Altered endothelium-dependent relaxations in lambs with high pulmonary blood flow and pulmonary hypertension

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The regulation of normal pulmonary vascular tone is a complex process that is regulated in part by vasoactive substances produced by the vascular endothelium (7). Nitric oxide (NO), a gaseous free radical, is produced from the terminal nitrogen of L-arginine by NO synthase (NOS). There are three known isoforms of NOS in mammals, and all three isoforms are present and developmentally regulated in the fetal rat lung (13, 18). Specific stimuli such as shear stress and the receptor binding of specific endothelium-dependent vasodilators activate a constitutively expressed endothelial NOS (eNOS) to synthesize and release NO. The best-described vascular action of NO is its activation of soluble guanylate cyclase, a heterodimer with α1- and β1-subunits. This in turn results in increased smooth muscle cell concentrations of cGMP and activation of protein kinase G. Cyclic nucleotide phosphodiesterases constitute the only known pathway for the hydrolysis of cGMP and therefore control the intensity and duration of its signal transduction. A cGMP-specific phosphodiesterase (PDE5) is found in especially high concentrations in the lung (14). In addition to properties of vasodilation, NO and cGMP inhibit smooth muscle mitogenesis (8).

Congenital heart disease associated with increased pulmonary blood flow and/or increased pulmonary venous pressure commonly leads to the development of pulmonary hypertension and its associated increased vascular reactivity (11). The risk and timing of developing pulmonary hypertension is dependent on a variety of factors, including the age of the patient and the particular heart defect. Endothelial injury induced by increases in flow or pressure has been proposed as an important factor in the development of pulmonary hypertension. For example, adults with advanced pulmonary hypertension have impaired endothelium-dependent pulmonary vasodilation and decreased eNOS gene expression within pulmonary vascular endothelial cells (5, 9). However, because most patients who undergo histological evaluation have advanced pulmonary hypertension, it has been difficult to investigate early aberrations in the NO-cGMP cascade and to determine the potential role of these aberrations in the development of pulmonary hypertension secondary to increased pulmonary blood flow.

We recently established a unique animal model of pulmonary hypertension that mimics congenital heart disease with increased pulmonary blood flow (15) by...
placing aortopulmonary shunts in the fetal lamb. In vivo these intact lambs have physiological alterations in the NO-cGMP cascade and selective impairment of endothelium-dependent pulmonary vasodilation by 4 wk of age (16). However, we recently reported that expression of eNOS is increased in pulmonary arteries (PAs) isolated from these lambs (2). The purpose of the present study was to further localize and determine potential mechanisms for the impairment in endothelium-dependent relaxation by assessing functional responses in fifth-generation PAs and pulmonary veins (PVs) isolated from control and shunted lambs.

METHODS

Surgical preparations and care. This study was approved by the State University of New York at Buffalo Laboratory Animal Care Committee. Ten pregnant ewes (137–141 days gestation, term = 145 days) were operated on under sterile conditions as previously described (15). Through a left lateral fetal thoracotomy, an 8.0-mm Gore-Tex vascular graft (½2 mm length) (W. L. Gore, Somerville, NJ) was anastomosed between the ascending aorta and the main PA of the fetus with 7.0 prolene (Ethicon, Somerville, NJ) using a continuous suture technique (15). After recovery from anesthesia the ewe was returned to the cage with free access to food and water. Antibiotics (2,000,000 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.

After spontaneous delivery, antibiotics (1,000,000 units of penicillin G potassium and 25 mg of gentamicin sulfate im) were administered to the lambs for 2 days. The lambs were weighed daily, and respiratory and heart rates were measured. Furosemide (1 mg/kg im) was administered daily. Elemental iron (50 mg im) was given weekly. At age 4 wk, pentobarbitol sodium (≤30 mg/kg iv) was given to lambs as needed to maintain adequate anesthesia. The lambs were immediately killed by rapid exsanguination through a cardia cut puncture.

Vessel isolation. The heart and lungs were removed en bloc from the thorax immediately after death and placed in Krebs-Ringer solution (in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25.5 NaHCO3, 5.6 glucose, and 0.026 calcium disodium EDTA). Fifth-generation intralobar PAs with inside diameters of 0.5–1.5 mm were isolated, dissected with care to preserve the integrity of the endothelium, and cut into rings 2–3 mm long and 1–3 mm in weight. Wet tissue weights were obtained at the end of each experiment after the rings were blotted dry on gauze pads. The force of contraction was normalized by the weight of each ring and is expressed as grams per gram of tissue (g/g).

