Increased oxidative stress in lambs with increased pulmonary blood flow and pulmonary hypertension: role of NADPH oxidase and endothelial NO synthase

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Increased oxidative stress in lambs with increased pulmonary blood flow and pulmonary hypertension: role of NADPH oxidase and endothelial NO synthase. Am J Physiol Lung Cell Mol Physiol 290: L1069–L1077, 2006; doi:10.1152/ajplung.00408.2005.—Although oxidative stress is known to contribute to endothelial dysfunction-associated systemic vascular disorders, its role in pulmonary vascular disorders is less clear. Our previous studies, using isolated pulmonary arteries taken from lambs with surgically created heart defect and increased pulmonary blood flow (Shunt), have suggested a role for reactive oxygen species (ROS) in the endothelial dysfunction of pulmonary hypertension, but in vivo data are lacking. Thus the initial objective of this study was to determine whether Shunt lambs had elevated levels of ROS generation and whether this was associated with alterations in antioxidant capacity. Our results indicate that superoxide, but not hydrogen peroxide, levels were significantly elevated in Shunt lambs. In addition, we found that the increase in superoxide generation was not associated with alterations in antioxidant enzyme expression or activity. These data suggested that there is an increase in superoxide generation rather than a decrease in scavenging capacity in the lung. Thus we next examined the expression of various subunits of the NADPH oxidase complex as a potential source of the superoxide production. Results indicated that the expression of Rac1 and p47phox is increased in Shunt lambs. We also found that the NADPH oxidase inhibitor diphenyliodonium (DPI) significantly reduced dihydroethidium (DHE) oxidation in lung sections prepared from Shunt but not Control lambs. As DPI can also inhibit endothelial nitric oxide synthase (eNOS) superoxide generation, we repeated this experiment using a more specific NADPH oxidase inhibitor (apocytochrome b558). Our results indicate that both inhibitors significantly reduced DHE oxidation in lung sections prepared from Shunt but not Control lambs. To further investigate the mechanism by which eNOS becomes uncoupled in Shunt lambs, we evaluated the levels of dihydrobiopterin (BH2) and tetrahydrobiopterin (BH4) in lung tissues of Shunt and Control lambs. Our data indicated that although BH4 levels were unchanged, BH2 levels were significantly increased. Finally, we demonstrated that the addition of BH2 produced an increase in superoxide generation from purified, recombinant eNOS. In conclusion our data demonstrate that the development of pulmonary hypertension in Shunt lambs is associated with increases in oxidative stress that are not explained by decreases in antioxidant expression or activity. Rather, the observed increase in oxidative stress is due, at least in part, to increased expression and activity of the NADPH oxidase complex and uncoupled eNOS due to elevated levels of BH2.

CHILDREN WITH CONGENITAL HEART DISEASE and increased pulmonary blood flow suffer significant morbidity and mortality due to pulmonary vascular remodeling and its resultant increase in pulmonary arterial pressure and/or resistance. Indeed, pulmonary morphometric analysis of the lungs of these patients shows altered pulmonary vascular growth and remodeling, which correlates with the hemodynamic state (32). In addition, these children have evidence of endothelial dysfunction as indicated by impaired endothelium-dependent relaxation in early disease and decrease endothelial nitric oxide synthase (eNOS) gene expression in late disease (9, 12). We have established a lamb model that mimics a congenital heart defect with increased pulmonary blood flow using in utero placement of an aorta-to-pulmonary artery vascular graft (33). This model is associated with increased pulmonary blood flow and pressure. At 4 wk of age, these Shunt lambs have clinical and pathologic sequelae similar to children with congenital heart defects associated with increased pulmonary blood flow and pulmonary hypertension, including a selective impairment of endothelium-dependent pulmonary vasodilation.

