparent molecular mass of 52 kDa (Fig. 7); and the antibody to thromboxane synthase detected thromboxane synthase in all homogenates at an apparent molecular mass of 60 kDa (Fig. 7). Values are means ± SE. eNOS, endothelial nitric oxide synthase; COX, cyclooxygenase; PA, pulmonary artery.

Table 3. Densitometry for eNOS, prostanoid enzymes, and actin

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>eNOS</th>
<th>COX-1</th>
<th>COX-2</th>
<th>Prostacyclin synthase</th>
<th>Thromboxane synthase</th>
<th>Reprobed actin</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham whole lung</td>
<td>1.9 ± 0.2</td>
<td>0.8 ± 0.04</td>
<td>0.8 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Shunt whole lung</td>
<td>1.8 ± 0.1</td>
<td>0.9 ± 0.02</td>
<td>0.5 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.8 ± 0.1</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Sham small PA</td>
<td>2.9 ± 0.6</td>
<td>1.5 ± 0.04</td>
<td>0.7 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>2.9 ± 0.1</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Shunt small PA</td>
<td>3.0 ± 0.1</td>
<td>1.4 ± 0.04</td>
<td>0.4 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.3</td>
<td>3.0 ± 0.1</td>
<td>1.9 ± 0.1</td>
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<tr>
<td>Sham larger PA</td>
<td>3.0 ± 0.1</td>
<td>1.0 ± 0.05</td>
<td>0.5 ± 0.3</td>
<td>1.8 ± 0.1</td>
<td>0.4 ± 0.3</td>
<td>3.1 ± 0.1</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Shunt larger PA</td>
<td>3.2 ± 0.1</td>
<td>1.1 ± 0.09</td>
<td>0.5 ± 0.3</td>
<td>1.6 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>3.2 ± 0.1</td>
<td>2.2 ± 0.1</td>
</tr>
</tbody>
</table>

Comparative sizes of pulmonary arteries: small pulmonary arteries: 100–600 μm in diameter; larger pulmonary arteries: 700–1,200 μm in diameter. Positive control for COX-2 (obtained from Cayman Laboratories). Positive control for COX-1 (obtained from Cayman Laboratories).
lung homogenates and both size groups of small pulmonary artery homogenates using samples containing total protein amounts within the dynamic range of the actin immunoblot analyses. The antibody to actin detected actin in all homogenates at an apparent molecular mass of 42 kDa as determined from an exponential fit of molecular mass standards. For the reprobed homogenate samples, the absorbance of the actin bands did not differ between shunted and sham piglets for either reprobed whole lung homogenates or for either size group of reprobed small pulmonary arteries (Table 3). Moreover, there was no difference in the absorbance of bands for actin for whole lung homogenates or for homogenates of either size group of small pulmonary arteries from samples of shunted compared with sham piglets containing total protein amounts within the dynamic range for actin analysis (Table 3). Thus, actin abundance was unaltered in lung tissue samples from shunted piglets, and normalization of the eNOS and prostanoid enzyme data for actin amounts does not change the results (Table 3).

DISCUSSION

The major findings in this study were that 5–6 wk after placement of an aortopulmonary shunt in newborn piglets, amounts of eNOS and of the prostanoid enzymes COX-1, COX-2, prostacyclin synthase, and thromboxane synthase were unaltered in distal lung tissue and in two different size groups of small pulmonary arteries (100–600 μm in diameter) and larger pulmonary arteries (700–1,200 μm in diameter). Other important findings were that pulmonary vascular resistance was greater and pulmonary vascular dilation to both an endothelium-dependent and an endothelium-independent agent was less in isolated lungs of shunted piglets than in isolated lungs of sham-operated piglets. Moreover, in vivo measurements in anesthetized piglets showed that pulmonary arterial pressure was elevated in the shunted animals. Together, these findings suggest that mechanisms other than altered amounts of either eNOS or the prostanoid enzymes COX-1, COX-2, prostacyclin synthase, and thromboxane synthase underlie the elevated pulmonary arterial pressure and the blunted dilation that develop in lungs of newborn piglets 5–6 wk after aortopulmonary shunt placement.

Other investigators have studied animals with aortopulmonary shunts to investigate the development of pulmonary hypertension resulting from high pulmonary blood flow. Previous investigators surgically placed aortopulmonary shunts in piglets aged 1–3 mo and studied them 1–3 mo later (10, 22, 26). Their findings provided evidence that pulmonary hypertension develops when aortopulmonary shunts are placed in growing piglets (10, 22, 26). In contrast, experimental attempts to induce high-flow pulmonary hypertension with surgically placed shunts in adult animals have met with less success (16, 20). The presence of low pulmonary vascular resistance at surgical placement, making the animals susceptible to developing fatal congestive heart failure, is believed to be one of the reasons that pulmonary hypertension is difficult to induce with shunts in adult animals. We chose to surgically place aortopulmonary shunts in piglets at an age before their pulmonary vascular resistance decreases to adult levels (25) but at an age allowing for...
acclimation to the experimental environment before surgery.

