Increased Superoxide Generation Is Associated With Pulmonary Hypertension in Fetal Lambs
A Role for NADPH Oxidase


Abstract—Ligation of the ductus arteriosus in utero produces pulmonary hypertension and vascular remodeling in fetal and newborn lambs. However, the mechanisms producing these vascular changes are not well defined. Because reactive oxygen species (ROS) have been implicated as mediators of smooth muscle cell proliferation, we hypothesized that increased formation of ROS may be involved in the pathophysiology of pulmonary hypertension after in utero ductal ligation. Using ethidium fluorescence, we demonstrated an increase in superoxide levels after 9 days of ductal ligation compared with control lungs ($P<0.05$) that was localized to the adventitia and smooth muscle cells of hypertensive vessels. SOD-1 and SOD-2 protein levels and activities in lung, vein, and artery of hypertensive lambs were unchanged relative to controls after 2 days of ductal ligation. However, after 9 days, superoxide dismutase (SOD) activity was significantly decreased in arteries from ligated lambs without associated changes in SOD protein expression ($P<0.05$).

Examination of NADPH oxidase expression as a potential source of the superoxide production indicated that the levels of p67phox, a subunit of the NADPH oxidase complex, were significantly increased in the pulmonary arteries, but not veins, from the ligated lung as early as 2 days ($P<0.05$). Functional analyses demonstrated that reducing superoxide levels significantly increased the NO-mediated relaxation of pulmonary arteries isolated after 9 days, but not 2 days, of ductal ligation ($P<0.05$). These results suggest that increased NADPH oxidase expression may increase levels of superoxide in persistent pulmonary hypertension of the newborn lung tissue, and that increased superoxide blunts vascular relaxations to exogenous NO while stimulating smooth muscle cell growth. (Circ Res. 2003;92:683-691.)

Key Words: reactive oxygen species $\blacklozenge$ vascular remodeling $\blacklozenge$ smooth muscle

With initiation of ventilation and oxygenation at birth, pulmonary vascular resistance decreases and pulmonary blood flow increases. In some neonates, this normal transition does not occur, resulting in persistent pulmonary hypertension of the newborn (PPHN). PPHN can occur idioopathically or as a complication of a variety of cardiorespiratory disorders, including hyaline membrane disease, asphyxia, meconium aspiration, and congenital diaphragmatic hernia. In newborns that die with PPHN, there is an increase in both the thickness of the smooth muscle layer within small pulmonary arteries and an extension of this muscle to nonmuscular arteries. There is also proliferation of adventitial tissue. These structural changes indicate that in utero events have altered the pulmonary circulation. However, the mechanism underlying these changes is unclear. Surgical ligation or constriction of the ductus arteriosus in the fetal lamb several days before delivery at term results in PPHN after delivery. This lamb model closely resembles the human condition, with suprasystemic pulmonary arterial pressure and anatomic changes including muscularization of the normally nonmuscular intra-acinar arteries.

Recently, production of reactive oxygen species (ROS) has been demonstrated in fibroblasts, endothelial cells, and smooth muscle cells (SMCs). This oxidant production plays an important role both in the normal functioning of vascular systems as well as in the pathogenesis of vascular disease. Every cell type in the vascular wall has been shown to produce and be regulated by ROS. We, and others, have demonstrated that a vascular equivalent of the phagocytic NADPH oxidase plays an important role in generating ROS in cells of the vessel wall. This vascular NAD(P)H oxidase enzyme complex appears to be membrane associated, catalyzing the one electron reduction of oxygen using NADPH or NADH as the electron donor: NAD(P)H $+2O_2\rightarrow$ NAD(P)$^+$ as the electron donor: NAD(P)$^+$ $+2O_2^-$ $+2H^+$. One of the important attributes of the vascular oxidase is that it responds to external signals to generate superoxide. However, the role of superoxide and NADPH oxidase in the development of PPHN has not been evaluated. Thus, the purpose...
of our study was to determine whether the development of PPHN is associated with increased superoxide generation, determine a possible mechanism for this effect, and establish whether superoxide scavenging could restore NO-mediated vessel relaxation and reduce SMC proliferation.

Materials and Methods
The Laboratory Animal Care Committee at the State University of New York at Buffalo and Northwestern University approved all protocols.

Prenatal Ligation of the Ductus Arteriosus
The technique for creating pulmonary hypertension by prenatal ligation of the ductus arteriosus has been described previously. Briefly, time-dated pregnant ewes (mixed breed; Swartz family sheep farm, Attica, NY) were operated on at 127 days of gestation (term=146 days). After either 2 or 9 days, the fetus was delivered by cesarean section and the umbilical cord was clamped (n=8). Twin fetuses served as controls (n=8). Before the first breath, lambs were killed by rapid exsanguination through a direct cardiac puncture.

