Alterations in Endogenous Nitric Oxide Production After Cardiopulmonary Bypass in Lambs With Normal and Increased Pulmonary Blood Flow

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Background—After cardiopulmonary bypass (CPB), altered vascular reactivity is a major source of complications, particularly for children with increased pulmonary blood flow. Although changes in agonist-induced NO activity are well described after CPB, potential changes in basal NO production and their role in post-CPB pulmonary hypertension remain unclear. By using aortopulmonary vascular graft placement in the fetal lamb (shunt lambs), we established a unique model of pulmonary hypertension that mimics congenital heart disease with increased pulmonary blood flow. The objective of the present study was to investigate potential alterations in endogenous NO production after CPB in lambs with normal and increased pulmonary blood flow.

Methods and Results—Vascular pressures and blood flows were monitored in 1-month-old lambs (n = 7) with increased pulmonary blood flow and 6 age-matched control lambs. After shunt closure, hypothermic CPB (25°C) was performed for 2 hours. The hemodynamic variables were monitored for 4 hours after CPB. Before, during, and after CPB, peripheral lung biopsies were performed to determine tissue NO, nitrite, nitrate, and cGMP concentrations; total NO synthase (NOS) activity; and endothelial NOS protein levels. Hypothermic CPB increased both mean pulmonary arterial pressure and left pulmonary vascular resistance (P < 0.05). The increase in pulmonary arterial pressure induced in shunt lambs was greater than that induced in control lambs (P < 0.05). Four hours after CPB, tissue concentrations of NO, nitrite, nitrate, and cGMP were decreased to ≈70% of pre-CPB levels in both control and shunt lambs (P < 0.05). Total NOS activity and endothelial NOS protein levels were unchanged.

Conclusions—Modest decreases in basal NO production, the inability to increase NO production, or both may play a role in the altered pulmonary vascular reactivity after CPB. The decrease in NO is independent of gene expression. However, other mechanisms for this decrease, such as substrate or cofactor availability, warrant further study. (Circulation. 2000;102[suppl III]:III-172-III-178.)

Key Words: nitric oxide ■ cardiopulmonary bypass ■ hypertension ■ lungs ■ endothelium

The development of pulmonary hypertension is a common accompaniment of congenital heart disease with increased pulmonary blood flow.1 Although early surgical repair of these congenital heart defects has decreased the incidence of irreversible pulmonary vascular disease, children with reversible vascular changes experience complications and death in the postoperative period secondary to elevations in pulmonary vascular resistance and increased pulmonary vascular reactivity immediately after cardiopulmonary bypass (CPB).2 Although increases in pulmonary vascular resistance and pulmonary vascular reactivity after CPB are well described, their mechanisms are not completely understood.

Recent evidence suggests that pulmonary vascular tone is regulated through a complex interaction of vasoactive substances that are locally produced by the vascular endothelium.3 During CPB, pulmonary vascular endothelial injury secondary to a variety of factors, including the disruption of normal pulmonary blood flow, complement activation, and neutrophil activation, may contribute to post-CPB pulmonary hypertension.4 NO is a labile humoral factor that is synthesized from the oxidation of the guanidino nitrogen moiety of l-arginine after the activation of NO synthase (NOS).5 Three isofoms of NOS are known. Constitutive forms are present in endothelial cells (endothelial NOS [eNOS]) and neurons (neuronal NOS), and a third inducible isoform is present in macrophages (inducible NOS [iNOS]).6 Pulmonary vascular endothelial cells synthesize NO after certain stimuli, such as shear stress and the receptor binding of specific vasodilators, which activate eNOS. Once released from endothelial cells, NO diffuses into adjacent vascular smooth muscle cells, where it activates soluble guanylate cyclase. This results in an increase in intracellular cGMP and initiates a cascade that leads to smooth muscle relaxation.7

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Endogenously produced endothelium-derived NO is an important mediator of normal pulmonary vascular tone and vascular reactivity, and aberrations in basal NO production have been implicated in the pathophysiology of pulmonary hypertensive disorders. Decreases in agonist-induced NO activity have been demonstrated after CPB, suggesting a role for decreased NO in post CPB pulmonary hypertension. However, previous studies of the changes in basal NO production induced by CPB, as determined on the basis of plasma levels of NO metabolites, have been inconsistent.