Similar to previous findings with shunted growing piglets (10, 22, 26), we found that pulmonary arterial pressure was significantly elevated in anesthetized piglets studied 5–6 wk after aortopulmonary shunt placement. A limitation of our study is that pulmonary blood flow was not measured in vivo. Our finding that cardiac output was 75% greater in shunted piglets than in sham-operated piglets supports the presence of elevated pulmonary blood flow in the shunted piglets. That pulmonary hypertension developed is supported by the elevated pulmonary vascular resistance that we measured in isolated, perfused lungs of the shunted piglets. Structural remodeling in the shunted lungs (22, 26) may contribute to the elevated pulmonary arterial pressure in isolated, perfused lungs of the shunted piglets that persists after removal of vasomotor tone with papaverine.

Our finding of reduced responses to both the endothelium-dependent agent ACh and the endothelium-independent agent papaverine suggests that impaired smooth muscle dilation and not pulmonary vascular endothelial dysfunction underlies altered pulmonary vascular responses in the shunted piglets. By comparison, impaired responses to endothelium-dependent agents but preserved responses to endothelium-independent agents have been demonstrated both in children aged 3–12 yr with left-to-right shunts and increased pulmonary blood flow (7) and in 4-wk-old newborn lambs that had undergone in utero surgical placement of aortopulmonary shunts (24).

The mechanisms underlying the diminished dilation to papaverine, i.e., impaired smooth muscle dilation, in the shunted piglets are not clear. Papaverine is a nonselective phosphodiesterase inhibitor (21). It is possible that the ability to elevate cAMP and/or cGMP is impaired in pulmonary vascular smooth muscle of the shunted piglets. However, papaverine may also cause dilation by nonselective inhibition of voltage-activated calcium channels (18). Either possibility is consistent with our findings of unaltered amounts of either eNOS or prostanoid enzymes in lung and vessel homogenates of the shunted piglets. In other words, our findings suggest that a mechanism downstream from altered synthesis of NO, prostacyclin, and thromboxane underlies the blunted dilation that develops in newborn piglets with shunt-induced pulmonary hypertension.

Like us, other investigators found no change in lung tissue eNOS amounts resultant from altered flow in studies with 6-wk-old piglets (22), adult rats (12), and fetal lambs (29). In comparison, eNOS abundance was increased in distal lung tissue and in fifth-generation pulmonary arteries of 4-wk-old newborn lambs who had undergone in utero surgical placement of aortopulmonary shunts (6). The different results could be due to use of different species and ages of animals and differences in experimental conditions, such as the magnitude and duration of changes in pulmonary blood flow.

Although a number of studies have evaluated eNOS abundance in both high flow-induced (6, 12, 22) and other models of pulmonary hypertension (14, 27, 32), the influence of prostanoid enzymes has received little attention (8, 30). Yet, it has long been hypothesized that altered production of prostanoid metabolites could contribute to the pathogenesis of pulmonary hypertension in both newborns and adults (1–3, 5, 8, 9, 15, 30). In this regard, it was recently reported that amounts of COX-2, but not of COX-1, were increased in lungs of adult rats with chronic hypoxia-induced pulmonary hypertension (8). Other investigators recently reported decreased prostacyclin synthase expression in lungs from patients with severe pulmonary hypertension (30). Our findings do not support an important role for either a decrease or an increase in the abundance of either COX-1, COX-2, prostacyclin synthase, or thromboxane synthase and the elevation in pulmonary arterial pressure found in newborn piglets with aortopulmonary shunts for 5–6 wk.

Limitations in use of the immunoblot technique to assess protein abundance must be acknowledged. For example, potential changes in lung tissue composition in remodeled lungs make it difficult to know what...
protein to use as an internal control. It is important that the internal control remain unaltered with the experimental conditions. Our finding that actin did not differ between lung tissue samples from shunted and sham lungs supports the use of actin as an internal control. Unfortunately, the amount of total protein needed to detect eNOS and prostanoid enzymes was outside the dynamic range for actin immunoblot analysis. Thus it is possible, although unlikely, that differences in actin amounts were undetected in the “re-probed” samples.

In summary, our findings suggest that blunted pulmonary vascular responses and pulmonary hypertension develop in piglets 5–6 wk after placement of an aortopulmonary shunt because of impaired smooth muscle dilation and not because of altered abundances of eNOS or the prostanoid enzymes COX-1, COX-2, prostacyclin synthase, and thromboxane synthase. Future studies are needed to clarify the mechanisms for the impaired smooth muscle dilation.

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