Tissue Preparation and Western Blotting
Peripheral lung parenchyma and fifth-generation pulmonary arteries and veins were rapidly isolated and frozen in liquid nitrogen. Protein extracts, prepared as previously described, were separated on 12% (superoxide dismutase [SOD] enzymes) or 4% to 20% (copper CCS-1 protein (1:250), and p67phox (1:500). After washing blots to polyacrylamide gels, then transferred onto enhanced chemiluminescence nitrocellulose (Amersham Pharmacia). After blocking, the membranes were incubated at room temperature for 1 hour with the appropriate dilution of antibody: CuZnSOD and MnSOD (1:1000), CCS-1 protein (1:250), and p67phox (1:500). After washing blots to remove excess primary antibody binding, blots were incubated for 1 hour with the appropriate horseradish peroxidase–linked secondary antibody: rabbit (1:1000) for SOD and CCS-1 and mouse (1:1000) for p67phox. Antibody binding was detected using enhanced chemiluminescence (Amersham Pharmacia).

Immunohistochemistry
Immunohistochemistry was performed as previously described. Studies were done on serial sections of control and ductal ligation lung, using polyclonal antiserum raised against the p67phox subunit of NADPH oxidase. The tissue sections were incubated in antiserum (1:500 dilution) for 12 to 18 hours at 4 °C. Appropriate biotin-conjugated secondary antibodies were used and detected with an anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC). Sections were then viewed under a fluorescent microscope (Olympus BX40) and imaged using a digital camera (Pixera DiRactor).

In Situ Analysis of Superoxide Generation
Lung segments and vessels were obtained from control and PPHN lambs as early as 2 days of ductal ligation, and these increases

Isolated Vessel Protocol
We have previously described the techniques for study of isolated vessels in detail. In the present study, we used fifth-generation pulmonary arteries with inside diameters of≤500 μm. Vessel rings were mounted on stainless steel hooks and placed in water-jacketed chambers containing Krebs-Ringer solution. The buffer was maintained at 37 °C and aerated with a gas mixture of 94% O2 and 6% CO2 to maintain a pH of 7.40, a Pco2 of 38 torr, and a Po2 of >500 torr. Vessels were pretreated for 20 minutes with 10−5 mol/l indomethacin to prevent the formation of vasoactive prostaglandins and with 10−5 mol/l propranolol to block activation of β-adrenergic receptors. Vessels were preconstricted with a submaximal concentration of norepinephrine. Cumulative concentration-response curves for N-nitrosyl-acyetyl-penicillamine (SNAP, 10−4 to 3×10−6 mol/L) were developed. Some vessels were pretreated for 20 minutes with polyethylene glycol–bound SOD (37.5 U/mL), Tiron (10−3 mol/L), or the NADPH oxidase inhibitor diphenyliodonium (2×10−6 mol/L) before relaxation with SNAP.

Statistical Analysis
All data are expressed as mean±SE. Band intensities from Western blot analysis were analyzed densitometrically using the Kodak 1SD software. Comparisons of Western blot band densities, ethidium fluorescence intensities, and SOD activity were compared by ANOVA. Comparisons of vascular responses were made by repeated-measures ANOVA. A value of P<0.05 was considered statistically significant.

Results
We measured superoxide levels in lung sections from control and PPHN lambs using the superoxide-sensitive dye dihydroethidium (Figure 1A). We found that there was a 2-fold increase in ethidium fluorescence intensity in PPHN sections compared with lung sections from control lambs after 9 days, but not after 2 days, of ducatal ligation (Figure 1B; P<0.05). Using whole lung and isolated vessel homogenates, we determined whether there were changes in lung antioxidant enzymes. Western blot analysis showed that CuZnSOD (Figures 2A and 2B) and MnSOD (Figures 2C and 2D) protein levels were not different in lung and isolated vessels from control lambs compared with PPHN lambs after either 2 days or 9 days of ducatal ligation. Total SOD activity was unchanged in lung parenchyma or isolated pulmonary vessels after 2 days of ducatal ligation (Figure 3). However, pulmonary arteries, but not pulmonary veins, isolated from the 9-day ducatal ligation lambs exhibited a 44% decrease in SOD activity compared with control lambs (Figure 3; P<0.05). CuZnSOD activity is dependent on copper transfer from the CCS-1 protein. Thus, a potential mechanism for the apparent decrease in SOD activity in the pulmonary arteries from ligated lambs could be a decrease in CCS-1 expression. However, results demonstrated that CCS-1 was abundantly expressed in lung and isolated vessels and that only the significant change in CCS-1 expression was a decrease in the pulmonary veins of the PPHN lambs after 2 days of ducatal ligation (Figure 4).