We established a model of pulmonary hypertension with increased pulmonary blood flow in the lamb, after in utero placement of an aorta-to-pulmonary vascular graft. At 1 month of age, these lambs (shunt lambs) have a pulmonary-to-systemic blood flow ratio of ~2:2:1, a mean pulmonary arterial pressure that is 75% of mean systemic arterial pressure, and pulmonary vascular remodeling characteristic of that of children with pulmonary hypertension and increased pulmonary blood flow. Previously, we have shown that shunt lambs have increased basal NO production compared with age-matched control animals and a greater increase in pulmonary vascular resistance after CPB. The purpose of the present study was to characterize potential alterations in endogenous NO production after hypothermic CPB in lambs with normal and increased pulmonary blood flow and to assess their potential role in post-CPB pulmonary hypertension. To accomplish this, we monitored the hemodynamic response of the pulmonary circulation in seven 1-month-old shunt lambs and 6 age-matched control animals for 120 minutes during hypothermic CPB and for 4 hours after CPB. Before, during, and after CPB, peripheral lung biopsy samples were obtained to measure tissue NO, nitrate, and nitrate (NOx) concentrations (a measure of total NO production), tissue cGMP concentrations, exhaled NO concentrations, tissue NOS activity (by the conversion of 3H-arginine to 3H-citrulline), and eNOS protein levels (by Western blot analysis).

Methods

Surgical Preparations and Care

Ewes

Seven mixed-breed Western pregnant ewes (136.6±3.2 days' gestation, term = 145 days) were operated on under sterile conditions as previously described. Through a left lateral fetal thoracotomy, an 8.0-mm expanded polytetrafluoroethylene (Gore-Tex) vascular graft (~2-mm length; W.L. Gore and Associates) was anastomosed between the ascending aorta and main pulmonary artery of the fetus with 7-0 prolene (Ethicon Inc), with a continuous suture technique as previously described. After recovery from anesthesia, the ewe was returned to the cage with free access to food and water. Antibiotics (1 million U penicillin G potassium and 100 mg gentamicin sulfate) were administered to the ewes during surgery and daily thereafter until 2 days after spontaneous delivery of the lamb.

Lambs

After spontaneous delivery, antibiotics (1 million units of penicillin G potassium and 25 mg of gentamicin sulfate IM) were administered for 2 days. The lambs were weighed daily, and the respiratory rate and heart rate were obtained. Furosemide (1 mg/kg IM) was administered daily.

At 1 month of age, 7 shunted lambs and 6 age-matched control lambs were anesthetized with ketamine hydrochloride (~1 mg/kg) and diazepam (0.002 mg · kg⁻¹ · min⁻¹) and mechanically ventilated as previously described. An intravenous infusion of lactated Ringer’s and 5% dextrose (75 mL/h) was begun and continued throughout the study period. Cefazolin (500 mg IV) and gentamicin (3 mg/kg IV) were administered before the first surgical incision. The lambs were maintained normothermic (37°C) with a heating blanket. With strict aseptic technique, a midsternotomy incision was then made, and the pericardium was incised. Polyurethane catheters were inserted into the left and right atria and the main pulmonary artery distal to the vascular graft. An ultrasonic flow probe (Transonic Systems Inc) was placed around the left pulmonary artery to measure left pulmonary blood flow after a 30-minute recovery. Blood was obtained from the left and right atria, distal pulmonary artery, right ventricle, and descending aorta for hemoglobin and oxygen saturation determinations. In shunted lambs, a vascular clamp was then placed on the graft to completely occlude it, and oxygen saturation determinations were made from the right ventricle and distal pulmonary artery to document shunt closure. The sternum was then temporarily approximated with towel clamps.

CPB Procedure

The bypass circuit was similar to the standard neonatal circuit. It consisted of a membrane oxygenator (Minimax; Medtronic), an infant venous reservoir (Medtronic), an arterial filter (40 μm; Bentley), and a cardiotomy reservoir and suction. An ultrasonic flow probe (Transonic Systems) was incorporated into the circuit to continuously monitor pump flows. The circuit was primed with fresh heparinized sheep whole blood (400 mL), electrolyte solution (600 mL Normosol; Abbott Labs), heparin (2500 U), sodium bicarbonate (10 mEq), prednisolone sodium succinate (30 mg/kg Solu-Medrol), and cefazolin sodium (25 mg/kg Kefzol). The bypass methodology was similar to standard neonatal methods. Heparin (300 U/kg) was administered to the lamb into the right atrium. Right atrial venous cannulation was performed with a 16F to 20F venous cannula (DLB Inc). The ascending aorta was cannulated with a 14F cannula (Electro-catheter Corp). CPB was begun, and surface and core cooling was initiated. The sternum was again temporarily approximated. Normothermic flows ranged from 150 to 200 mL · kg⁻¹ · min⁻¹. The lambs were cooled to 25°C, and flow was reduced to 100 mL · kg⁻¹ · min⁻¹. After 120 minutes at 25°C, rewarming was started. Throughout the CPB period, an α-stat blood gas strategy was maintained, whereby the temperature-uncorrected PaCO₂ was maintained near 40 mm Hg (measured at 37°C) and the temperature-uncorrected pH was maintained near 7.40, regardless of body temperature. Mannitol (0.5 g/kg) and furosemide (0.5 mg/kg) were added to the prime at the onset of rewarming. After the core temperature reached 32°C, calcium gluconate (1 g) was added to the prime. After the lambs were rewarmed, ventilation was resumed, and the animals were weaned off of CPB. Heparin was completely reversed with protamine (3 mg/kg) administered into the left atrium.

