

Inhaled Nitric Oxide Effects on Lung Structure and Function in Chronically Ventilated Preterm Lambs

Richard D. Bland, Kurt H. Albertine, David P. Carlton, and Amy J. MacRitchie

Department of Pediatrics, Stanford University School of Medicine, Stanford University, Stanford, California; Department of Pediatrics, University of Utah School of Medicine, Salt Lake City, Utah; and Department of Pediatrics, University of Wisconsin School of Medicine, Madison, Wisconsin

Rationale: Inhaled nitric oxide (iNO) can reverse neonatal pulmonary hypertension and bronchoconstriction and reduce proliferation of cultured arterial and airway smooth muscle cells.

Objectives: To see if continuous iNO from birth might reduce pulmonary vascular and respiratory tract resistance (PVR, Re) and attenuate growth of arterial and airway smooth muscle in preterm lambs with chronic lung disease.

Methods: Eight premature lambs received mechanical ventilation for 3 weeks, four with and four without iNO (5–15 ppm). Four term lambs, mechanically ventilated without iNO for 3 weeks, served as additional control animals.

Measurements: PVR and Re were measured weekly. After 3 weeks, lung tissue was processed for quantitative image analysis of smooth muscle abundance around small arteries (SM_{art}) and terminal bronchioles (SM_{tb}). Radial alveolar counts were done to assess alveolar number. Endothelial NO synthase (eNOS) protein in arteries and airways was measured by immunoblot analysis.

Main Results: At study's end, PVR was similar in iNO-treated and untreated preterm lambs; PVR was less in iNO-treated preterm lambs compared with term control animals. Re in iNO-treated lambs was less than 40% of Re measured in preterm control animals. SM_{art} was similar in iNO-treated and both groups of control lambs; SM_{tb} in lambs given iNO was significantly less (~50%) than in preterm control animals. Radial alveolar counts of iNO-treated lambs were more than twice that of preterm control animals. eNOS was similar in arteries and airways of iNO-treated preterm lambs compared with control term lambs.

Conclusions: iNO preserves structure and function of airway smooth muscle and enhances alveolar development in preterm lambs with chronic lung disease.

Keywords: airway smooth muscle; bronchopulmonary dysplasia; endothelial nitric oxide synthase; lung growth and development; neonatal chronic lung disease; pulmonary vascular and respiratory tract resistances

Infants who are born at a very early stage of development, notably at less than 28 weeks of gestation, often have respiratory failure because of their immature lungs, primitive respiratory drive, and susceptibility to infection. The frequent need for prolonged assisted ventilation in such infants typically leads to a form of chronic lung injury that was first described by Northway

and colleagues (1) as bronchopulmonary dysplasia. The pathologic features of this condition include impaired lung vascular and alveolar development, with excess abundance, tone, and reactivity of pulmonary arterial and airway smooth muscle (2–9). These structural abnormalities of the pulmonary circulation and conducting airways are associated with high resistance to blood flow in the lung vascular bed (10, 11) and high resistance to airflow through the respiratory tract, especially during expiration (12, 13). The etiology of these changes is unknown, but may relate to chronic inflammation that develops in the premature lung exposed to repetitive stretch with oxygen-enriched gas, often complicated by infection in the airways and lung parenchyma.

Most research related to nitric oxide (NO) during fetal and early postnatal lung development has focused on the pulmonary circulation, notably the key role of NO in lowering pulmonary vascular resistance (PVR) after birth, and the benefit of inhaled NO (iNO) in reducing PVR in neonates with persistent pulmonary hypertension. NO plays an important role in regulating smooth muscle tone in the blood vessels of the newborn lung. Blocking NO production lessens the normal postnatal decrease of PVR in newborn sheep (14, 15). Several studies have shown that iNO causes a rapid drop in PVR and improves arterial blood oxygenation in both newborn animals and human infants with pulmonary hypertension (16–23). A recent report indicated that iNO coupled with oxygen breathing caused marked pulmonary vasodilation in children with long-standing pulmonary hypertension from advanced lung vascular disease that began as bronchopulmonary dysplasia (24).

Long-term iNO has been shown to reduce the lung vascular remodeling that occurs in chronically hypoxic rats with pulmonary hypertension (25, 26). At least two studies have shown that NO inhibits proliferation of vascular smooth muscle cells in culture (27, 28). Low-dose iNO has been shown to increase arterial oxygenation, decrease PVR, and lessen lung neutrophil accumulation in preterm lambs with acute respiratory failure (29). In a neonatal piglet model of ischemia-reperfusion lung injury, iNO decreased lung fluid leak and inhibited endothelial dysfunction (30). Because pulmonary hypertension and edema are characteristic features of neonatal chronic lung disease (CLD), early postnatal treatment with iNO might be expected to prevent or at least reduce severity of the lung vascular component of this disease.