Vessel rings were mounted on stainless steel hooks and placed in water-jacketed chambers. Tissues were bathed with 6 mL of Krebs-Ringer solution, which was maintained at 37°C and aerated with a gas mixture of 94% O2-6% CO2 to maintain pH 7.40, P CO2 = 38 Torr, and PO2 > 500 Torr. A continuous recording of isometric force generation was obtained by tying each vessel ring to a force-displacement transducer (Statham UC 2, Statham Instruments, Hato Rey, PR) that was connected to an oscillographic recorder. Once mounted, the vessel rings were allowed to equilibrate for 20 min in the bathing solution. A micrometer was then used to stretch the tissues repeatedly in small increments over the next 45 min until resting tone remained stable at a passive tension of 1.0 g for control arteries and 1.2 g for arteries from hypertensive lambs. Preliminary experiments determined that this was the optimal length for generation of active tone in response to exogenous norepinephrine (NE).

Experimental protocols. The following pharmacological agents were used: L-norepinephrine hydrochloride, indomethacin, d-propranolol hydrochloride, Nω-nitro-l-arginine (L-NNA), calcium ionophore A23187, ACh, S-nitroso-N-acetyl-penicillamine (SNAP), atrial natriuretic peptide (ANP), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), zaprinast, L-arginine, sepiapterin, polyethylene glycol-superoxide dismutase (PEG-SOD), and polyethylene glycol-catalase (PEG-CAT). All drugs were purchased from Sigma Chemical (St. Louis, MO) except for zaprinast, which was a gift from Rhone-Poulenc (Dagenham Essex, UK). Drugs were dissolved in distilled H2O, except L-NNA, which was dissolved directly in Krebs-Ringer solution due to the high bath concentrations used, and indomethacin, which was dissolved in ethanol. Ethanol, at the concentrations used in these experiments, did not alter the preexisting tone of PAs or PVs. Drugs were made fresh daily.

All vessels were pretreated for 20 min with 10−5 M indomethacin to prevent the formation of vasoactive prostaglandins and with 10−6 M propranolol to block β-adrenergic receptors. To examine the effect of dilator agents, PVs were first preconstricted with an EC50 (the concentration required to achieve 50% of maximum constriction) of NE (3 × 10−7 M). The EC50 for NE was determined from preliminary studies in which cumulative concentration-response curves for NE (10−8–10−5 M) were developed in both arteries and veins. Once the response to NE had reached a steady level, cumu-
relative concentration-response curves to dilator agents were obtained by increasing the bath concentration of these drugs in successive steps: the next concentration was added only when the response to the prior concentration had reached a plateau. Vessel rings were used for one experimental protocol and then discarded.

In some protocols tissues were pretreated with the competitive NOS inhibitor L-NNA (10^{-3} M) in addition to prostaglandin inhibitors. Because of the sensitivity of L-NNA to light, all experiments were performed in a darkened room and tissue baths were wrapped in aluminum foil.

In all experiments, n represents the number of animals from which vessel rings were studied. Data are expressed as the means ± SE. Statistical analysis was performed with StatView 4.5 software (Abacus Concepts, Berkeley, CA). Statistical comparisons were performed on concentration-response curves using repeated-measures ANOVA and Student-Newman-Keuls test for post hoc testing of multiple comparisons as appropriate. Individual data points between curves were compared using one-way ANOVA and Student-Newman-Keuls testing for multiple comparisons as needed. Significance was accepted at P < 0.05.

RESULTS

Relaxations to A-23187 and zaprinast were used to test for responsiveness to endogenously produced NO. Figure 1 shows that PAs from shunted lambs relaxed significantly less to 3 × 10^{-7} M A-23187 than did PAs isolated from control lambs (32 ± 7% vs. 79 ± 8%; P < 0.05). In the PVs, relaxations induced by A-23187 were similar in shunted and control lambs. Figure 2 shows a similar pattern of responsiveness to zaprinast. In addition, preliminary experiments (n = 3 for control and shunt, data not shown) revealed that PA relaxation responses to the receptor-dependent agonist ACh were similar in pattern to the receptor-independent agonist A-23187.

PAs and PVs from both control and shunted lambs relaxed similarly and completely to SNAP (Fig. 3). In addition PAs and PVs from control and shunted lambs relaxed similarly to increasing concentrations of ANP (Fig. 4).