Experimental Protocol

Sixty minutes after shunt and chest closure, baseline measurements of the hemodynamic variables (pulmonary and systemic arterial pressure, heart rate, pulmonary blood flow, left and right atrial pressures) and systemic arterial blood gases and pH were measured. A peripheral lung wedge biopsy was obtained to determine nitrate and cGMP concentrations, eNOS activity, and eNOS protein (before CPB), and CPB was begun. A side-biting vascular clamp was used to isolate peripheral lung tissue from a randomly selected lobe, and the incision was cauterized. Approximately 500 mg of peripheral lung was obtained for each biopsy. The hemodynamic variables were monitored continuously before, during, and 4 hours after CPB. Systemic arterial blood gases were determined intermittently, and ventilation was adjusted to achieve a PaCO₂ between 35 and 45 mm Hg and a PaO₂ of >50 mm Hg. Sodium bicarbonate was administered intermittently to maintain pH >7.30. Normal saline and red blood cells were administered to maintain atrial pressures and hemoglobin concentrations at pre-CPB levels. Peripheral lung wedge biopsies were again performed during and after CPB. In 5 shunt lambs, breath-to-breath determinations of exhaled NO concentrations were also determined. Four hours after CPB, all lambs were killed with a lethal injection of sodium pentobarbital followed by bilateral thoracotomy as described in the NIH Guidelines for the
Measurements
Pulmonary and systemic arterial and right and left atrial pressures were measured with Statham Instruments P23Db pressure transducers. Mean pressures were obtained through electrical integration. Heart rate was measured with a cardiograph triggered from the phasic systemic arterial pressure pulse wave. Left pulmonary blood flow and bypass flows were measured with an ultrasonic flowmeter (Transonic Systems). All hemodynamic variables were recorded continuously with a Gould multichannel electrostatic recorder. Systemic arterial blood gases and pH were measured with a Corning 158 pH/blood gas analyzer (Corning Medical and Scientific). Hemoglobin concentration and oxygen saturation were measured with a hemoximeter (model OSM 2; Radiometer). The ratio of pulmonary to systemic blood flow (Qp/Qs) was calculated with the Fick equation. Pulmonary vascular resistance was calculated with standard formulas.

NOx Determinations
Peripheral lung tissue was snap frozen in liquid nitrogen and stored at −70°C. Lung tissue was homogenized in 6% trichloroacetic acid at 4°C to provide a 10% weight-to-volume homogenate and centrifuged at 3000 rpm for 15 minutes at 4°C. The supernatant was recovered and used immediately for analysis. In solution, NO reacts with molecular oxygen to form nitrite and with oxyhemoglobin and superoxide anion to form nitrate. The nitrite and nitrate were reduced with vanadium(III) and hydrochloric acid at 90°C. NO was then purged from solution, resulting in a peak of NO. Therefore, this value represents a combination of total NO, nitrite, and nitrate (NOx).

Exhaled NO Determinations
Expiratory gas was sampled continuously from a side port attached to the ventilator circuit at a rate of 200 mL/min. Exhaled NO was measured with a chemiluminescence analyzer (NOA280; Sievers Instruments Inc). The detection limit is 1 nmol L−1·ml nitrate−1.

Exhaled NO Determinations
Peripherally lung tissue was snap frozen in liquid nitrogen and stored at −70°C. Tissue was homogenized in 6% trichloroacetic acid at 4°C to provide a 10% weight-to-volume homogenate and centrifuged at 2000 rpm for 15 minutes at 4°C. The supernatant was recovered and washed 4 times with 5 vol of water-saturated diethyl ether. Aqueous extracts were then lyophilized, and the dried pellet was resuspended in assay buffer before analysis. The samples were assayed with a cGMP radioimmunoassay kit (Amersham International plc) according to the manufacturer's instructions. Cross-reactivity for other nucleotides is <0.001.