Although most studies of iNO have focused on the lung circulation, some investigators have assessed the influence of iNO on airway resistance and bronchiolar smooth muscle. One group showed that iNO decreased respiratory tract resistance (Re) in anesthetized adult guinea pigs with induced bronchoconstriction (31). Another group found that iNO decreased Re in anesthetized newborn piglets (32). NO has been shown to reduce proliferation of airway smooth muscle cells in culture (33). Taken together, these studies indicate that NO can influence smooth muscle abundance and tone in the conducting airways during postnatal development.

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Correspondence and requests for reprints should be addressed to Richard D. Bland, M.D., Professor of Pediatrics, Stanford University School of Medicine, CCSR Building, Room 1225, 269 Campus Drive, Stanford, CA 94305-5162. E-mail: rbland@stanford.edu

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We previously created an animal model of CLD, in which fetal sheep were delivered prematurely by cesarean section, given intrapulmonary surfactant, and mechanically ventilated for 3 weeks with sufficient inspired oxygen to maintain normal arterial oxygenation. Physiologic studies showed sustained elevation of PVR and RE, which was associated with postmortem histologic evidence of increased smooth muscle and elastin in the lung circulation and distal airways, a paucity of alveoli and small pulmonary arteries, reduced capillary surface density, and decreased abundance of endothelial NO synthase (eNOS) in blood vessels and airways when compared with lungs of control lambs that were born at term (34–37). We subsequently found that iNO given at 15 ppm for 1 hour consistently reduced PVR by approximately 20% at the end of the first week of mechanical ventilation, but that continued mechanical ventilation was associated with loss of the pulmonary vasodilator response to iNO, possibly related to diminished abundance of eNOS and soluble guanylate cyclase that was noted in the lung circulation at the end of the 3-week studies (38).

These findings, together with accumulating evidence that iNO reduces smooth muscle proliferation and relaxes smooth muscle tone in the lung circulation and conducting airways, led us to consider the possibility that continuous, low-dose iNO, beginning immediately at birth and sustained during long-term mechanical ventilation, might reduce the severity of neonatal lung injury in our ovine model of bronchopulmonary dysplasia. Our hypothesis was that prolonged iNO starting at birth would inhibit vascular and airway smooth muscle growth and thereby reduce PVR and RE. To test this hypothesis, eight preterm lambs were mechanically ventilated for 3 weeks, four of them with iNO at 5 to 15 ppm beginning at birth, and four of them without iNO. PVR after 3 weeks was not significantly different between iNO-treated and control lambs. RE, however, was significantly less in the lambs that received iNO, and postmortem histopathology showed less airway smooth muscle in the lambs treated with iNO compared with control animals. Immunoblot analysis for eNOS in excised small arteries and airways taken from iNO-treated lambs and control lambs born at term showed no difference in abundance of eNOS protein. This result contrasted with previous studies showing reduced eNOS protein in lung blood vessels and airways of chronically ventilated preterm lambs compared with control lambs born at term (36).

We were surprised to discover that alveolarization was significantly greater in iNO-treated lambs than in control lambs, analogous to the beneficial effect on lung septation that was seen with prolonged nasal application of continuous positive airway pressure in very premature baboons (39). These findings indicate that iNO, begun at birth and sustained during 3 weeks of mechanical ventilation, may help to prevent impaired alveolar development, increased lung resistance, overgrowth of airway smooth muscle, and diminished pulmonary expression of eNOS observed in preterm lambs with CLD. Some of the results of this investigation were reported previously in abstract form (40–42).

METHODS

Animals and Experimental Protocol

Animals. We used eight lambs that were delivered prematurely by cesarean section at 124 ± 3 days' gestation (term, 147 days). Four lambs had mechanical ventilation and received iNO continuously for 3 weeks beginning immediately after birth, and four lambs had mechanical ventilation for 3 weeks without iNO (preterm control lambs). Table 1 lists descriptive data for the two groups of lambs. Gestational age and weights at birth and death were similar in both groups. Detailed descriptions of operative procedures and early postnatal management have been published previously (35) and can be viewed in the online supplement.

Experimental protocol. All eight of the studies with iNO-treated and control preterm lambs were done within a 15-month period at the University of Utah. Before delivery, four lambs were preassigned to receive iNO continuously beginning immediately after birth, and four control lambs were preassigned not to receive iNO during 3 weeks of mechanical ventilation. For lambs assigned to receive iNO, the gas was allowed to flow into the inspiratory limb of the ventilator circuit from a cylinder of NO (Scott Specialty Gases, Plumsteadville, PA) that contained 2,200 ppm, with nitrogen as the balance gas. The NO concentration in the inspired gas was measured frequently by a chemiluminescent analyzer (Model 2108; Dasibi Environmental Corp., Glendale, CA), which was calibrated before and intermittently during each 3-week study using a gas cylinder containing a known concentration of NO. The flow of NO was adjusted to keep measured NO concentrations between 5 and 15 ppm. NO flow was reduced if blood methemoglobin concentration, which was measured frequently (Model 300; Radiometer, Copenhagen, Denmark), exceeded 2%.