To investigate potential sources of superoxide, we carried out Western blot analysis of lung and isolated vessels using an antibody to the p67phox subunit of NADPH oxidase (Figure 5). We found significant increases in p67phox expression in lung and pulmonary arteries from PPHN relative to control lambs as early as 2 days of ducatal ligation, and these increases
in expression were maintained at 9 days (P<0.05). There was no difference in p67phox expression in pulmonary veins at either time point examined. Immunostaining of lung sections confirmed this Western blot data with increased p67phox expression observed in vessels in the lung sections prepared from PPHN lambs relative to controls after both 2 days and 9 days of ductal ligation (Figure 6).

We then performed functional assays using fifth-generation pulmonary arteries from control and PPHN lambs. Pulmonary arteries from PPHN lambs showed significantly blunted relaxations to the NO donor SNAP (Figure 7A), but only after 9 days of ductal ligation. Pretreatment with the cell-permeable superoxide scavengers Tiron (Figure 7B) and PEG-SOD (Figure 7C) enhanced the relaxations to SNAP in pulmonary arteries from PPHN lambs after 9 days of ductal ligation but had no effect on SNAP-induced relaxations in pulmonary arteries isolated from PPHN lambs after 2 days of ductal ligation. Similarly, pretreatment with diphenyliodonium (DPI) to inhibit NADPH oxidase also enhanced relaxations to SNAP in pulmonary arteries isolated from PPHN lambs but only after 9 days of ductal ligation (Figure 7D).

Pretreatment with Tiron, SOD, and DPI had no significant effect on SNAP relaxations in pulmonary arteries isolated from control lambs at either time point (Figures 7B through 7D). In addition, the relaxation curves obtained from pulmonary arteries isolated from lambs after 2 days of ductal ligation were equivalent to those obtained from control lambs (Figures 7A through 7D).

**Discussion**

In the present study, we used a lamb model of PPHN to investigate the potential role of superoxide in the pathophysiology. We found a 2-fold increase in superoxide formation in lungs from PPHN lambs compared with controls after 9 days of ductal ligation (Figure 1B). Superoxide formation in the hypertensive lambs was evident in the SMCs and the adventitia of pulmonary arteries and arterioles, sites where the anatomic changes of vascular remodeling are most evident.6 PPHN is also associated with endothelial dysfunction, and endothelial cells have been shown to increase their generation of superoxide both from the “uncoupling” of endothelial NOS and from the activation of NADPH oxidase.21 Thus, it is
possible that some of the increased superoxide we have observed in the ligated lung may be generated by the endothelium. Further studies will be required to examine this possibility.

Little is known about the levels and activity of lung antioxidant enzymes in PPHN. We investigated whether the enzymes responsible for clearance of superoxide were altered in PPHN. These enzymes, CuZnSOD and MnSOD, are metalloenzymes that catalyze the dismutation of the superoxide anion to hydrogen peroxide and oxygen. Using Western blotting, we demonstrated that, while both CuZnSOD (Figures 2A and 2B) and MnSOD (Figures 2C and 2D) were present in fetal lung, there was no difference in PPHN lambs compared with controls at either time point examined. Therefore, we conclude that PPHN is associated with an overproduction of the oxidant superoxide without a simultaneous increase in cellular antioxidant capacity. This hypothesis is strengthened when we compared the SOD activities in fetal and adult lung. Our preliminary results indicate that SOD activity is 4-fold less in fetal compared with adult lung. Although these data will need to be confirmed at the protein expression levels, they suggest that the fetal lung may be especially sensitive to oxidative stress. Further studies will be required to investigate the mechanism by which increased oxidative stress reduces SOD activity during the progression of pulmonary hypertension in the fetal lung.

Activity of CuZnSOD requires copper. To limit spontaneous fenton-type reactions, copper availability is limited to very low levels in vivo, and delivery of copper to this enzyme is the task of the CCS-1 protein. Because SOD protein levels were not altered in the PPHN lung, we determined whether alterations in CCS-1 expression explained the increased superoxide formation seen in the PPHN lungs. Recent evidence has shown that there is an oxygen requirement for copper transfer to CuZnSOD, and it has been suggested that excess superoxide may inhibit copper transfer by CCS-1 and inactivate the CuZnSOD enzyme. These changes would enhance superoxide formation in the cytosol. However, our data indicate that CCS-1 expression was not altered in lung or isolated pulmonary arteries in PPHN lambs, although a reduction in CCS-1 was observed after 2 days of ductal ligation in pulmonary veins, but this was not maintained after 9 days (Figures 4A and 4B). Thus, although SOD activity is significantly less than that in adult sheep lung, this is not likely to be due to a lack of CCS-1.

