Increased endothelial NOS in lambs with increased pulmonary blood flow and pulmonary hypertension

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The development of pulmonary hypertension and its associated increased vascular reactivity commonly accompanies congenital heart disease with increased pulmonary blood flow (5, 40). After birth, the presence of a systemic-to-pulmonary communication (i.e., truncus arteriosus) results in increasing pulmonary blood flow as pulmonary vascular resistance normally decreases. This abnormal postnatal hemodynamic state results in progressive structural and functional abnormalities of the pulmonary vascular bed (30). After surgical correction, early vascular changes are reversible; however, more severe changes are irreversible and progressive. Therefore, the status of the pulmonary vasculature is often the principal determinant of the clinical course and feasibility of surgical treatment. Although the vascular morphology is well described, the mechanisms of vascular remodeling and altered vascular reactivity associated with pulmonary hypertension secondary to increased pulmonary blood flow remain incompletely understood.

Recent evidence suggests that normal pulmonary vascular tone is regulated by a complex interaction of vasoactive substances that are produced locally by the vascular endothelium (4, 10, 14, 41). Nitric oxide (NO) is an endothelium-derived relaxing factor synthesized by the oxidation of the guanido nitrogen moiety of L-arginine after activation of NO synthase (NOS; see Ref. 29). Three isoforms of NOS are known. Constitutive forms are present in endothelial cells and neurons, and a third inducible isoform is present in macrophages (20, 36, 22). After certain stimuli, such as shear stress and the receptor binding of specific vaso dilators (endothelium-dependent vasodilators), NO is synthesized and released from the endothelial cell by the activation of endothelial NOS (eNOS; see Refs. 24, 34). Once released from endothelial cells, NO diffuses into vascular smooth muscle cells and activates soluble guanylate cyclase, a heterodimer with α3- and β3-subunits that catalyzes the production of guanosine 3′,5′-cyclic monophosphate (cGMP) from GDP. cGMP induces vascular smooth muscle relaxation through activation of a cGMP-dependent protein kinase, although the exact mechanism remains unclear (15, 16, 25). Cyclic nucleotide phosphodiesterases regulate intracellular levels of cGMP by catalyzing cGMP to GMP (2, 23).

Evidence that NO mediates normal pulmonary vascular tone has led to the hypothesis that endothelial injury induced by congenital heart disease with increased pulmonary blood flow disrupts these regulatory mechanisms and participates in the development of pulmonary hypertension and its associated altered vascular reactivity. For example, adults with advanced pulmonary hypertension have impaired endothelium-dependent pulmonary vasodilation and decreased eNOS gene expression within pulmonary vascular endothelial cells (9, 12). However, because most patients who undergo histological evaluation have advanced pulmonary hypertension, it has been difficult to investigate early aberrations in NO activity and its potential role in the development of pulmonary hypertension secondary to increased pulmonary blood flow.

Utilizing aortopulmonary shunt placement in the fetal lamb, we recently established a unique animal...
model of pulmonary hypertension that mimics congenital heart disease with increased pulmonary blood flow (31). We have previously shown that these lambs have physiological alterations in NO activity as early as 4 wk of age. For example, shunted lambs display a selective impairment of endothelium-dependent pulmonary vasodilation, suggestive of decreased NO activity; the pulmonary vasodilating effects of acetylcholine and ATP are attenuated compared with control lambs, but inhaled NO dilates normally (32). However, shunted lambs also display an augmented pulmonary vasconstricting response to N-nitro-L-arginine (a competitive inhibitor of NO synthesis) and increased plasma concentrations of cGMP, suggestive of increased basal NO activity (32). Therefore, with the use of the physiological results alone, the early alterations in NO activity induced by increased pulmonary blood flow remained unclear.

The purpose of the present study was to further characterize, at the molecular level, the potential early alterations in eNOS gene expression, localization, and activity induced by increased pulmonary blood flow. Utilizing RNase protection assays and Western blotting, we compared eNOS mRNA and protein in 4-wk-old lambs with increased pulmonary blood flow (shunt lambs) with age-matched controls (control lambs). These changes were normalized for potential differences in endothelial cell density with platelet endothelial cell adhesion molecule (PECAM)-1, a 130-kDa member of the immunoglobulin superfamily that is a major constituent of the endothelial cell intercellular junction (27), and localized utilizing in situ hybridization and immunohistochemistry. Finally, tissue cGMP concentrations in isolated pulmonary arteries and total lung NOS activity were determined in both shunt and control lambs to determine if increased pulmonary blood flow affects eNOS enzyme activity.