Assay for NOS Activity
This assay was performed with the conversion of 3H-arginine to 3H-citrulline as a measure of NOS activity essentially as described by Bush et al.21 Briefly, lung tissues were homogenized in NOS assay buffer (50 mmol/L Tris-HCl, pH 7.5, containing 0.1 mmol/L EDTA and 0.1 mmol/L EGTA) with a protease inhibitor cocktail. Enzymatic reactions were carried out at 37°C in the presence of total lung protein extracts (500 μg), 1 mmol/L NADPH, 14 μmol/L tetrahydribopterin, 100 μmol/L FAD, 1 mmol/L MgCl2, 5 μmol/L unlabeled L-arginine, 15 mmol/L 3H-arginine, 25 μmol/L HCl, 25 μmol/L L-citrulline, 2 mmol/L EDTA, and 0.2 mmol/L EGTA) and then applied to columns containing 1 mL of Dowex AG50W-X8 resin, Na+ form, pre-equilibrated with 1 N NaOH. H-Citrulline was then quantified with scintillation counting. In 5 shunt lambs, parallel assays with and without calcium were performed to distinguish between Ca2+-dependent, constitutive NOS activity and Ca2+-independent, iNOS activity.

Preparation of Protein Extracts and Western Blot Analysis
Lung protein extracts were prepared by homogenizing peripheral lung tissues in Triton lysis buffer (50 mmol/L Tris-HCl, pH 7.6, 0.5% Triton X-100, 20% glycerol) containing a protease inhibitor cocktail. Extracts were then clarified through centrifugation (15 000g for 10 minutes at 4°C). Supernatant fractions were then quantified for protein concentration with the Bradford reagent (BioRad). Extracts were then diluted in a 1:1 dilution with assay buffer (20 mmol/L sodium acetate, pH 5, 1 mmol/L L-citrulline, 2 mmol/L EDTA, and 0.2 mmol/L EGTA) and then applied to columns containing 1 mL of Dowex AG50W-X8 resin, Na+ form, pre-equilibrated with 1 N NaOH. H-Citrulline was then quantified with scintillation counting. In 5 shunt lambs, parallel assays with and without calcium were performed to distinguish between Ca2+-dependent, constitutive NOS activity and Ca2+-independent, iNOS activity.

Statistical Analysis
The mean±SD values were calculated for the baseline hemodynamic variables, systemic arterial blood gases and pH, lung nitrate and cGMP concentrations, and lung NOS activity. Comparisons over time (within each group of lambs) were made by ANOVA for repeated measures with multiple comparison testing. Comparisons between the 2 groups of lambs (control and shunt) were made by the unpaired t-test. Correlations between the mean pulmonary arterial pressure and nitrate concentrations, induced by CPB, were sought by linear regression analysis.

Quantification of autoradiographic results was performed through scanning (SCA Jet IICX; Hewlett Packard Inc) the bands of interest into an image editing software program (Adobe Photoshop; Adobe Systems). Band intensities from Western blot analysis were analyzed densitometrically on a Macintosh computer (model 9500; Apple Computer, Inc) using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). Results from pre-NO CPB were assigned the value of 1 (relative eNOS protein). The mean±SEM values were calculated for the relative protein at each time point after the start of CPB. Comparisons over time were made by ANOVA for repeated measures. A values of P<0.05 was considered statistically significant.

Results
All shunted lambs had an audible continuous murmur and an increase in oxygen saturation between the right ventricle and distal pulmonary artery. Their pulmonary-to-systemic blood flow ratio was 2.65±0.8. Before closure of the shunt, shunted lambs had increased mean pulmonary arterial pressure
TABLE 1. Hemodynamic Changes After CPB in Shunt and Control Lambs