Physiologic studies included weekly measurements of steady-state pulmonary arterial and left atrial pressures and cardiac output (measured in triplicate by thermodilution using Model SP 1425, Cardiac Output Computer; Gould Inc., Oxnard, CA) to determine PVR. We measured vascular pressures with calibrated pressure transducers (BT3DC; Statham Instruments, Oxnard, CA) connected to an eight-channel amplifier-recorder (Model 7D; Grass Instruments, Quincy, MA). Respiratory variables were assessed weekly from simultaneous measurements of proximal airway and pleural pressures and gas flow, which was measured using a calibrated pneumotachograph connected to a PEDS Pulmonary Evaluation and Diagnosis System (Medical Associated Services, Hatfield, PA) (43).

Studies performed with control term lambs. To better assess the impact of iNO on lung structure and function of mechanically ventilated preterm sheep, we did four additional studies on newborn lambs that were born vaginally at term gestation and mechanically ventilated for 3 weeks without iNO. This enabled us to compare physiologic and histologic variables of premature versus mature newborn lambs with ventilator-induced lung injury, and to determine the extent to which iNO attenuates the abnormalities that occur in the incompletely developed lung exposed to prolonged cyclic stretch. The design of the studies performed with term lambs was similar to the design of the studies done with preterm lambs, except that the term lambs were born vaginally after spontaneous onset of labor at an average gestational age of 144 ± 1 days, and cardiac output was measured with a flow meter (Model T106; Transonic Systems, Inc., Ithaca, NY) connected by a cable to a 12-mm ultrasonic flow probe (Transonic Systems) that was surgically implanted around the main pulmonary artery.

TABLE 1. DEMOGRAPHIC DATA (MEAN \pm SD) FOR THE TWO GROUPS OF FOUR PRETERM LAMBS

	Gestation (d)	Birth Weight (kg)	Death Weight (kg)	Male:Female
Control lambs, no iNO	124 \pm 4	2.85 \pm 0.45	3.36 \pm 0.95	1:3
Treated with iNO	124 \pm 3	2.70 \pm 0.86	3.46 \pm 0.73	1:3

Definition of abbreviation: iNO = inhaled nitric oxide.

All surgical procedures and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Utah School of Medicine.

Postmortem Studies

At the end of each 3-week study, the lambs were anesthetized with intravenous pentobarbital sodium, 35 mg/kg, followed by a thoracotomy to excise the lungs for subsequent histopathologic, biochemical, and molecular assays. We used quantitative methods, as previously described (34, 35), to study postmortem lung histopathology.

Quantitative histology was used to estimate the following: (1) smooth muscle thickness in the walls of small pulmonary arteries and airways, (2) numbers of small pulmonary blood vessels, (3) radial alveolar counts, (4) surface density of capillaries in the walls of distal airspaces, and (5) elastin content of distal lung parenchyma. The results reflect two-dimensional area measurements. Three-dimensional estimates were not made because the entire lung was not available for volume displacement measurements or structural analysis. The alveolar number across terminal respiratory units was estimated by the radial alveolar count method described by Emery and Mithal (44). We used immunohistochemistry and morphometry to estimate the surface density of capillaries in the walls of distal airspaces (35). Details of the quantitative histologic methods can be viewed in the online supplement.

Immunoblot analysis of eNOS protein. The caudal lobe of the right lung was used to dissect third- and fourth-generation intrapulmonary arteries and airways, which were stored at -80°C for subsequent immunoblot analysis of eNOS protein as previously described (36). We used lungs from three spontaneously born term lambs that were killed 1 day after birth to measure eNOS protein abundance in lung blood vessels and airways of control term lambs for comparison with measurements made on lung arteries and airways of iNO-treated preterm lambs. We previously reported results of eNOS protein measurements made on excised intrapulmonary arteries and airways of chronically ventilated preterm lambs that did not receive iNO (the control group for this study) and of control term lambs that breathed spontaneously for either 1 day or 3 weeks after birth (36). The dissections were performed at 4°C . The excised lung lobe was rinsed with cold sterile saline, followed by dissection of third- and fourth-generation intrapulmonary arteries and adjacent airways, which were placed immediately in liquid nitrogen for later processing, as described in the online supplement.

Portions of both lungs were used for measurement of extravascular lung water by a previously described gravimetric method (45).

Statistical Analysis

Data in the text, tables, and figures are expressed as mean \pm SD. When comparing datasets that displayed a normal Gaussian distribution, we used Student's unpaired *t* test to assess for significant differences in physiologic and histologic data between the two groups of preterm lambs that were mechanically ventilated for 3 weeks with or without iNO (46). For datasets in which there was a skewed non-Gaussian distribution, we applied the nonparametric Mann-Whitney test to assess for significant differences. We used one-way analysis of variance and Student-Newman-Keuls multiple comparison tests to identify differences in physiologic and histologic variables between the two groups of preterm lambs and the group of term lambs that were mechanically ventilated for 3 weeks, and for assessment of differences in the immunoblot measurements of eNOS protein between experimental and control groups. Statistical analysis was done using a commercially available computer program (StatView SE+ Graphics; Abacus Concepts, Inc.,

Berkeley, CA) and standard statistical tables (46). Differences were considered statistically significant if the *p* value was less than 0.05.