METHODS

Surgical Preparations and Care

Ewes. Ten mixed-breed Western pregnant ewes (137–141 days gestation, term = 145 days) were operated on under sterile conditions as previously described (31). Through a left lateral fetal thoracotomy, an 8.0-mm Gore-tex vascular graft (2 mm length; W. L. Gore, Milpitas, CA) was anastomosed to the fetal aorta and main pulmonary artery of the fetus with 7.0 prolene (Ethicon, Somerville, NJ), using a continuous suture technique as previously described (31). Unoperated twin fetuses served as controls. We have previously shown that unoperated and sham-operated control lambs have similar physiology and morphology (31). After spontaneous delivery, antibiotics (1×10^6 units of penicillin G potassium and 100 mg of gentamicin sulfate) were administered to the ewe during surgery and daily thereafter until 2 days after spontaneous delivery of the lamb.

Lambs. After spontaneous delivery, antibiotics (1×10^6 units of penicillin G potassium and 25 mg of gentamicin sulfate intramuscularly) were administered for 2 days. The lambs were weighed daily, and the respiratory rate and heart rate were obtained. Furosemide (1 mg/kg intramuscularly) was administered daily. Elemental iron (50 mg intramuscularly) was given weekly. At 4 wk of age, the lambs were killed by an intravenous injection of pentobarbital sodium (Euthanasia CII; Central City Medical, Union City, CA) followed by bilateral thoracotomy. An autopsy was performed to confirm patency of the vascular graft. The lungs were removed and prepared for RNase protection assays, Western blot analysis, in situ hybridization, and immunohistochemistry. All procedures and protocols were approved by the Committee on Animal Research of the University of California, San Francisco.

Tissue Preparation

The heart and lungs were removed en bloc. The lungs were dissected with care to preserve the integrity of the vascular endothelium. Two- to three-gram sections from each lobe of the lung and sections from third- to fifth-generation intralobar pulmonary arteries and veins, with inside diameters of 0.5–2.0 mm, were removed. These tissues were snap-frozen in liquid nitrogen and stored at –70°C until used.

For RNA isolation, the snap-frozen lung tissue was pulverized and then briefly homogenized in 4 M guanidinium isothiocyanate. Total RNA was extracted with acid phenol and precipitated in isopropanol (3). For protein isolation, the snap-frozen lung tissue and intralobar pulmonary arteries were homogenized using a Tissuemizer (2×15 s at 80% power) at 4°C and then centrifuged (14,000 g for 20 min), and the supernatant was removed for protein determination and Western blot analysis (3).

For in situ hybridization and 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 of 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 h at 4°C by immersion in 4% paraformaldehyde. Representative slices from each lobe were removed, placed in 30% sucrose until they sank, placed in optimum cutting temperature compound, frozen on dry ice, and stored at –70°C until sectioned. Ten- to twenty-micrometer sections were cut using a cryostat, transferred to aminoalkylsilane-treated slides (Superfrost Plus; Fisher Scientific, Santa Clara, CA), and stored at –70°C (3).

Generation of Ovine eNOS cDNA

Total ovine fetal lung RNA was used in RT-PCR reactions (kit from Perkin-Elmer, Foster City, CA). A region within the home-binding domain was identified as a region of minimal homology between the three isoforms of NOS (3). Oligonucleotides were synthesized (using the bovine eNOS as a template) to allow amplification of this region within the ovine eNOS sequence. The sequences of the oligonucleotides were 5’-CCTCCGGAGGGCCCAATTCCCTCGC-3’ for oligonucleotide 1 and 5’-CAGTCCAGGCGCC TTCTCAGGGGT-3’ for oligonucleotide 2. The 681-bp region amplified corresponds to amino acids 62 to 288 of the eNOS protein. The generated cDNA fragment of interest was then cloned directly into the pCR II vector (Invitrogen, San Diego, CA), sequenced (Sequenase kit from USB, Cleveland, OH), and then subcloned into pBluescript KS+ (Stratagene, La Jolla, CA).
RNA Probe Synthesis, RNAse Protection Assay, and In Situ Hybridization

The plasmid containing the ovine cDNA fragment of interest was linearized with the appropriate restriction enzyme (GIBCO-BRL, Grand Island, NY). Antisense and sense radiolabeled single-stranded ovine eNOS riboprobes were synthesized by in vitro transcription using either T3 or T7 RNA polymerases (Boehringer-Mannheim, Indianapolis, IN) in the presence of cold rCTP, rGTP, and rATP. RNA probes labeled with [32P]UTP (New England Nuclear, Boston, MA) were used for RNAse protection assays (3); RNA probes labeled with [35S]UTP were used for in situ hybridization (3, 26, 37).