Using isolated pulmonary arteries taken from shunt lambs, we have previously found that the removal of superoxide enhanced endothelium-dependent relaxations (38). Thus the endothelial dysfunction associated with pulmonary hypertension may be due, in part, to excessive superoxide production. However, the mechanisms by which this increased oxidative stress are produced remains unresolved. The primary source of reactive oxygen species in vascular cells is superoxide-generating NADPH oxidase (39, 42, 43, 45, 46), whereas eNOS can produce superoxide when uncoupled (18, 20). This occurs when the consumption of NADPH is uncoupled from nitric oxide (NO) synthesis resulting in the production of superoxide anions and hydrogen peroxide (H2O2). This uncoupling can occur in conditions in which there is reduced availability of the cofactor, tetrahydrobiopterin (BH4) or the substrate, L-arginine. Under normal conditions, eNOS produces NO in the vasculature that can rapidly combine with superoxide to produce the...
oxidant peroxynitrite, which can oxidize a variety of biological molecules (18, 35). \( \text{H}_2\text{O}_2 \), produced via the dismutation of superoxide, can also serve as a signaling molecule within the vasculature. Antioxidant enzyme systems play a role in the regulation of oxidant levels in the vasculature and could potentially play a role in the development of diseases (including hypertension), and dysregulation of these systems could lead to imbalances in the normal signaling, resulting in pathological manifestations (45). There are several antioxidant systems, including superoxide dismutases (SOD) and catalase, which regulate the presence of oxidant species in vivo. There are two SOD in cells, Cu/ZnSOD (SODI) in the cytoplasm and MnSOD (SODII) in the mitochondria, whereas extracellular SOD (SODIII) is present in the extracellular spaces. These enzymes catalyze the rapid conversion of superoxide into \( \text{H}_2\text{O}_2 \) and oxygen, whereas \( \text{H}_2\text{O}_2 \) is converted within the cell to oxygen and water by the action of catalase (45). These systems serve to keep oxidant levels low, thereby reducing oxidant stress. Thus the purpose of this study was to explore the mechanisms underlying the increase in oxidative stress in lambs with pulmonary hypertension secondary to increased pulmonary blood flow (33).

**MATERIALS AND METHODS**

**Surgical preparations and care.** Twelve mixed-breed Western pregnant ewes (137–141 days gestation, term = 145 days) were operated on under sterile conditions with the use of local anesthesia (2\% lidocaine hydrochloride) and inhalational anesthesia (1–3\% isoflurane). A midline incision was made in the ventral abdomen, and the pregnant horn of the uterus was exposed. Through a small uterine incision, the left fetal forelimb and chest were exposed, and a left thoracotomy was performed. The thoracotomy incision was then closed in layers. This procedure was previously described in detail (28).

Four weeks after spontaneous delivery, the lambs were fasted for 24 h, with free access to water. The lambs were then anesthetized with ketamine hydrochloride (15 mg/kg im). With the lambs under additional local anesthesia with 1\% lidocaine hydrochloride and ketamine hydrochloride (20 mg im). With the use of side-biting vascular clamps, an 8.0-mm Gore-tex vascular graft (~2 mm length; W. L. Gore and Associates, Milpitas, CA) was anastomosed between the ascending aorta and main pulmonary artery with 7.0 prolene (Ethicon, Somerville, NJ) by a continuous suture technique. The thoracotomy incision was then closed in layers. This procedure was previously described in detail (28).

**SOD activity assay.** Frozen (~80°C) lung tissue was weighed, 5x the weight of 10 mM EDTA was added, and the tissue was minced and sonicated with 3× 10-s pulses at 40% power (High Intensity Ultrasonic Processor, Autotune series; A. Daigger, Vernon Hills, IL). The samples were centrifuged at 20,000 g, 4°C for 15 min, the supernatant was collected, and aliquots were frozen at -80°C until needed. Tissue SOD activity was determined by a modification of the method of Elstner and Heupel (11) using bovine liver SOD (Sigma Chemical) to generate the standards.