RESULTS

Table 2 shows results of key respiratory variables that were measured at the end of Week 3 in the two groups of preterm lambs. There was considerable variability in the severity of pulmonary dysfunction in the lambs that did not receive iNO. Consequently, there were no significant differences between the two groups with respect to FIO_2 , arterial blood gas tensions, peak inflation pressure, or dynamic lung compliance (CL). RE, however, was significantly less in preterm lambs that received iNO than it was in untreated preterm lambs. RE was not significantly different in iNO-treated preterm lambs compared with control term lambs that did not receive iNO (Figure 1). Although CL was not significantly different between the two groups of preterm lambs, it is noteworthy that CL was significantly less in preterm lambs that did not receive iNO (0.51 ± 0.21 [ml/cm H_2O]/kg bodyweight) compared with term lambs (0.98 ± 0.15 [ml/cm H_2O]/kg bodyweight), whereas CL was similar in iNO-treated preterm lambs (0.88 ± 0.35 [ml/cm H_2O]/kg bodyweight) and term lambs that did not receive iNO.

Table 3 shows results of important cardiovascular variables that were measured at the end of Week 3 in the two groups of preterm lambs. There was marked variability of mean pulmonary arterial blood pressure (Ppa) among the four preterm lambs that did not receive iNO. Consequently, there was no statistically significant difference ($p > 0.15$) in either Ppa or PVR between treated and untreated preterm lambs. It is noteworthy that PVR was less in preterm lambs that received iNO than it was in control term lambs that did not receive iNO (Figure 1).

Although there was a tendency for extravascular lung water to be greater in the lambs that received iNO, this difference between the two groups of lambs was not statistically significant (5.35 ± 0.91 g/g dry lung in lambs that did not receive iNO, compared with 6.62 ± 1.31 g/g dry lung in lambs that received iNO, $p > 0.15$). Extravascular lung water was significantly greater in both sets of preterm lambs than it was in term lambs that were ventilated for 3 weeks without iNO (their extravascular lung water was 4.56 ± 0.38 g/g dry lung).

Quantitative assessment of lung histopathology showed significantly less airway smooth muscle in preterm lambs that received iNO than in the control group of preterm lambs (Figures 2 and 3). Airway smooth muscle in iNO-treated preterm lambs also was less than it was in control term lambs that did not receive iNO. There was no significant difference in pulmonary artery smooth muscle abundance between the two groups of preterm lambs, nor was there a difference in vascular smooth muscle between either group of preterm lambs when compared with the group of control term lambs (Figure 2).

Lambs that received iNO had significantly more alveoli, as assessed by radial alveolar counts, than control preterm lambs (Figures 4 and 5). Capillary surface density tended to be greater

TABLE 2. RESPIRATORY DATA (MEAN \pm SD) FOR THE TWO GROUPS OF PRETERM LAMBS AT THE END OF THE 3-WEEK STUDY

iNO	FIO_2	PaO_2 (mm Hg)	PaCO_2 (mm Hg)	pH	Airway Pressure (cm H_2O)			Dynamic Compliance ([ml/cm H_2O]/kg BW)	Expiratory Resistance (cm H_2O /[L/s])
					Peak	Mean	V_T (ml/kg)		
-	0.48 ± 0.35	70 ± 11	43 ± 30	7.35 ± 0.13	41 ± 24	13 ± 5	13 ± 5	0.51 ± 0.21	106.8 ± 37.3
+	0.29 ± 0.09	70 ± 9	31 ± 3	7.41 ± 0.04	24 ± 8	9 ± 2	14 ± 4	0.88 ± 0.35	$40.3^* \pm 22.4$

Definition of abbreviations: BW = bodyweight; iNO = inhaled nitric oxide.

* Significant difference compared to preterm lambs not treated with iNO, $p < 0.05$.

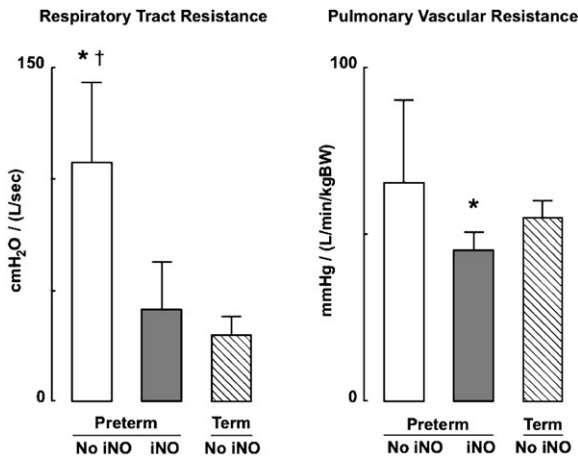


Figure 1. Respiratory tract and pulmonary vascular resistances (mean and SD) in four preterm lambs that received iNO at 5–15 ppm (dark bars) compared with four preterm lambs that did not receive inhaled nitric oxide (iNO; white bars) and four term lambs that did not receive iNO (hatched bars) during 3 weeks of mechanical ventilation. *Significant difference compared with the group of four term lambs, $p < 0.05$; †significant difference compared with the group of four preterm lambs given iNO, $p < 0.05$. BW = bodyweight.