RNAse protection assays were performed as previously described (3). Studies were done on total RNA prepared from control and shunt lung, using the antisense radiolabeled cRNA eNOS probe. Antisense radiolabeled single-stranded ovine RNA probes were hybridized overnight at 42°C with total RNA isolated from control and shunt lung (50 µg) in 80% formamide, 50 mM Pipes (pH 6.4), 0.4 M NaCl, and 1 mM EDTA. Single-stranded RNA was digested with an RNAse A-T1 mixture (Ambion, Austin, TX) for 1 h at 37°C. After phenol/CHCl3 extraction and ethanol precipitation, the protected fragments were analyzed by electrophoresis on a 6% denaturing polyacrylamide gel. 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. In all experiments, sufficient counts were added such that the eNOS and 18S riboprobes were always in molar excess.

In situ hybridization was performed as previously described (3, 26, 37). Studies were done on serial sections of control and shunt ovine lung, using the antisense radiolabeled ovine cRNA eNOS probe and the corresponding nonhybridizing sense radiolabeled ovine cRNA eNOS probe. Frozen tissue sections were allowed to come to room temperature. All sections were fixed in 4% paraformaldehyde in PBS, treated with proteinase K, and acetylated [0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride (pH 7.5)]. After being washed in 0.5× saline-sodium citrate (SSC), the sections were covered with hybridization solution [50% deionized formamide, 0.3 M NaCl, 20 mM Tris, pH 8, 5 mM EDTA, 1× Denhardt’s solution, 10% dextran sulfate, and 10 mM dithiothreitol (DTT)] and prehybridized for 1–3 h at 55°C. In the presence of trNa, the radiolabeled RNA probe (600,000 counts·min⁻¹·slide⁻¹) was applied to the hybridization solution, and the sections were then hybridized for 12–18 h at 55°C. After hybridization, the sections were washed for 20 min (2× SSC, 10 mM β-mercaptoethanol, and 1 mM EDTA), treated with RNase A (20 mg/ml), and washed for 20 min (2× SSC, 10 mM β-mercaptoethanol, and 1 mM EDTA). The sections were then washed in high-stringency buffer (50% deionized formamide, 2× SSC, and 0.1 M DTT) for 30 min at 65°C. The final wash was in 0.5× SSC for 20 min. The sections were dehydrated, and the slides were dipped in photographic emulsion (Ilford, St. Louis, MO), stored at 4°C, developed after 2–10 wk of exposure, and counterstained with hematoxylin-eosin. For each experiment, four antisense and four sense slides (containing 1–2 sections) were studied. For each experiment, new radiolabeled probes were synthesized.

Immunohistochemistry

Immunohistochemistry was performed as previously described (3). Studies were done on serial sections of control and shunt ovine lung, using a polyclonal eNOS antiserum (3). The specificity of the antiserum for endothelial cells was assessed by Western blot analysis on protein extracts prepared from a variety of tissues. Frozen tissue sections were allowed to come to room temperature. To eliminate nonspecific binding of the primary antiserum to tissue proteins and to block endogenous peroxidase activity, the tissue sections were first incubated in 1× PBS, 0.3% Triton X-100, and 1% horse serum and then incubated in 0.3% H2O2 in methanol. Next, the tissue sections were incubated in the polyclonal eNOS antiserum (1:2,000 dilution) for 12–18 h at 4°C. Appropriate biotin-conjugated secondary antibodies were used and detected with avidin-horseradish peroxidase ( vectastain Elite ABC-HRP, anti-rabbit IgG, PK-6101; Vector Laboratories, Burlingame, CA) with reagents provided and used according to the manufacturer’s instructions.