**Catalase assay.** Catalase activity was quantified by a modification of the method of Aebi (2) in 50 mM potassium phosphate buffer, pH 7.0. For each sample 40 μg of protein were added to buffer, 3\% \( \text{H}_2\text{O}_2 \) (~10 mM) was added, and the consumption followed at 240 nm for 60 s using the Shimadzu UV-1700 Pharmaspec and UV Probe software (Shimadzu, Columbia, MD). The change in absorbance was used to calculate catalase-specific activity using the extinction coefficient of 43.6 m\( \text{M}^{-1} \cdot \text{cm}^{-1} \).

**High-performance liquid chromatography determination of tissue BH4.** Dihydrobiopterin (BH4) and BH2 content were measured by high-performance liquid chromatography (HPLC) with fluorescent detection after differential iodine oxidation as described by Milstien et al. (28) with minor modifications. In brief, ~200 μg of lung tissue were sonicated in 500 μl of 50 mM Tris buffer (pH 7.4) with 1 mM DTT and 1 mM EDTA and centrifuged at 20,000 g for 15 min at 4°C. Protein content of the supernatants was determined by the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). For the acidic oxidation, 100 μl of supernatant were added to 50 μl of 1 M H3PO4 containing 5 μl of 1% I2 in 2% KI.
Detection of BH₂ and BH₄ was performed using an Amersham Biosciences AKTA purifier system (GE Healthcare, Piscataway, NJ) with a Spherisorb 5 μM ODS 1 reverse phase column (4.6 x 25 mm; Waters, Milford, MA), equipped with a Jasco FP-2020 fluorescence detector (Jasco, Tokyo, Japan). BH₂ and BH₄ levels were quantified by fluorescence detection at 450 nm (emission) and 350 nm (excitation) using an isocratic elution in 5% methanol/95% H₂O over 20 min at a flow rate of 0.8 ml/min. Retention time for reduced pterins was ~14 min as confirmed by a standard. We calculated total BH₄ level by subtracting the amount of reduced pterins present following oxidation in base by the amount of reduced pterins present following oxidation in acid. Total BH₂ was determined as the amount of reduced pterins present following oxidation in base.

Purification of recombinant human eNOS. The poly-His-pCWeNOS vector (a gift from Paul Ortiz de Montellano, UCSF) was transformed in to the protease-deficient Escherichia coli strain BL21 (DE3) pLysS (Novagen). Cells were grown in Luria broth with 1% glyceral containing 200 μg/ml ampicillin and 40 μg/ml chloramphenicol. Cultures were grown at 28°C until an optical density at 600 nm (OD₆₀₀) of 0.8 was reached. Approximately 1 h before that, heme precursor was added. Cells were then induced by addition of isopropyl-β-D-thiogalactoside (0.8 mM final concentration); 350 μM ATP and 3 μM riboflavin were also added, and the cells were then grown at 22°C for a further 48 h in the dark. Cells were then harvested by centrifugation (15 min at 4,000 g at 4°C) and resuspended in lysis buffer (40 mM N-(2-hydroxyethyl)piperazine-N-(3-propane sulfonic acid) (EPPS), pH 7.6, containing 1 mg/ml lysozyme, 150 mM NaCl, 0.5 mM t-arginine, 4 μM BH₁, 2 μM flavin adenine dinucleotide (FAD), 10% glycerol) and protease inhibitor cocktail (Sigma). The bacterial suspension was incubated with mild shaking at 4°C for 30 min to ensure complete cell lysis. Cells were broken by sonication with three 25-s pulses followed by three cycles of freezing and thawing. Cell debris was removed by centrifugation at 30,000 g for 30 min at 4°C. The supernatant was then applied to a Ni-NTA His-Bind Superflow (Novagen) column pre-equilibrated with buffer A (40 mM EPPS, pH 7.6, containing 150 mM NaCl, 10% glycerol, and 0.5 mM L-arginine). The column was washed with five bed volumes of buffer A followed by buffer B (buffer A with 25 mM imidazole). The bound protein was then eluted with buffer C (buffer A + 200 mM imidazole). The heme-containing fractions were pooled and concentrated using centricon-100 YM-10 (Millipore). The concentrated proteins were dialyzed against three changes of buffer A containing 4 μM BH₄ and 1 mM DTT. The protein was further purified by using a 2.5’-ADP-Sepharose column equilibrated with 40 mM Tris buffer pH 7.6, containing 1 mM t-arginine, 3 mM DTT, 4 μM BH₄, 4 μM FAD, 10% glycerol, and 150 mM NaCl (buffer D) and washed with buffer D containing 400 mM NaCl to prevent nonspecific binding. eNOS was then eluted with buffer E (buffer D with 5 mM 2’-adenine monophosphate). The heme-containing fractions were pooled, concentrated; dialyzed at 4°C against buffer D containing 1 mM DTT, 4 μM BH₄, 4 μM FAD, and 10% glycerol; and stored at -80°C until used. The DTT, BH₄, and FAD were removed by repeated buffer exchange using Centricron centrifugal filters before use.