Plateau contractile responses to NE were not significantly different in PAs or PVs isolated from shunted lambs first incubated with indomethacin and propranolol and then constricted with increasing concentrations of NE (Fig. 5). In further experiments, pretreatment with 10^{-3} M L-NNA was used to test for the effect of endogenously produced NO. Constrictions to NE were significantly enhanced after pretreatment with L-NNA in control and shunt PVs and control PAs but not in PAs from shunted lambs (Fig. 5). Further experiments used increasing concentrations of the soluble guanylate cyclase inhibitor ODQ after preconstriction with NE. Control vessels constricted significantly to increasing concentrations of ODQ, however, shunt vessels did not (Fig. 6).
To begin to elucidate the mechanism for the impairment in endothelium-dependent relaxation, PAs were pretreated with L-arginine (10⁻³ M) or sepiapterin (10⁻⁴ M). Neither agent enhanced relaxations to threshold or maximal concentrations of A-23187 in shunt PAs (Fig. 7) or control PAs (data not shown). Finally, PAs from control and shunted lambs were pretreated with the combination of PEG-SOD (37.5 U/ml) and PEG-CAT (1,200 U/ml) before relaxation with A-23187. Pretreatment with SOD-CAT did not alter relaxations to A-23187 in control PVs but did significantly enhance relaxations to A-23187 in PVs from shunted lambs (Fig. 8).

**DISCUSSION**

The development of pulmonary hypertension and its associated altered reactivity is a major source of morbidity and mortality in children with congenital heart disease. There are two major types of defects that induce pulmonary hypertension: those with increased pulmonary blood flow that result in increased arterial pressure, and those with left-sided obstruction that result in increased venous pressure. Although the vascular smooth muscle morphology is similar in advanced disease, early functional differences between arteries and veins during pulmonary hypertension have not been well studied. Increasing data suggest that aberrations in endothelial function participate in the pathophysiology of pulmonary hypertension. To begin to investigate early aberrations in endothelial function during the development of pulmonary hypertension, we developed an animal model of increased pulmonary blood flow in the lamb after in utero placement of an aorta-to-pulmonary vascular graft. Previously we demonstrated a selective impairment in endothelium-dependent pulmonary vasodilation in intact 4-wk-old lambs (16). In the present study we utilized isolated vessel techniques to determine functional differences between PAs and PVs as well as their potential mechanisms. The present data suggest that increased pulmonary blood flow induces an impairment of endothelium-dependent relaxation that is selective to the PAs. In addition, the impaired relaxation is not secondary to altered L-arginine or cofactor availability but is mediated in part by excess superoxide production.

To assess potential early alterations in endothelium-dependent vasodilation secondary to increased pulmonary blood flow, we evaluated NO-dependent vasoactive responses in PAs and PVs isolated from control...
lambs and lambs with high pulmonary blood flow due to an aortopulmonary shunt at age 4 wk. In vivo we previously demonstrated that the pulmonary vasodilating effects of ACh and ATP, both endothelium-dependent vasodilators, were significantly attenuated compared with age-matched controls with a similar degree of pulmonary hypertension induced by U-46619 (16). The effects of inhaled NO were similar in both groups of lambs. In the current isolated vessel study, we chose A-23187 instead of ACh as an agonist for NOS because its action is independent of receptor function.

In vivo both the arteries and veins are exposed to similar amounts of increased pulmonary blood flow. However, there is a large pressure drop across the pulmonary capillary bed, such that the pressure in the arteries is much greater than the veins. These data therefore suggest that the impairment in endothelium-dependent relaxation is secondary to exposure to increased pressure in addition to increased flow. To begin to further determine potential mechanisms for the selective impairment in endothelium-dependent relaxation, we attempted to restore relaxations to A-23187 by the addition of L-arginine, sepiapterin (a precursor for synthesis of the essential NOS cofactor tetrahydrobiopterin), or the combination of SOD-CAT. Several biological systems have suggested that depletion of critical substrates is a potential mechanism for decreased NOS activity (3, 4, 10, 12). However, in the present study pretreatment with an excess of L-arginine or sepiapterin did not restore endothelium-dependent relaxations, suggesting that substrate depletion was not the mechanism for decreased NO activity in our isolated vessels. However, it is still possible that longer term in vivo supplementation might produce different results (10).