Western Blot Analysis

Western blot analysis was performed as previously described (3). Protein extracts, prepared from control and shunt lung (100 µg) and intralobar pulmonary arteries and veins (25 µg), were separated on 7.5% SDS-polyacrylamide gel and electrophoretically transferred to Hybond-polyvinyliden difluoride membranes (Amersham, Arlington Heights, IL). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween. After blocking, the membranes were incubated at room temperature with the appropriate dilution of the appropriate antiserum [1:2,500 dilution (Transduction Laboratories, Lexington, KY) or 1:100 for PECAM (a gift from Dr. Steven M. Albelda)], washed with TBS containing 0.1% Tween, and then incubated with the appropriate species anti-IgG-horseradish peroxidase conjugate. After washing was completed, chemiluminescence (Pierce Laboratories) was used to detect the protein bands of interest.

Assay for NOS Activity

This was performed using the conversion of [3H]arginine to [3H]citrulline as a measure of NOS activity essentially as described by Bush et al. (6). Briefly, lung tissues were homogenized in NOS assay buffer (50 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA and 0.1 mM EGTA) with a protease inhibitor cocktail. Enzyme reactions were carried out at 37°C in the presence of total lung protein extracts (500 µg), 1 mM NADPH, 14 µM tetrahydrobiopetost, 100 µM FAD, 1 mM MgCl2, 5 µM unlabeled L-arginine, 15 nM [3H]arginine, 25 units calmodulin, and 5 mM calcium to produce conditions that drive the reaction at maximal velocity. The reactions were stopped by the addition of iced stop buffer (20 mM sodium acetate, pH 5.1, 1 mM citrulline, 2 mM EDTA, and 0.2 mM EGTA) and then were applied to columns containing 1 ml of Dowex AG50W-X8 resin, Na+ form, prerelibrated with 1 N NaOH. [3H]citrulline was then quantitated by scintillation counting.

Data Analysis

Quantitation of autoradiographic results was performed by scanning (Hewlett-Packard SCA Jet IICX; Hewlett-Packard, Palo Alto, CA) the bands of interest into an image-editing software program (Adobe Photoshop; Adobe Systems, Mountain View, CA). Band intensities from RNAse protection assays and Western blot analysis were analyzed densitometrically on a Macintosh computer (model 9500; Apple Computer, Cupertino, CA) using the public domain National Institutes of Health Image program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). For RNAse protection assays, to control for the amount of input RNA and the recovery of protected probe fragments, the eNOS mRNA signal was normalized to the corresponding 18S signal for each lane. The
signal for 18S was significantly higher than that of eNOS. Thus exposure times were varied to ensure that each signal was in the linear range of the autoradiographic film. Results from control lungs were assigned the value of one (relative eNOS mRNA). For Western blot analysis, to ensure equal protein loading, duplicate polyacrylamide gels were run. One was stained with Coomassie blue. To control for the number of endothelial cells in the lung, the eNOS protein signal was normalized to the corresponding PECAM signal for each lane. Results from control lungs and of third- to fifth-generation intralobar pulmonary arteries and veins were assigned the value of one (relative eNOS protein). The means ± SE were calculated for the relative mRNA, the relative protein, and NOS activity. Comparisons between control and shunt lambs were made by the unpaired \( t \)-test. \( P < 0.05 \) was considered statistically significant.

**Measurement of cGMP**

For cGMP content, the snap-frozen lung sections of intralobar pulmonary arteries were homogenized in cold (4°C) 6% trichloroacetic acid (10% wet wt/vol) and centrifuged for 15 min at 4°C. cGMP was measured using an \(^{125}\)I radioimmunoassay kit with reagents provided and used according to the manufacturer's instructions (Amersham cGMP \(^{125}\)I assay system RPA 525; Amersham). The supernatant was washed four times with 5 vol of diethyl ether. The remaining aqueous extract was dried under a stream of air and then resuspended in assay buffer (1 ml). A 500-µl sample and cGMP standards (2–128 fmol/tube) were acetylated with triethylamine-acetic anhydride (25 µl, 2:1). To a 100-µl aliquot, 100 µl of antiserum were added and incubated for 1 h at room temperature. Next, 100 µl of \(^{125}\)I-labeled cGMP were added and incubated for 18 h at 4°C. Next, Amerlex-M second antibody reagent (500 µl) was added and incubated for 10 min at room temperature. The antibody-bound fraction was separated by centrifugation and counted using a gamma scintillation counter.