Electron spin resonance spectroscopy and spin trapping. Spin trapping measurements of oxygen radical generation were performed using a MiniScope MS200 instrument (Magnettech, Berlin, Germany). The reaction mixture consisted of 50 mM HEPES, pH 7.4, containing 1 mM NADPH, 1 mM Ca²⁺, 1 mM DTPA, 10 μg/ml calmodulin, and 4 μM BH₄ in 50 μl. Purified eNOS (2 μg) and BH₂ (250 nM–100 μM) were added to the reaction with 10 mM 5-diethoxyphosphoryl-5-methyl-1-pyrrrole-n-oxide (DEPMPO) as the spin trap. The samples (35 μl) were loaded into a 50-μl capillary tube, and spectra were obtained after 20 min with the following parameters: microwave power of 80 mW, modulation amplitude of 1.000 mG, and modulation frequency of 100 kHz. Quantitation of the amplitude of free radical signals was performed using ANALYSIS version 2.02 software (Magnettech).

Statistical analysis. Statistical calculations were performed using the software GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego CA; www.graphpad.com). Means ± SE were calculated for all samples, and significance was determined either by the unpaired t-test (for 2 groups) or ANOVA with Dunnett’s post hoc test (for ≥3 groups). A value of P < 0.05 was considered significant (29).

RESULTS

Superoxide but not H₂O₂ levels are increased in Shunt compared with control lambs. Initially we used the fluorescent dyes DHE and H₂DCF-DA to determine whether superoxide and H₂O₂ were altered in pulmonary vessels of Shunt compared with Control lambs at 4 wk of age (n = 6 for each). The results obtained indicate that DHE oxidation was 2.5-fold higher in the pulmonary arteries isolated from Shunt lambs relative to controls (Fig. 1, A and B, P < 0.05), whereas H₂DCF-DA fluorescence was unchanged (Fig. 1, C and D; P > 0.05). Pretreatment of the tissue sections with either PEG-SOD (Fig. 1B) or PEG-catalase (Fig. 1D) significantly reduced the quantifiable DHE or H₂DCF-DA fluorescence in respective tissue sections.

Catalase, SODI, and SODII expression is unchanged in Control and Shunt lambs. Changes in lung antioxidant systems could produce the observed differences in superoxide (determined by DHE) or H₂O₂ (determined by DCFDA), and Western blot analysis was used to evaluate the expression of catalase, SODI, and SODII. The data obtained indicate that there were no differences in expression of catalase (Control = 264,693 ± 30,256; Shunt = 248,938 ± 29,481), SODI (Control = 601,146 ± 86,351; Shunt = 910,558 ± 150,825) or SODII (Control = 166,299 ± 20,547; Shunt = 232,149 ± 29,606) between Control and Shunt lambs (all P > 0.05).