Previously we demonstrated an upregulation of NOS expression in shunted lambs (2). However, in the current study we observed blunted relaxations to zapri-
nast as well as blunted constrictions to L-NNA and ODQ in PAs from shunted lambs. Constrictions to L-NNA reflect removal of NO, and constrictions to ODQ reflect inhibition of cGMP production by soluble guanylate cyclase. Therefore, we found no functional evidence for increased basal NO production by NOS in vessels isolated from shunted lambs relative to controls. Our data indicate decreased functional basal and stimulated activity of NOS despite our previous findings of increased expression. We therefore considered the possibilities that NOS was not functional, or that NOS was functional and that the NO produced was inactivated by another agent.

When endothelial cells are subjected to shear stress, a complex set of responses is initiated that includes changes in gene expression (17). 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. Because the pulmonary circulation of shunted lambs is exposed to increased pulmonary blood flow and the PAs have a decreased internal radius secondary to vascular remodeling, shear stress is most likely increased, and this increase is sustained over time in shunted lambs. Increases in shear stress stimulate endothelial cells to produce several modulators of vascular tone in addition to NO. One such agent is superoxide, a free radical implicated in producing endothelial dysfunction in a variety of biological systems. Superoxide production is possible from several sources, including xanthine oxidase, NADPH oxidase, or even NOS under specific conditions (1, 22). In addition, increased exogenous NO has been reported to increase cellular superoxide generation, which in turn inhibits NOS activity (19). We found that pretreatment of vessels with membrane-permeable SOD-CAT enhanced relaxations to A-23187 in PAs from shunted but not control lambs. These data suggest superoxide and/or peroxynitrite formation are at least partially responsible for the impairment in endothelium-dependent relaxations displayed in shunted lambs.

Previously we demonstrated that mRNA expression and protein content of eNOS were significantly increased in shunted lambs relative to controls (2). In situ hybridization and immunohistochemistry localized these increases to the endothelium of small and large PAs. The size of vessels used in our current study was well within the vessel size used to determine NOS expression. In fact, Western blot analysis of these isolated vessels demonstrated that the increase in eNOS protein was isolated to the PAs and was unchanged in the PVs. These data suggest that eNOS may be a source for the superoxide-mediated impaired relaxations. All vessels were aerated with a gas mixture that produced hyperoxic conditions. Although pretreatment with SOD-CAT did not alter relaxations in the control PAs, it is possible that these hyperoxic conditions amplified superoxide production in the shunt PAs.

Relaxations to exogenous NO in the form of SNAP and to ANP, which produces cGMP by the particulate guanylate cyclase pathway, were not different in either the PAs or PVs of shunted lambs. These data suggest that no difference in functional activity of soluble guanylate cyclase or in mechanisms of relaxation to cGMP exists in either the PAs or PVs. Vascular relaxations to cGMP-specific phosphodiesterase inhibitors such as zaprinast are dependent on basal endothelial NO production as well as on endogenous phosphodiesterase activity (6). We previously reported an 80% increase in expression of PDE5 in shunted lambs (2). Increased functional activity of PDE5 would be expected to enhance cGMP clearance and therefore blunt relaxations to cGMP generated in response to NO and ANP. However, we found no difference between PAs isolated from control and shunted lambs in relaxations to NO and ANP in the current study or in previous studies; therefore it is unlikely that the decreased relaxations to zaprinast were the result of altered phosphodiesterase activity. Instead we conclude that these decreased relaxations are most likely due to decreased functional eNOS activity in the shunt vessels, leading to decreased NO and cGMP production. Finally, because A-23187 is a receptor-independent agonist, the impairment in endothelium-dependent relaxations we previously demonstrated to ACh in vivo does not appear to be secondary to alterations in receptor function.

Although we were not able to directly compare NO production in our isolated PVs, it seems likely that endothelial dysfunction associated with excess superoxide production interferes with the vascular relaxing effects of NO produced from NOS. By clearing excess superoxide, SOD and CAT restored normal reactivity. There is evidence that reactive oxygen species diminish NO bioactivity in human diseases including systemic hypertension (20). Our data indicate a potential role in pulmonary hypertension associated with congenital heart disease as well. Further studies can be focused on determining vascular NO production, potential sources of excess vascular superoxide production, and the interactions between these as potential mechanisms of early endothelial dysfunction in this model of pulmonary hypertension.

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