**RESULTS**

There were changes in the expression of the eNOS gene and in eNOS enzyme activity after in utero insertion of an aortopulmonary vascular graft. eNOS mRNA expression was increased by 140% in shunt lambs (\( P < 0.05 \); Fig. 1). In situ hybridization showed that this increase in eNOS mRNA expression was confined to the endothelium of small and large pulmonary arteries (Fig. 2).

Previously we have found that there is a 2.1-fold increase in pulmonary vessel number in shunt lambs compared with controls (31). Thus we attempted to normalize for endothelial cell density differences between shunt and control lambs by Western blot using a specific antiserum to the endothelial cell specific marker PECAM-1. eNOS protein levels after normalization for endothelial cell density were calculated (Fig. 3B). When normalized for endothelial cell density using the endothelial cell-specific marker PECAM-1, shunt lambs had a 108% increase in eNOS protein (\( P < 0.05 \)). Immunohistochemistry localized this increase in protein expression to the endothelium of small and large pulmonary arteries (Fig. 4). To further ensure that differences in eNOS expression were independent of differences in endothelial cell density between shunt and control lambs and to determine if there were differential alterations in eNOS protein between the arterial and venous systems, we measured eNOS protein from isolated third- to fifth-generation intralobar pulmonary arteries and veins. Western blot analysis for eNOS protein expression demonstrated that there was still a significant increase (64%, \( P < 0.05 \)) in isolated third- to fifth-generation intralobar pulmonary arteries of shunt...
lambs. However, there were no significant alterations in eNOS protein expression in the pulmonary veins (Fig. 5).

There was also an increase in NOS activity in the lungs of shunt lambs compared with age-matched controls. We found that shunt lambs had a 155% ($P < 0.05$) increase in eNOS activity as determined using the conversion of $[^3H]$arginine to $[^3H]$citrulline (Fig. 6). However, this assay determines the maximal capacity of the tissue to metabolize L-arginine and not the activity of the eNOS protein in vivo. To determine the net effects of the alterations in eNOS gene expression, in vivo, we measured tissue cGMP content in third- to fifth-generation intralobar pulmonary arteries. cGMP was increased by 112% in the pulmonary arteries prepared from shunt lambs ($P < 0.05$; Fig. 7).

**DISCUSSION**

Children with early, reversible pulmonary hypertension secondary to increased pulmonary blood flow suffer morbidity from enhanced pulmonary vascular reactivity (5, 13, 40). NO is an important modulator of pulmonary vascular reactivity and smooth muscle mitogenesis. Although eNOS gene expression is decreased in the lungs of patients with late, irreversible pulmonary hypertension, potential early alterations in the NO-cGMP cascade that may play a crucial role in the pathophysiology of pulmonary hypertension have not been examined (12). Therefore, the purpose of the present study was to determine whether pulmonary hypertension with increased pulmonary blood flow induces early changes in the expression, localization, or activity of eNOS. For this study, we utilized a novel model of pulmonary hypertension with increased pulmonary blood flow in the lamb after in utero placement of an aorta-to-pulmonary artery vascular graft. At 4 wk of age, these lambs have a pulmonary-to-systemic blood flow ratio of $\sim 2:1$, a mean pulmonary arterial pressure that is 50–75% of mean systemic arterial pressure, and pulmonary vascular remodeling characteristic of children with pulmonary hypertension with increased pulmonary blood flow (31). In contrast to advanced pulmonary vascular disease, we found that eNOS mRNA, protein, and activity were upregulated in the lungs of 4-wk-old shunt lambs. Because NO produces vasodilation and inhibits smooth muscle cell mitogenesis, increased NO may represent an early adaptive response...
of the pulmonary circulation to minimize pulmonary vascular resistance in the setting of increased pulmonary blood flow.