<table>
<thead>
<tr>
<th></th>
<th>Before CPB</th>
<th>15</th>
<th>30</th>
<th>60</th>
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<tr>
<td>Pulmonary arterial pressure, mm Hg</td>
<td></td>
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<tr>
<td>Control</td>
<td>15.3 ± 3.7</td>
<td>20.5 ± 2.6*</td>
<td>19.0 ± 1.6*</td>
<td>18.3 ± 1.5*</td>
<td>16.5 ± 1.5</td>
</tr>
<tr>
<td>Shunt</td>
<td>16.4 ± 4.8</td>
<td>26.6 ± 5.5*</td>
<td>24.3 ± 3.5*</td>
<td>23.4 ± 4.4*</td>
<td>20.3 ± 5.7*</td>
</tr>
<tr>
<td>Left pulmonary vascular resistance, mm Hg · mL⁻¹ · min⁻¹ · kg⁻¹</td>
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<tr>
<td>Control</td>
<td>0.297 ± 0.05</td>
<td>0.266 ± 0.13*</td>
<td>0.288 ± 0.09*</td>
<td>0.316 ± 0.05*</td>
<td>0.300 ± 0.05</td>
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<tr>
<td>Shunt</td>
<td>0.196 ± 0.05</td>
<td>0.335 ± 0.15*</td>
<td>0.348 ± 0.16*</td>
<td>0.344 ± 0.10*</td>
<td>0.310 ± 0.07*</td>
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<tr>
<td>Left pulmonary blood flow, mL · kg⁻¹ · min⁻¹</td>
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<tr>
<td>Control</td>
<td>36.0 ± 17.2</td>
<td>53.4 ± 13.9*</td>
<td>46.4 ± 10.1</td>
<td>38.7 ± 6.2</td>
<td>36.6 ± 9.2</td>
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<tr>
<td>Shunt</td>
<td>64.2 ± 17.3</td>
<td>66.8 ± 19.8</td>
<td>61.4 ± 19.6</td>
<td>54.0 ± 11.1</td>
<td>47.0 ± 14.5*</td>
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<td>Systemic arterial pressure, mm Hg</td>
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<tr>
<td>Control</td>
<td>68.1 ± 10.0</td>
<td>70.0 ± 15.0</td>
<td>68.0 ± 15.9</td>
<td>63.0 ± 17.8</td>
<td>51.0 ± 11.0*</td>
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<td>Shunt</td>
<td>76.6 ± 9.6</td>
<td>66.6 ± 12.9*</td>
<td>61.4 ± 14.8*</td>
<td>63.7 ± 16.3*</td>
<td>51.4 ± 12.8*</td>
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<td>Heart rate, bpm</td>
<td></td>
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<tr>
<td>Control</td>
<td>155.5 ± 21.9</td>
<td>161.0 ± 34.6</td>
<td>159.8 ± 35.3</td>
<td>163.6 ± 29.2</td>
<td>154.8 ± 29.3</td>
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<td>Shunt</td>
<td>159.4 ± 24.9</td>
<td>153.6 ± 30.0</td>
<td>148.1 ± 27.0</td>
<td>134.4 ± 25.6*</td>
<td>133.4 ± 28.9*</td>
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<tr>
<td>Left atrial pressure, mm Hg</td>
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<tr>
<td>Control</td>
<td>5.0 ± 1.1</td>
<td>7.6 ± 1.5*</td>
<td>6.3 ± 1.5*</td>
<td>6.0 ± 1.7</td>
<td>5.8 ± 1.2</td>
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<tr>
<td>Shunt</td>
<td>4.0 ± 1.1</td>
<td>6.0 ± 1.9*</td>
<td>5.3 ± 1.5</td>
<td>6.3 ± 2.1*</td>
<td>5.9 ± 2.4*</td>
</tr>
<tr>
<td>Right atrial pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.2 ± 1.0</td>
<td>6.0 ± 0.9*</td>
<td>5.0 ± 0.9*</td>
<td>4.8 ± 1.2</td>
<td>4.5 ± 1.0</td>
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<tr>
<td>Shunt</td>
<td>5.4 ± 1.9</td>
<td>7.0 ± 1.8*</td>
<td>6.3 ± 1.2</td>
<td>6.7 ± 1.7</td>
<td>6.0 ± 2.0</td>
</tr>
</tbody>
</table>

Values are mean ± SD for 6 control lambs and 7 shunt lambs. *P<0.05 vs corresponding pre-CPB values (ANOVA). Pre-CPB values in shunt lambs were obtained after graft closure.

(24.0 ± 7.7 versus 15.3 ± 3.7 mm Hg), left pulmonary blood flow (157.6 ± 24.2 versus 36.0 ± 17.1 mL · kg⁻¹ · min⁻¹), and left atrial pressures (8.0 ± 3.1 versus 5.0 ± 1.0 mm Hg) compared with control lambs (P<0.05). Left pulmonary vascular resistance (0.100 ± 0.03 versus 0.297 ± 0.05 mm Hg · ml⁻¹ · min⁻¹ · kg⁻¹), mean systemic arterial pressure (52.6 ± 9.6 versus 68.1 ± 10.0 mm Hg), and body weight (12.3 ± 2.7 versus 14.2 ± 3.1 kg) were decreased in shunt lambs (P<0.05). There were no differences in systemic arterial blood gases and pH between the 2 groups, and all values were within the normal range for the laboratory. There also were no differences in total CPB time (152.1 ± 6.3 versus 149.5 ± 10.8 minutes).