SOD and catalase activities are unchanged between Control and Shunt lambs. Because the levels of catalase, SODI, and SODII were unchanged, we next evaluated whether the activity of these proteins was altered in Shunt compared with Control lambs. Again, we observed no differences in either catalase (Control = 5.23 ± 0.47 × 10⁻⁴ to 0.57 × 10⁻⁴ IU/μg protein; Shunt = 6.3 ± 0.98 × 10⁻⁴ IU/40 μg protein, P > 0.05) or total SOD activity (Control = 6.1 ± 0.54 U/mg protein; Shunt = 7.63 ± 0.78 U/mg protein, P > 0.05) between Control and Shunt lambs.

NADPH oxidase subunit expression is increased in Shunt compared with Control lambs. As the increase in superoxide levels in Shunt lambs was not accounted for by decreases in antioxidant proteins, we next evaluated other potential sources of superoxide. The primary source of superoxide in the vasculature is believed to be NADPH oxidase a multisubunit protein that requires the action of a number of subunits to become functional. Our previous data have suggested that Rac1, p47phox, and p67phox are important in the generation of super-
oxide in the pulmonary vasculature, and we used Western blot analysis to determine whether the expression of these subunits was altered in Shunt compared with Control lambs. Our results indicate that the expression of Rac1 (Fig. 2, A and B; \( P < 0.05 \)) and p47^{phox} (Fig. 2, C and D; \( P < 0.05 \)) were elevated in Shunt lambs, whereas p67^{phox} expression was unchanged (Fig. 2, E and F; \( P > 0.05 \)).

**NADPH oxidase and eNOS are significant sources of the increased superoxide observed in Shunt compared with Control lambs.** We utilized pharmacologic inhibitors of NADPH oxidase to further confirm the role played by NADPH oxidase activity in the increased superoxide observed in Shunt lambs. Slides of cryosectioned lung tissues were pretreated with either PBS or inhibitors of NADPH oxidase (apocynin and DPI) before exposure to DHE. When we evaluated DHE oxidation of multiple vessels within the lungs of Control lambs, no significant differences were observed between inhibitor-treated and untreated sections (Fig. 3A). However, in sections prepared from Shunt lambs, DHE oxidation was decreased by 53\% for apocynin (\( P < 0.01 \)) and ~71\% for DPI (\( P < 0.01 \)) (Fig. 3B). As DPI can also inhibit eNOS activity, we also examined the potential for an uncoupled eNOS to be playing a role in the increased superoxide generation observed in Shunt compared with Control lambs. To accomplish this, we pretreated slides of cryosectioned lung tissues with either PBS or the NOS inhibitor 3-ETU. Again 3-ETU had no effect on DHE oxidation in sections prepared from Control lambs (Fig. 3A). However, in sections prepared from Shunt lambs, DHE oxidation was decreased by ~54\% by pretreatment with 3-ETU (\( P < 0.01 \)) (Fig. 3B). These data suggest that both NADPH oxidase and eNOS are responsible, at least in part, for the increased superoxide generation observed in the lungs of Shunt compared with Control lambs.
One potential mechanism by which eNOS becomes a significant source of superoxide is by uncoupling under conditions where BH₄ levels are decreased. Thus we next evaluated the potential role of altered biopterin metabolism in Shunt lambs to evaluate its role in eNOS uncoupling. However, when lung tissue levels of BH₄ were quantified by HPLC, levels were found to be unchanged in Shunt compared with Control lambs (Fig. 4A). As the rate-limiting step in BH₄ biosynthesis is controlled by GTP cyclohydrolase I, we also evaluated the expression of this protein by Western blot analysis. Confirming the HPLC analysis, we found no significant differences between Shunt and Control lambs (Control/H₁₁₀₀⁵ 38,789/Control/H₁₁₀₀⁶ 8,932; *P < 0.05). However, we did find that the tissue levels of BH₂ (an oxidized form of BH₄) were significantly elevated in the lungs of Shunt compared with Control lambs (Fig. 4B, *P < 0.05 vs. control).