We did find that SOD activity was decreased in pulmonary arteries, but not pulmonary veins, isolated from PPHN lambs relative to controls after 9 days, but not 2 days, of ductal ligation (Figure 3). The reason for this decrease in activity after 9 days, which occurred without reduction in the level of SOD protein, is unclear. However, Cys55 in bovine CuZn-
SOD (or Cys57 in human) is involved in the formation of an internal disulfide bond that is essential for activity. Without the Cys55 to Cys144 disulfide bond, a key hydrogen bond (essential for SOD activity) cannot form between the carbonyl group of Cys55 and the amine group of Arg141. It has been suggested that this cysteine residue is sensitive to oxidative modification, which can result in enzyme inhibition. Because the sheep CuZnSOD protein has not yet been analyzed, it is unclear whether the Cys55 equivalent residue in the ovine CuZnSOD enzyme would be sensitive to this type of oxidative inhibition.

We next investigated the potential sources of the increased superoxide generated in the PPHN lungs identifying an increase in the p67phox subunit of the NADPH oxidase enzyme complex by Western blot analysis (Figure 5) or immunohistochemistry (Figure 6). The presence of a superoxide-generating enzyme similar to neutrophil NADPH oxidase has been identified in cultured calf pulmonary artery SMCs. Vascular smooth muscle cell accumulation and hypertrophy are characteristic of hypertensive vascular diseases, and it is also thought that many other cells that comprise the vessel wall generate ROS. In phagocytic cells, the NADPH oxidase system consists of the cytosolic components, p47phox and p67phox, a low molecular weight G protein, Rac1 or Rac2, and a membrane-associated cytochrome b558. In phagocytes, cytochrome b558 functions as the final electron transporter from NADPH to molecular oxygen, and this protein consists of a 22-kDa subunit (p22phox) and a glycosylated 91-kDa subunit (gp91phox). Messenger RNAs for p22phox, p47phox, and p67phox, gp91phox, and Rac1 have been identified in endothelial cells while smooth muscle cells appear to express p22phox, p47phox, p67phox, and Rac1 with the expression of gp91phox less certain. It has been suggested that the recently identified mox-1 protein (for mitogenic oxidase) may represent the gp91phox homologue in SMCs.
Cultured endothelial cells have been shown to produce ROS in response to LDL exposure. In these cell lysates, increased expression of gp91 phox and p67 phox was demonstrated by Western blotting. Upregulation of p67 phox and gp91 phox has also been shown in the aorta of mice with hypertension after infusion of angiotensin II. NADPH oxidase is a highly regulated enzyme complex, and its activation requires the assembly of both cytosolic- and membrane-bound factors. Using Western blot analysis, we demonstrated that the p67 phox subunit was increased in whole-lung homogenates and pulmonary arteries of PPHN lambs as early as 2 days after ductal ligation, and this increase was maintained at 9 days (Figures 5A and 5B). Further, this increase appeared to be localized to the adventitia and smooth muscle layers (Figure 6), the same layers where we localized the increases in superoxide and the areas that have been previously reported to exhibit vascular remodeling. Although our studies are highly suggestive that the increased superoxide generated in the fetal lung during the development of pulmonary hypertension is due to an increase in the NADPH oxidase expression further studies will be required to confirm that there is an increase in NADPH oxidase activity. In addition, although NADPH oxidase expression was found to be elevated after only 2 days of ductal ligation, this did not correlate with an increase in the levels of superoxide (Figure 1). This suggests that at this early time period the antioxidant capacity of the lung is sufficient to scavenge the excess superoxide generated by the increase in NADPH oxidase expression. In agreement, our vessel studies (Figure 7) determined that pulmonary arteries isolated after only 2 days of ductal ligation relaxed normally to SNAP.
Figure 7. Cumulative concentration-response curves for the NO donor SNAP in isolated fifth-generation pulmonary arteries in near-term fetal lamb lungs: Control and after ligation of the ductus arteriosus in utero (Ductal Ligation). A, Isolated fifth-generation pulmonary artery rings from PPHN lambs exhibit an attenuated response to SNAP-induced relaxation after 9 days but not 2 days of ductal ligation. All rings were pretreated with indomethacin and propranolol. Relaxations are expressed as percent of a plateau constriction in response to norepinephrine (NE; 100%). Data are mean±SE, n=4 lambs for each data point. *P<0.05 vs Control. B, Pretreatment of fifth-generation pulmonary artery rings with Tiron (10 μmol/L) significantly enhanced relaxations in response to SNAP only in lambs with PPHN induced by 9 days (B-2) but not 2 days (B-1) of ductal ligation. All rings were pretreated with indomethacin and propranolol. Relaxations are expressed as percent of a plateau constriction in response to norepinephrine (100%). Data are mean±SE, n=4 lambs for each data point. *P<0.05 vs SNAP alone. C, Pretreatment of fifth-generation pulmonary artery rings with PEG-SOD (75 U/mL) significantly enhanced relaxations in response to SNAP only in lambs with PPHN induced by 9 days (C-2) but not 2 days (C-1) of ductal ligation. All rings were pretreated with indomethacin and propranolol. Relaxations are expressed as percent of a plateau constriction in response to norepinephrine (100%). Data are mean±SE, n=4 lambs for each data point. *P<0.05 vs SNAP alone. D, Pretreatment of fifth-generation pulmonary artery rings with DPI (4 μmol/L) significantly enhanced relaxations in response to SNAP only in lambs with PPHN induced by 9 days (D-2) but not 2 days (D-1) of ductal ligation. All rings were pretreated with indomethacin and propranolol. Relaxations are expressed as percent of a plateau constriction in response to norepinephrine (100%). Data are mean±SE, n=4 lambs for each data point. *P<0.05 vs SNAP alone.
One of the important attributes of the vascular oxidase is that it appears to respond to external signals to generate superoxide. We have previously demonstrated that ET-1 stimulates the activity of the vascular NADPH oxidase complex in pulmonary arterial SMCs from fetal sheep, which leads to an increase in proliferation.18 PPHN is associated with increased levels of ET-1 in both infants and in the ductal ligation model.9 Thus, we speculate that the elevated ET-1 concentrations activate NADPH oxidase, which in turn increases superoxide generation. Superoxide could lead to an increase in smooth muscle proliferation, producing the anatomic findings of increased thickness in the smooth muscle layer of the pulmonary arteries, as well as muscularization of the nonmuscularized arteries. Further studies will be required to determine whether ET-1 also increases the expression of the subunits of NADPH oxidase.