In control lambs, CPB induced mild increases in mean pulmonary arterial pressure and left pulmonary vascular resistance within the first hour (P<0.05) but were unchanged from baseline after 4 hours. Left pulmonary blood flow was increased at 15 minutes, and mean systemic arterial pressure was decreased at 4 hours (P<0.05) (Table 1). In contrast, mean pulmonary arterial pressure and left pulmonary vascular resistance increased significantly in shunt lambs after CPB and remained increased throughout the study period. In addition, left pulmonary blood flow and systemic arterial pressure decreased in shunt lambs (P<0.05) (Table 1). After CPB, the mean pulmonary arterial pressure was greater in shunt lambs than in control lambs (P<0.05) (Figure 1). In both groups of lambs, systemic arterial blood gases and pH remained within normal limits for the laboratory (data not shown).

In both control and shunt lambs, tissue NOx and cGMP concentrations decreased after CPB (P<0.05) (Table 2, Figures 2 and 3). The tissue NOx concentration did not correlate with the mean pulmonary arterial pressure or pulmonary vascular resistance after CPB. In shunt lambs, exhaled NO concentrations decreased from 3.6 ± 3.4 to 0.2 ± 0.2 ppb after CPB (P<0.05) (Figure 4).

To determine potential mechanisms for the decrease in NO production after CPB, lung tissue NOS activity and eNOS protein levels were obtained. In both control and shunt lambs, total NOS activity was unchanged after CPB (Table 2) (Figure 5). Parallel assays with and without Ca²⁺ in 5 shunt lambs also demonstrated no changes in either the Ca²⁺-dependent or the Ca²⁺-independent NOS activity fractions after CPB (data not shown). Western blot analysis demonstrated no change in eNOS protein levels after CPB in both shunt and control lambs (Figures 6A and 6B). Additional Western blot analysis determined that the iNOS isof orm was unchanged after CPB (data not shown).

We previously demonstrated that instrumentation, mechanical ventilation, and serial lung biopsies alone, without CPB, do not change the hemodynamic variables or NO-cGMP production during a 24-hour study period.²²

Discussion

The present study demonstrates that hypothermic CPB induces modest decreases in endogenous NO production in both the normal and abnormal pulmonary circulation. In
1-month-old lambs with normal or preexisting increased pulmonary blood flow, lung tissue concentrations of NO metabolites and cGMP (the second messenger of NO-mediated vasodilation) decreased after CPB to \( \frac{70}{\text{pre-CPB values}} \). In addition, exhaled NO (an indicator of pulmonary NO production) decreased to \( \frac{20}{\text{pre-CPB values}} \) in shunt lambs. Lung tissue NOS activity and eNOS protein levels were unchanged, suggesting that the decrease in NO production is independent of changes in NOS activity or gene expression. The decrease in basal NO-cGMP concentrations did not correlate with the increase in mean pulmonary arterial pressure after CPB, suggesting that the decrease in NO plays a minor, if any, role in the increase in pulmonary vascular tone after CPB. However, because increases in NO production modulate pulmonary vasoconstriction, these moderate decreases in NO or the inability to increase NO production, or both, may participate in the altered pulmonary vascular reactivity noted after CPB.

The role of altered endothelial function in the pathophysiology of post-CPB pulmonary hypertension is well established.\textsuperscript{4} For example, endothelin-1 (ET-1) levels are consistently increased after CPB. Their levels correlate with the degree of post-CPB pulmonary hypertension, and ET receptor blockade completely blocks the increase in pulmonary vascular resistance after CPB.\textsuperscript{18} Studies on the role of endogenous NO production in post-CPB pulmonary hypertension have been less convincing. For example, human studies have shown either moderate decreases, no change, or increases in plasma NO metabolites after CPB.\textsuperscript{11-14} There are many potential reasons for these inconsistent results, including differences in age, underlying disease, duration and type of CPB, and use of NO donor agents. In addition, plasma NO metabolites are an indirect determination of whole body NO production, whose values in the post-CPB setting may be difficult to interpret because of changes in extracellular volume and renal function.