Elevated BH₂ levels lead to an uncoupling of eNOS. Finally, we evaluated whether our observed increases in BH₂ (an oxidized form of BH₄) could uncouple eNOS. Using ESR, we found that the addition of BH₂ dose dependently uncoupled eNOS and led to an increase in superoxide generation by purified recombinant human eNOS (Fig. 5). Furthermore, this uncoupling could be achieved using the ratio of BH₂/BH₄ (1:7) we determined in vivo.

**DISCUSSION**

An increasing number of studies implicate oxidative stress in the development of endothelial dysfunction and the pathogenesis of cardiovascular disease (8, 15). Furthermore, an increasing body of evidence suggests that endothelial injury and the resulting alterations in the balance of vasoactive substances play a significant role in the development of pulmonary hypertension (9, 10, 31). Support for this hypothesis is strengthened by observations that endothelial injury precedes the vascular remodeling seen in several animal models of pulmonary hypertension (1, 26). In humans, endothelial dysfunction, demonstrated by histological abnormalities of the endothelium, impairment of endothelium-dependent pulmonary vasodilation, as well as increased plasma endothelin-1 concentrations, has been described in children with congenital heart defects and pulmonary hypertension before the development of significant vascular remodeling (9, 31, 48). There is also increasing evidence that alterations in the production and/or ability to scavenge reactive oxygen species (ROS) participate in the endothelial dysfunction associated with pulmonary hypertension. We have previously shown that both superoxide (6) and H₂O₂ (47) are increased in an ovine ductal ligation model of persistent pulmonary hypertension of the newborn (PPHN). In
addition, our previous data indicated that superoxide scavenging via SOD increased NO-mediated signaling in pulmonary vessels isolated from Shunt lambs, suggesting that the underlying endothelial dysfunction in these lambs is also associated with increased oxidative stress (38). In this study, our initial data confirmed an increase in superoxide generation although interestingly not H$_2$O$_2$. There are two mechanisms by which the increase in superoxide levels could occur in the Shunt lambs: either by decreased scavenging or by increased generation. To determine whether the observed differences in oxidant levels in Shunt lambs could be explained by alterations in SOD expression or activity, we next investigated the involvement of superoxide-generating enzymes.

Fig. 3. Both NADPH oxidase and endothelial nitric oxide synthase (eNOS) contribute to the increased superoxide generation in Shunt lambs. A: unfixed frozen sections (5 μm) of peripheral lung prepared from Control lambs were incubated pretreated with inhibitors of NADPH oxidase (apocynin), eNOS (3-ETU), or both (DPI); then the effects on ethidium fluorescence were visualized by fluorescent microscopy. Relative changes in DHE oxidation relative to untreated (UTD) sections were then determined. None of the inhibitors significantly altered DHE oxidation. Values are means ± SE from 6 Control and 6 Shunt lambs. B: unfixed frozen sections (5 μm) of peripheral lung prepared from Shunt lambs were incubated pretreated with inhibitors of NADPH oxidase (apocynin), eNOS (3-ETU), or both (DPI); then the effects on DHE oxidation were visualized by fluorescent microscopy. Relative changes in ethidium fluorescence relative to UTD sections were then determined. DHE oxidation was significantly decreased by pretreatment with apocynin, ETU, and DPI in the pulmonary vessels of the Shunt lambs. Values are means ± SE from 6 Control and 6 Shunt lambs. *P < 0.05 vs. untreated.