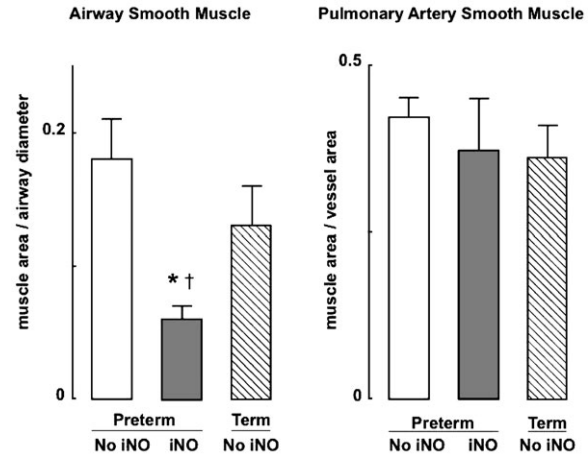


Figure 2. Airway and pulmonary artery smooth muscle area (mean and SD) in four preterm lambs that received iNO at 5–15 ppm (dark bars) compared with four preterm lambs that did not receive iNO (white bars) and four term lambs that did not receive iNO (hatched bars) during 3 weeks of mechanical ventilation. *Significant difference compared with the group of four preterm lambs not given iNO, $p < 0.05$; †significant difference compared with the group of four term lambs, $p < 0.05$.

in preterm lambs that received iNO ($p = 0.06$), consistent with the apparent difference in radial alveolar counts between the two groups of preterm lambs. Both alveolar number and capillary surface density were less in the two groups of preterm lambs than in the group of term lambs (Figure 5). These structural differences between preterm and term lambs are not surprising, because alveolar and lung vascular development progresses rapidly in sheep during late gestation.

Elastin content in lung parenchyma, which was assessed by quantitative image analysis of 6 to 10 histologic tissue sections per animal, was not different in the two groups of preterm lambs ($15.8 \pm 6.4\%$ with iNO vs. $19.1 \pm 2.6\%$ without iNO, expressed as a percentage of parenchymal area). Compared with the four term lambs that were mechanically ventilated without iNO for 3 weeks, however, both groups of preterm lambs had greater elastin accumulation in their distal lung tissue than term lambs ($3.7 \pm 1.2\%$).

Immunoblot analysis of eNOS protein abundance in excised airways and small pulmonary arteries of preterm lambs that received iNO, compared with unventilated control lambs born at term, showed no significant differences (Figure 6). This finding contrasts with previous results that demonstrated reduced eNOS protein abundance, as assessed both by immunohistochemistry and by immunoblot analysis, in small pulmonary arteries and airways of chronically ventilated preterm lambs (similar to the control lambs reported here) compared with unventilated con-

trol lambs born at term (36). We compared eNOS protein measured in small pulmonary arteries and airways of chronically ventilated preterm lambs, with or without iNO treatment from birth, versus eNOS protein measured in small pulmonary arteries and airways of control lambs that were 1 day old (same postconceptional age as the preterm lambs) and 3 weeks old (same postnatal age as the preterm lambs) after birth at term gestation: eNOS protein was significantly less in blood vessels and airways obtained from preterm lambs that did not receive iNO, but there was no difference between preterm lambs that received iNO versus either group of term lambs (Figure 7).

DISCUSSION

When we began this study, we expected that iNO would have its greatest effect on the lung vasculature in this ovine model of neonatal CLD, because we previously had discovered that PVR consistently decreased by approximately 20 to 25% when chronically ventilated preterm lambs received iNO for 1 hour early in the course of evolving CLD. The pulmonary vasodilator response to iNO was either diminished or lost after 3 weeks of mechanical ventilation, a finding that we attributed to deficient eNOS and soluble guanylate cyclase protein abundance measured in the lungs of preterm lambs with established CLD (36, 38). We speculated that early and continuous administration of iNO to chronically ventilated preterm lambs might inhibit early lung inflammation, as previous studies had suggested (29),

TABLE 3. CARDIOVASCULAR DATA (MEAN \pm SD) FOR THE TWO GROUPS OF PRETERM LAMBS AT THE END OF THE 3-WEEK STUDY

	Pulmonary Artery Pressure (mm Hg)	Left Atrial Pressure (mm Hg)	Pulmonary Blood Flow ([L/min]/kg BW)	Pulmonary Vascular Resistance (mm Hg/[L/min]/kg BW)
Controls, no iNO	26 \pm 13	5 \pm 2	0.35 \pm 0.17	65.2 \pm 25.1
Treated with iNO	16 \pm 3	6 \pm 2	0.21 \pm 0.03	44.8 \pm 5.4

For definition of abbreviation, see Table 2. There were no significant differences.