### Figure 1
Changes in mean pulmonary arterial pressure (top) and left pulmonary vascular resistance (bottom) before and after CPB in both control and shunt lambs. Increase in mean pulmonary arterial pressure was greater in shunt lambs than in control animals. Values are mean±SEM (n=6 control and n=7 shunt lambs). \( ^*P<0.05 \) vs before CPB (ANOVA). \( +P<0.05 \) vs corresponding control lamb.

### Figure 2
Relative changes in lung tissue NOx concentrations before, during (mid), and after CPB in both control and shunt lambs. Values are mean±SEM (n=6 control and n=7 shunt lambs). \( ^*P<0.05 \) vs before CPB (ANOVA).

### Table 2: Biochemical Changes After CPB

<table>
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<tr>
<th>Time After CPB, min</th>
<th>Before CPB</th>
<th>Mid</th>
<th>15</th>
<th>60</th>
<th>240</th>
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<tr>
<td><strong>Tissue NOx, ( \mu \text{mol/L} )</strong></td>
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</tr>
<tr>
<td>Control</td>
<td>7.1±4.5</td>
<td>5.2±2.2</td>
<td>4.8±1.4*</td>
<td>4.5±3.2*</td>
<td>5.5±2.0*</td>
</tr>
<tr>
<td>Shunt</td>
<td>4.6±2.0</td>
<td>4.0±1.6</td>
<td>3.7±1.6</td>
<td>4.5±3.4</td>
<td>3.4±2.0*</td>
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<tr>
<td><strong>Tissue cGMP, pmol/g</strong></td>
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<tr>
<td>Control</td>
<td>111.1±38.3</td>
<td>55.4±16.0*</td>
<td>75.5±29.9</td>
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<td>Shunt</td>
<td>202.1±88.3</td>
<td>123.5±22.9*</td>
<td>139.8±42.5*</td>
<td>127.6±56.0*</td>
<td>153.9±101.5*</td>
</tr>
<tr>
<td><strong>NOS activity, pmol · min(^{-1}) · mg(^{-1})</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.134±0.05</td>
<td>0.151±0.09</td>
<td>0.138±0.06</td>
<td>0.135±0.03</td>
<td>0.132±0.05</td>
</tr>
<tr>
<td>Shunt</td>
<td>0.272±0.09</td>
<td>0.258±0.09</td>
<td>0.245±0.06</td>
<td>0.264±0.11</td>
<td>0.313±0.11</td>
</tr>
</tbody>
</table>

Values are mean±SD for 6 control lambs and 7 shunt lambs. \( ^*P<0.05 \) vs corresponding pre-CPB values (ANOVA).
In the current investigation, we studied age-matched lambs with either normal or preexisting increased pulmonary blood flow under identical conditions and similar CPB times. We found that the response to CPB was dependent on the preexisting status of the pulmonary circulation. Pulmonary vascular resistance increased after CPB in lambs with preexisting increased pulmonary blood flow and pulmonary hypertension but remained unchanged in normal lambs. These data correlate with our previous studies and clinical observations that children with preexisting pulmonary hypertension are at a greater risk for acute increases in pulmonary vascular resistance after CPB.2,18

Plasma NO metabolites are an indirect determination of whole body NO production, whose values in the post-CPB setting may be difficult to interpret because of changes in extracellular volume and renal function. Therefore, in the present study, we determined concentrations in peripheral lung tissue. We found a ≈30% decrease in NO metabolites after CPB in both normal and shunt lambs. The decrease in tissue cGMP concentrations paralleled the decrease in NO metabolites, suggesting that hypothermic CPB does modestly decrease basal NO-cGMP production. Because the decreases in normal and shunt lambs were similar, the change in NO-cGMP did not correlate with the change in pulmonary vascular tone after CPB, which brings into question the role of decreased NO in the increased pulmonary vascular resistance noted after CPB. However, it should be noted that shunt lambs have increased basal NO-cGMP production before CPB, which likely represents an early adaptive response to increased pulmonary blood flow and pressure17 (Table 2). In addition, shunt lambs have vascular smooth muscle cell remodeling, which includes medial hypertrophy.16 Therefore, similar decreases in NO-cGMP in shunt lambs may have a different physiological effect than in normal lambs. It should also be mentioned that other potential sources of cGMP, such as the natriuretic peptides, may be altered during CPB and were not measured in the present study.