A similar increase in superoxide levels independent of changes in SOD activity has been previously described in a rodent model of hypertension by Landmesser et al. (20). Because the increases in superoxide levels observed in Shunt lambs could not be explained by alterations in SOD expression or activity, we next investigated the involvement of superoxide-generating enzymes. Sources of superoxide within the vasculature include lipoxygenase, cyclooxygenase, xanthine oxidase, NOS, and NADPH oxidase (24); however, it is generally recognized that NADPH oxidase is the predominant explained by decreases in SOD protein expression or activity.

Fig. 4. Determination of dihydro (BH$_2$)- and tetrahydrobiopterin (BH$_4$) levels in peripheral lung tissue from Control and Shunt Lambs. The levels of BH$_4$ (A) and BH$_2$ (B) were determined in peripheral lung homogenates by HPLC. There were no significant differences in BH$_4$ levels in peripheral lung tissue from Control and Shunt lambs (A). However, BH$_2$ levels were found to be significantly higher in peripheral lung tissue from Shunt compared with Control and lambs. Values are means ± SE from 6 Control and 6 Shunt lambs. *P < 0.05 vs. Control.

Fig. 5. BH$_2$ stimulates eNOS superoxide generation. Purified human eNOS (2 μg) containing BH$_4$ (4 μM), NADPH (1 mM), Ca$^{2+}$ (1 mM), or calmodulin (10 μg/ml) was incubated with increasing concentrations of BH$_2$ (250 nM–100 μM), and the superoxide generated was trapped with 5-diethoxyphosphoryl-5-methyl-1-pyrroline-n-oxide (10 mM). Increasing concentrations of BH$_2$ led to a significant increased in eNOS-derived superoxide. Values are means ± SE from 6 determinations for each BH$_2$/BH$_4$ ratio. *P < 0.05 vs. no BH$_2$. 

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source of ROS in the vasculature (7). Thus we initially focused our studies on the NADPH oxidase system. NADPH oxidase is a multicomponent system that is composed of cytoplasmic and membrane-bound components that are assembled into an active complex upon stimulation. Therefore, increases in the expression of individual components should correspond to an increase in overall NADPH oxidase activity. Our Western blotting data indicate that the expression of both Rac1 and p47phox was increased in peripheral lung tissue prepared from Shunt compared with Control lambs. This correlates well with our recent in vitro studies in which we showed important roles for Rac1 and p47phox in regulating NADPH oxidase activity in cells isolated from the pulmonary vasculature (23, 30). Interestingly, we did not observe an increase in the expression of the p67phox subunit in peripheral lung tissue prepared from Shunt compared with Control lambs. This is in contrast to our recent studies using the ductal ligation model of PPHN in which we observed an increase in NADPH oxidase activity that was associated with increased expression of the p67phox subunit (6). The differences observed, in which NADPH oxidase subunits are increased during pulmonary hypertension, suggest that there may be different stimuli for the various NADPH oxidase subunits in these two models. It is also possible that some of the increases in superoxide we have observed in Shunt lambs may be due to increased activity of the NADPH oxidase complex independent of increases in Rac1 and p47phox. The pulmonary circulation of the Shunt lambs is exposed to increased in fluid shear, stress, cyclic stretch, and pressure due to the increase in pulmonary blood flow, and both shear stress and mechanical stretch have been previously shown to activate NADPH oxidase in endothelial and smooth muscle cells independently of changes in NADPH oxidase subunit expression. Rather, it is likely that translocation of the subunits from the cytosolic to membrane compartments also plays an important role in NADPH oxidase assembly and activation.