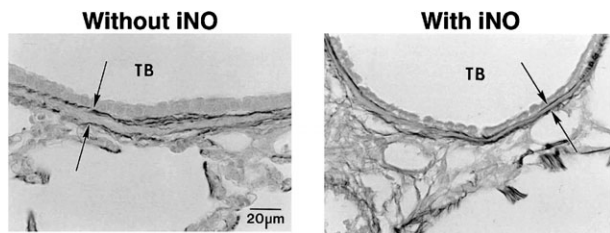


Figure 3. Representative photomicrographs showing smooth muscle wall thickness between the internal and external elastic laminae (arrows; Hart’s elastin stain) of terminal bronchioles (TB) in lungs of preterm lambs that were mechanically ventilated for 3 weeks either without iNO (control, left) or with iNO (5–15 ppm, right).

thereby preventing the adverse effects of long-term mechanical ventilation on the premature pulmonary circulation during post-natal development. The observation that iNO had a greater effect on the structure and function of the respiratory tract than it had on the pulmonary circulation of our lambs with established CLD was unexpected, perhaps reflecting direct exposure of the respiratory tract to iNO, as opposed to the potential barrier between airspaces and the pulmonary circulation resulting from lung inflammation, edema, and atelectasis in CLD.

We also were surprised to discover that continuous long-term administration of iNO did not reduce the expression of eNOS in the lungs of the lambs, which is the explanation sometimes given to account for the apparent rise of PVR and reduction of Pa_O₂ that often occur when iNO therapy is discontinued in infants with persistent pulmonary hypertension. Our findings that eNOS protein abundance remained at control levels in both arteries and airways of preterm lambs that received iNO during mechanical ventilation, however, are consistent with the previous report that NO donors increased NOS expression in cultured pulmonary artery endothelial cells derived from fetal sheep (47). Preservation of vascular eNOS, however, yielded no significant benefit with respect to either PVR or arterial smooth muscle abundance.

The importance that NO plays in modulating the structure and function of smooth muscle in the respiratory tract has received much less attention than has the role of NO on smooth muscle abundance, tone, and reactivity in lung blood vessels. A number of recent studies have indicated that NO may be a key regulatory element within the developing respiratory tract, just

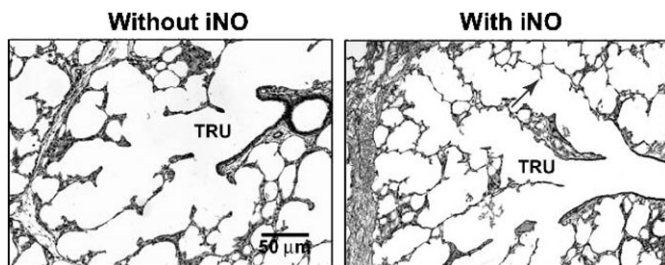


Figure 4. Lung tissue images illustrating differences in radial alveolar counts for preterm lambs that were mechanically ventilated for 3 weeks without iNO (left) or with iNO (right). Radial alveolar counts averaged 1.5 ± 0.3 alveoli/terminal bronchiole in lambs that did not receive iNO and 3.6 ± 1.5 alveoli/terminal bronchiole in lambs that received iNO. The arrow points to a secondary crest (alveolar septa). TRU = terminal respiratory unit, which is the landmark for measuring radial alveolar counts.

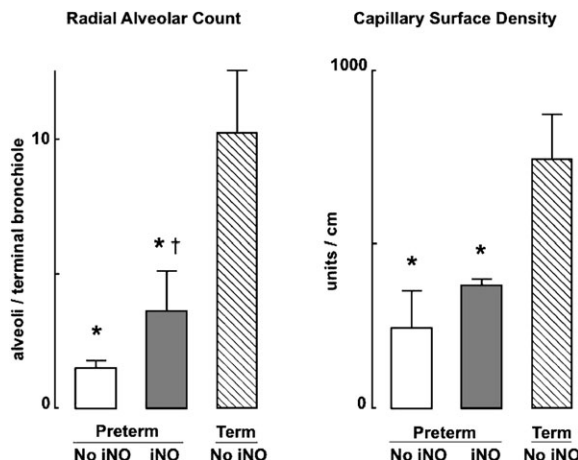


Figure 5. Radial alveolar count and capillary surface density (mean and SD) in lungs of four preterm lambs that received iNO at 5–15 ppm (dark bars) compared with four preterm lambs that did not receive iNO (white bars) and four term lambs that did not receive iNO (hatched bars) during 3 weeks of mechanical ventilation. *Significant difference compared with the group of four term lambs, $p < 0.05$; †significant difference compared with the group of four preterm lambs not given iNO, $p < 0.05$.

as it is in the vasculature. It is noteworthy that all three isoforms of NO synthase (eNOS, neuronal NOS, and inducible NOS) are expressed in the respiratory epithelium of both sheep and primates during fetal development (48, 49). We found that eNOS protein expression is reduced in both the pulmonary endothelium and respiratory epithelium of preterm lambs after 3 weeks of mechanical ventilation (36), an observation that recently was confirmed in a nonhuman primate model of CLD: both eNOS protein and total NOS activity were reduced in the lungs of preterm baboons with CLD (50). Lack of NOS activity could contribute to the increased abundance and tone of smooth muscle found in both airways and pulmonary arteries of preterm lambs with CLD compared with control lambs at the same post-conceptual age (34, 35), because NO has been shown to inhibit proliferation of vascular and airway smooth muscle cells in culture (27, 28, 33, 51) and to relax airway smooth muscle in newborn animals (32, 52).