When endothelial cells are subjected to shear stress, a diverse set of responses are initiated, some of which occur within minutes, others which occur within hours or days (33). Increases in shear stress stimulate endothelial cells to produce several modulators of vascular tone, including NO (17–19, 28). The increase in NO activity induced by shear stress is associated with rapid changes in endothelial cell calcium concentration, ionic conductance, adenylate cyclase activity, and inositol trisphosphate generation (17–19, 28, 33). Recent studies have also shown that shear stress regulation of eNOS is regulated by tyrosine phosphorylation of the enzyme and may not involve increased intracellular calcium (1, 8). If the shear stress is maintained, endothelial cells change their shape secondary to a structural reorganization of the cytoskeleton. There are also changes in gene expression. For example, in both bovine aortic and ovine pulmonary arterial endothelial cells, eNOS mRNA expression is increased by shear stress (3, 39). In addition, in vivo, lung eNOS mRNA...
and protein are increased in fetal sheep by the increased pulmonary blood flow associated with in utero ventilation (3). Shear stress is proportional to the velocity of blood and its viscosity and inversely proportional to the internal radius of the blood vessel to the third power (33, 39). Because the pulmonary circulation of shunt lambs is exposed to increased pulmonary blood flow, and the pulmonary arteries have a decreased internal radius secondary to vascular remodeling, shear stress is most likely increased in shunt lambs (31). Therefore, the increase in eNOS mRNA and protein expression in these lambs may be secondary to increases in shear stress. However, since shear stress was not directly measured in these studies, this conclusion is speculative and will require further analysis. In addition, other models of pulmonary hypertension have shown an upregulation of eNOS independent of increased flow and shear stress (21).

Previously, we have reported that 4-wk-old shunt lambs display a selective impairment of endothelium-dependent pulmonary vasodilation (32). Vasodilators (such as acetylcholine and ATP) that require the endothelial cell to produce NO have impaired vasodilating effects in shunt lambs, but vasodilators (such as sodium nitroprusside) that generate NO independent of the endothelial cell have normal vasodilating effects. In preliminary studies, the response to A-23187, a receptor-independent endothelium-dependent vasodilator, was also found to be attenuated in pulmonary arteries isolated from shunt lambs, suggesting that the effect is independent of receptor disruption (38). This selective impairment of endothelium-dependent pulmonary vasodilation has also been demonstrated in children with congenital heart disease and increased pulmonary blood flow (7). Although decreased endothelium-dependent vasodilation is suggestive of decreased eNOS activity, shunt lambs also display an augmented pulmonary vasoconstricting response to N-nitro-L-arginine (a com-
petitive inhibitor of NO synthesis) and increased plasma concentrations of cGMP, suggesting increased basal NO production. Because these physiological observations gave conflicting information regarding the eNOS activity in the lungs of shunt lambs, we directly measured NOS activity in the lungs of these animals and tissue concentrations of cGMP in isolated pulmonary arteries (as a measure of in vivo NO production). The results obtained clearly demonstrated that NOS activity and cGMP production were enhanced in shunt lambs compared with age-matched controls. Therefore, impaired endothelium-dependent vasodilation is not related to a posttranslational modification of eNOS that reduces enzyme activity.

On the basis of our previous physiological data and the present molecular data, we speculate that lambs with increased pulmonary blood flow may have increased basal NO production in the pulmonary vasculature that is refractory to further stimulation by agonist-induced endothelium-dependent vasodilators. In addition, preliminary in vitro data suggest that the impairment of endothelium-dependent vasodilation noted in vivo is selective to the pulmonary arteries; isolated pulmonary veins respond normally in shunt lambs (37). We speculate that the differences in eNOS expression noted above between the arteries and veins may explain, in part, the differences in the physiological responses; the NO activity in the veins is not maximally increased and is still capable of being increased by agonist-induced endothelium-dependent vasodilators. However, other, as yet uncharacterized, alterations in endothelial function may also contribute to the impairment in endothelium-dependent vasodilation noted in shunt lambs.

The present study demonstrates in a lamb model of pulmonary hypertension secondary to increased pulmo-

nary blood flow that eNOS gene expression and activity are upregulated at 4 wk of life. Increased pulmonary blood flow and pulmonary hypertension increased the expression of eNOS mRNA and protein. Utilizing in situ hybridization and immunohistochemistry, we found that the changes in eNOS were localized to the endothelium of the large and small pulmonary arteries. Because in vivo eNOS activity was also increased (as determined by an increase in tissue cGMP levels), the net result of these alterations is most likely increased production of NO. Therefore, upregulation of NO production may represent an early, and previously undescribed, adaptive response of the pulmonary circulation to maintain a low pulmonary vascular resistance in response to increased pulmonary blood flow and pressure. Finally, because NO is an important modulator of pulmonary vasoconstriction, we speculate that the enhanced pulmonary vascular reactivity noted in children with early, reversible, pulmonary hypertension may be secondary to a maximally stimulated eNOS enzyme that is unable to produce additional NO in response to vasoactive stimuli (4).

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