To further determine changes in endogenous NO production induced by CPB, we measured exhaled NO concentrations in 5 shunt lambs. We found that exhaled NO concentrations progressively decreased after CPB and were ≈20% of pre-CPB values after 4 hours. These data are consistent with a previous study in children and provide further evidence that endogenous NO production is decreased after CPB.23 However, the source of NO

**Figure 4.** Relative changes in relative exhaled NO concentrations before and after CPB in shunt lambs. Values are mean±SEM (n=5 shunt lambs). *P<0.05 vs before CPB (ANOVA).

**Figure 5.** Relative changes in relative NOS activity (with the conversion of [3H]-arginine to [3H]-citrulline) before, during (mid), and after CPB. Values are mean±SEM (n=6 control and n=7 shunt lambs).

**Figure 6.** Western blot analysis for eNOS protein in lung tissue before, during, and after hypothermic CPB. A, Representative Western blot from protein extracts (100 µg), prepared from lung tissue from control lambs (left) and shunt lambs (right), separated on a 7.5% SDS-polyacrylamide gel, electrophoretically transferred to Hybond membranes, and analyzed with a specific antiserum raised against eNOS. Ovine endothelial cell protein (ovine EC [20 µg]) was used as a positive control. B, Densitometric values for relative eNOS protein (normalized to control) from 6 control and 7 shunt lambs. Values are mean±SEM. eNOS protein expression is unchanged after CPB.
reduction within the lung cannot be determined with these measurements. The major sources of exhaled NO are the nasopharynx, sinuses, and respiratory tract. Because the tracheas of the lambs in this study were intubated, the sampling did isolate the source to the respiratory tract. However, because all 3 isoforms of NO are found throughout the respiratory tract, it is not possible to determine which isoform (including its location) is responsible for the decrease in exhaled NO. In fact, the disproportionate decrease in exhaled NO compared with the decrease in peripheral lung tissue NO metabolites suggests that NO from large airways is decreased after CPB. Further studies are needed to isolate the changes in NO production within the respiratory tract to particular lung regions and cell types.

To begin to determine potential mechanisms for the decrease in NO production after CPB, we measured total NOS activity and eNOS protein levels. We found that neither total NOS activity nor eNOS protein levels were changed. Because inflammatory states are associated with upregulation of the Ca\(^{2+}\)-independent, inducible NOS isoform (iNOS), we also performed parallel NOS activity assays with and without Ca\(^{2+}\) in shunt lambs to determine potential changes in eNOS activity. We found no changes in either the Ca\(^{2+}\)-dependent constitutive NOS activity or the Ca\(^{2+}\)-independent, iNOS activity, suggesting that eNOS and iNOS activities were unchanged. In addition, Western blot analysis detected no changes in iNOS protein levels. These data suggest that decreases in eNOS translation or posttranslational enzyme modifications do not account for the decreases in NO production after CPB, we measured total NOS activity and eNOS protein levels. We found that neither total NOS activity nor eNOS protein levels were changed. Because inflammatory states are associated with upregulation of the Ca\(^{2+}\)-independent, inducible NOS isoform (iNOS), we also performed parallel NOS activity assays with and without Ca\(^{2+}\) in shunt lambs to determine potential changes in eNOS activity. We found no changes in either the Ca\(^{2+}\)-dependent constitutive NOS activity or the Ca\(^{2+}\)-independent, iNOS activity, suggesting that eNOS and iNOS activities were unchanged. In addition, Western blot analysis detected no changes in iNOS protein levels. These data suggest that decreases in eNOS translation or posttranslational enzyme modifications do not account for the decreases in NO production after CPB.

In summary, the present study provides support that the endogenous production of NO is decreased after hypothermic CPB. By conducting intermittent lung biopsies in lambs with normal and increased pulmonary blood flow, we found a ≈30% decrease in lung tissue concentrations of NO metabolites and cGMP in both groups of lambs 4 hours after CPB. These decreases appear to be independent of changes in eNOS activity or gene expression. Because the decreases in NO-cGMP cascade did not correlate with the degree of post-CPB pulmonary hypertension, the role of NO in the mediation of the increase in pulmonary vascular resistance after CPB remains unclear. We speculate that other vasoactive substances, such as ET-1, play a more central role in the mediation of post-CPB pulmonary hypertension. However, the modest decrease in NO-cGMP or the inability to increase NO production may contribute to the alterations in vascular reactivity after CPB. Further studies are warranted to determine the exact cause for these decreases and their location within the lung.

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Alterations in Endogenous Nitric Oxide Production After Cardiopulmonary Bypass in Lambs With Normal and Increased Pulmonary Blood Flow
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