We were surprised to discover that the number of alveoli was significantly greater in lungs of preterm lambs that received continuous iNO from birth when compared with preterm lambs that did not receive iNO. Recent studies, however, have shown that NO may have a critical role in lung growth, affecting new

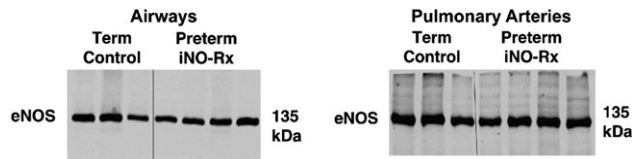


Figure 6. Immunoblot results for endothelial NO synthase (eNOS) in dissected third- and fourth-generation intrapulmonary airways (left) and arteries (right) excised from lungs of 1-day-old term lambs and preterm lambs given iNO, 5–15 ppm, during 3 weeks of mechanical ventilation. Term lambs were at the same postconceptional age as the preterm lambs, but did not receive mechanical ventilation or iNO. Densitometry showed no significant differences for either airways or arteries.

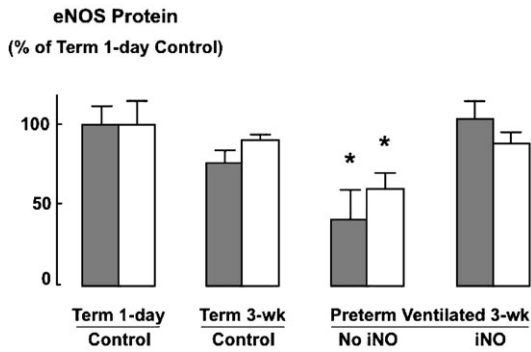


Figure 7. Summary data (mean and SD) for eNOS protein measured by immunoblot analysis of excised third- and fourth-generation intrapulmonary arteries (dark bars) and airways (light bars) from four groups of lambs: unventilated 1-day-old and 3-week-old lambs born at term (controls), and 3-week-old preterm lambs that received mechanical ventilation for 3 weeks either without iNO (third group from left) or with iNO (right). Results are plotted relative to 1-day-old term lambs (left) that were at the same postconceptional age as the ventilated preterm lambs. Note that eNOS protein abundance was similar in arteries and airways obtained from term control lambs and from preterm lambs that received iNO, whereas eNOS protein was significantly less in arteries and airways of preterm lambs that were ventilated for 3 weeks without iNO. Reference 36 displays all immunoblot results that are depicted in this figure except for those of iNO-treated preterm lambs, which are shown in Figure 6. *Significant difference compared with 1-day-old and 3-week-old term lambs and preterm lambs that were mechanically ventilated with iNO for 3 weeks, $p < 0.05$.

formation of both alveoli and pulmonary blood vessels. In a study of compensatory lung growth after left pneumonectomy, eNOS-deficient mice showed no increase of alveolar cell proliferation, alveolar surface density, or volume of the respiratory region in the right lung (53). Compensatory lung growth after pneumonectomy also was impaired in wild-type mice that were treated with an NOS inhibitor, N^G -nitro-L-arginine methyl ester (L-NAME). In another study comparing wild-type and eNOS-deficient mice, a 10-day exposure to mild hypoxia (FI_{O_2} , 0.16) during the postnatal period of rapid lung growth was associated with fewer alveoli and less blood vessel volume density in lungs of eNOS-deficient neonatal mice compared with age-matched control pups (54). This inhibition of hypoxia-induced lung septation and vasculogenesis was accompanied by reduced lung protein abundance of vascular endothelial growth factor receptor 2 (VEGF-R2), which led to speculation that NO may preserve normal growth of distal lung during hypoxia through preservation of VEGF-R2 signaling. A subsequent study by this group showed that pulmonary hypertension, induced in newborn rats by treatment with the VEGF-R2 inhibitor SU-5416, was reduced, and that lung growth, assessed by radial alveolar counts, was enhanced in newborn rats that were treated for 3 weeks after birth with 10 ppm iNO (55). Another recent study showed that eNOS-deficient mice display major defects in lung morphogenesis, which cause early postnatal death from respiratory failure (56). Lungs of transgenic newborn mice lacking eNOS, compared with wild-type pups, had thickened saccular septae and a paucity of distal lung arterioles, in addition to reduced surfactant phosphatidylcholine in their lung lavage fluid. These striking abnormalities of lung structure and function in eNOS-deficient mice underscore the critical role of NO in lung development and offer insights into why iNO beginning at birth, with preservation of lung eNOS in treated newborn sheep, might have facilitated

formation of alveoli and lung capillaries in the face of prolonged mechanical ventilation with oxygen-enriched gas.