When we utilized pharmacological inhibitors of the NADPH oxidase complex, we found that although these agents could reduce the superoxide generation in the cryosections prepared from Shunt lambs, the levels were not reduced to levels seen in Control lambs. This suggested that there were other sources of superoxide in the Shunt lambs, as suboptimal concentrations of cofactors have been shown to reduce the formation of NO and favor the “uncoupling” of NOS, leading to NOS-mediated reduction of oxygen and formation of superoxide anions. Indeed, recent findings suggest that reductions in BH4 may contribute to the pathogenesis of endothelial dysfunction in hypertension, hypercholesterolemia, diabetes, smoking, and ischemia-reperfusion (4, 14, 16, 27, 37, 41). BH4 is a cofactor essential for the catalytic activity of all three NOS isoforms (13, 19, 25, 40), and studies indicate that cellular BH4 levels have important consequences for the structure of NOS. These include the ability of NOS to shift its heme iron to a high spin state, increase arginine binding, and, at least in some NOS isoforms, stabilize the active dimeric form of the enzyme (13). However, accumulated evidence indicates that an optimal concentration of BH4 is of fundamental importance for normal function of eNOS in vascular endothelial cells. Thus given the importance of BH4 in regulating NO generation from NOS, we next evaluated the levels of BH4 in peripheral lung tissue prepared from Shunt and Control lambs. Our data indicate that there were no significant differences in BH4 levels in Shunt compared with Control lambs. These data are not unexpected as there is little evidence for the loss of BH4 in the dysfunctional blood vessel, although studies have shown that endothelial function can be normalized by BH4 supplementation in experimental animal models of insulin resistance and hypercholesterolemia (21, 36, 37, 41). However, the exact mechanisms responsible for the beneficial effects of BH4 remain unclear. The rate-limiting step in BH4 biosynthesis is controlled by GTP cyclohydrolase I, and alterations in the expression of this enzyme could affect BH4 levels. However, when we evaluated the expression of GTP cyclohydrolase by Western blot analysis, no difference was observed between Control and Shunt lambs, again confirming the BH4 data. However, our analysis of tissue bioppterin concentrations does demonstrate that Shunt lambs had significant increases in the levels of BH2, and we also found that exposing purified eNOS to increasing the levels of BH2 led to a significant increase in superoxide generation. These in vivo and in vitro data are in agreement with previous studies that found both peroxynitrite and superoxide could oxidize BH4 (18, 20, 22) and that increases in BH2 relative to BH4 (increased BH2/BH4 ratio) can result in increased superoxide production from eNOS (3, 44).

Increased oxidation of BH4 to BH2 leading to the uncoupling of eNOS and increasing the production of superoxide could also play a role, as both peroxynitrite and superoxide have been shown to oxidize BH4 (18, 20, 22). When BH4 and BH2 were quantified in the lung tissues of Control and Shunt animals, the levels of BH2 were increased in the Shunt animals while BH4 levels were unchanged. This suggests that the GTP cyclohydrolase activity and production of BH4 are not affected by the increased oxidant status but that there is an increased oxidation of BH2 to BH3 in this model. Increases in the level of BH2 have been shown to uncouple eNOS leading to the additional production of superoxide (44), and previously published work has implicated increases in BH2 relative to BH4 (increased BH2/BH4 ratio) with increased superoxide production from eNOS (3, 44). Landmesser et al. (20) has also demonstrated that hypertension led the production of ROS from NADPH oxidase an increase in the oxidation of BH4, uncoupling of eNOS, resulting in a reduction in NO production and increased ROS production from uncoupled eNOS (20). Our previous studies have indicated that, although eNOS expression is increased in Shunt lambs at 4 wk of age, NO-mediated signaling is diminished (5, 34). Thus our data suggest that the infusion of BH4 or its analogs may be beneficial in reducing both the oxidative stress and pulmonary endothelial dysfunction associated with increased pulmonary blood flow. However, further studies will be required to test this hypothesis.

In conclusion this study identifies superoxide as the primary oxidant molecule responsible for the increased oxidative stress in a lamb model of pulmonary hypertension secondary to increased pulmonary blood flow. We conclude that these increases are not due to a reduction in antioxidant scavenging capacity in the lung but, rather, to increased generation from NADPH oxidase and eNOS. Further studies are warranted to test the efficacy of increasing BH4 levels in reducing the endothelial dysfunction and vascular remodeling associated with the development of pulmonary hypertension in this model.
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