Our next set of studies investigated whether the increased levels of superoxide in the PPHN lung had functional consequences on the relaxation responses of pulmonary arteries (Figure 7). We have previously shown that pulmonary veins isolated from PPHN lambs relax normally to endogenous and exogenous NO.18 Therefore, we focused our present studies on pulmonary arterial responses. We demonstrated that fifth-generation pulmonary arteries isolated from PPHN lambs had significantly decreased relaxations to the NO donor SNAP compared with controls, but only after 9 days of ductal ligation (Figure 7A). The addition of the superoxide scavengers Tiron (Figure 7B) or PEG-SOD (Figure 7C) to pulmonary arteries isolated from PPHN lambs since significantly enhanced their relaxations to SNAP, but again only after 9 days of ductal ligation. Little effect of these scavengers was seen in control pulmonary arteries, indicating this enhancement was unique to PPHN. Further, the inhibition of NADPH oxidase with DPI enhanced relaxations to SNAP in pulmonary arteries from PPHN but not control lambs (Figure 7D). However, this was again limited to the lambs subjected to 9 days of ductal ligation. These data indicate that excess superoxide inhibits relaxations to exogenous NO in PPHN, and that scavengers of superoxide restore these relaxations. In addition, our studies indicate that pulmonary vessels isolated from lambs exposed to only 2 days of ductal ligation relax normally to SNAP, suggesting that the vascular remodeling normally associated with PPHN may not have occurred at this early time point.

ROS appear to be important for SMC proliferation and survival, and studies have demonstrated that suppression of endogenous ROS levels will inhibit SMC proliferation and promote apoptosis.38,39 Thus, the NADPH oxidase enzyme complex may play an important role in fetal pulmonary artery SMC proliferation by producing a required level of superoxide within the cell. Therefore, we speculate that antioxidant therapy could potentially be used to inhibit vascular SMC proliferation, although the concomitant effects of antioxidants on fetal pulmonary artery endothelial cell proliferation remain to be established.

In conclusion, although PPHN is a condition resulting from a diverse set of circumstances, and caution must be exercised when applying the results of experimental models to humans, this study provides a strong rationale to further explore...


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