The mechanism by which continuous exposure to iNO enhanced lung development during prolonged mechanical ventilation of preterm lambs remains unclear. Studies done with the fawn-hooded rat, an inbred strain in which lung expression of eNOS is reduced, showed not only abnormal lung vascular development and severe pulmonary hypertension when exposed to mild hypoxia (57), but these rats also displayed deficient postnatal alveolar development (58). Related studies conducted with cultured explants of fetal lung from Sprague-Dawley rats showed that NO donors induced a dose-dependent stimulation of lung branching, which was attenuated by NOS inhibition with either L-NAME or N^G -monomethyl-L-arginine (59). This observation was consistent with a previous report that NO contributes to the accelerated respiratory tract development that occurs with hypoxia in *Drosophila* (60). Because NO has been shown to inhibit alveolar epithelial cell apoptosis induced by either hyperoxia, stretch, or ischemia-reperfusion injury (61–63), it is possible that continuous exposure to iNO during prolonged mechanical ventilation of preterm lambs helped to preserve alveolar growth by preventing lung epithelial cell, and perhaps endothelial cell, death.

As noted above, recent reports have called attention to the possible role of VEGF and its receptors in regulating alveolar, as well as lung vascular, development (64, 65). Several studies have implicated NO as an important downstream mediator of VEGF, contributing to VEGF-induced angiogenesis in a number of *in vivo* and *in vitro* experimental models (66–69). There is also evidence that NO can induce synthesis of VEGF in vascular smooth muscle cells (70). Because pulmonary expression of VEGF and its receptors are reduced in lungs of infants dying of bronchopulmonary dysplasia and in animal models of this disease (71, 72), it is possible that delivery of iNO to the developing lung during long-term mechanical ventilation enabled VEGF signaling through preservation of the tyrosine kinase receptor Flk-1 (VEGF-R2), just as it preserved eNOS expression in lung blood vessels and airways.

Because of the arduous and costly nature of our lengthy life-support studies, the number of animals in each group was small (four/group), thus increasing the chance of missing important differences between groups. Despite this potential pitfall, significant differences in key respiratory variables between iNO-treated and untreated lambs were apparent after 3 weeks of mechanical ventilation.

Because of the instability of very premature lambs maintained in an intensive care setting over a prolonged period, there was considerable variability of respiratory and cardiovascular measurements at the end of Week 3 in the group of preterm lambs that did not receive iNO. This could have contributed to the absence of significant differences in physiologic variables that previously have been attributed to NO, such as the early postnatal improvement in CL and reduced pulmonary artery pressure reported in premature primates treated with iNO (73).

By comparing the two groups of preterm lambs to a group of term lambs that also were mechanically ventilated for 3 weeks, we discovered that CL was significantly less in preterm lambs that did not receive iNO than it was in term lambs, whereas CL was similar in preterm lambs that received iNO compared with term lambs without iNO. In addition, iNO-treated preterm lambs had significantly lower PVR values after 3 weeks of mechanical ventilation than term lambs, whereas untreated preterm lambs did not have lower values. These observations indicate that iNO may afford a major benefit in terms of inhibiting pulmonary hypertension, as well as reducing resistance and improving com-

pliance of the respiratory system during evolution of CLD after premature birth and lengthy mechanical ventilation.

A recent report described better lung function and lung growth, compared with control animals, in chronically ventilated preterm baboons that received continuous iNO at a lower concentration (5 ppm) and for a shorter duration of mechanical ventilation (2 weeks) than our lambs (73). In this study, iNO begun 1 hour after birth was associated with increased CL and reduced RE during the first week, and postmortem lung histology after 2 weeks showed longer secondary crests but no significant differences in the number of secondary crests or in lung volume measurements between iNO-treated and untreated baboons. In contrast to our results with preterm lambs, morphometric analysis of pulmonary arteries and bronchioles of preterm primates showed no significant difference in the amount of vascular or airway smooth muscle in iNO-treated compared with untreated baboons. In addition, excess accumulation of lung elastin, which is characteristic of CLD in human infants and newborn lambs (6, 37), including iNO-treated lambs, was absent in the baboons that received iNO. The reason for these histologic differences between the two species is unclear. It is possible that the greater concentration of iNO and the longer duration of mechanical ventilation in preterm lambs compared with baboons yielded a greater reduction in airway smooth muscle and allowed for improved alveolarization, without impacting lung elastin deposition in the lambs. Irrespective of these differences, however, the apparent benefit of iNO on structure and function of the developing lung in two different species and under two different sets of experimental conditions underscores the need to explore the mechanisms by which NO might contribute to lung development. These reports also provide a rationale for testing the therapeutic efficacy and safety of this intervention in disorders associated with impaired lung growth.

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