

## Pulmonary vascular dysfunction in preterm lambs with chronic lung disease

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**Bland, Richard D., Con Yee Ling, Kurt H. Albertine, David P. Carlton, Amy J. MacRitchie, Ronald W. Day, and Mar Janna Dahl.** Pulmonary vascular dysfunction in preterm lambs with chronic lung disease. *Am J Physiol Lung Cell Mol Physiol* 285: L76–L85, 2003. First published March 7, 2003; 10.1152/ajplung.00395.2002.—Chronic lung injury from prolonged mechanical ventilation after premature birth inhibits the normal postnatal decrease in pulmonary vascular resistance (PVR) and leads to structural abnormalities of the lung circulation in newborn sheep. Compared with normal lambs born at term, chronically ventilated preterm lambs have increased pulmonary arterial smooth muscle and elastin, fewer lung microvessels, and reduced abundance of endothelial nitric oxide synthase. These abnormalities may contribute to impaired respiratory gas exchange that often exists in infants with chronic lung disease (CLD). Nitric oxide inhalation (iNO) reduces PVR in human infants and lambs with persistent pulmonary hypertension. We wondered whether iNO might have a similar effect in lambs with CLD. We therefore studied the effect of iNO on PVR in lambs that were delivered prematurely at ~125 days of gestation (term = 147 days) and mechanically ventilated for 3 wk. All of the lambs had chronically implanted catheters for measurement of pulmonary vascular pressures and blood flow. During *week 2* of mechanical ventilation, iNO at 15 parts/million for 1 h decreased PVR by ~20% in 12 lambs with evolving CLD. When the same study was repeated in eight lambs at the end of *week 3*, iNO had no significant effect on PVR. To see whether this loss of iNO effect on PVR might reflect dysfunction of lung vascular smooth muscle, we infused 8-bromo-guanosine 3',5'-cyclic monophosphate (cGMP; 150  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  iv) for 15–30 min in four of these lambs at the end of *week 3*. PVR consistently decreased by 30–35%. Lung immunohistochemistry and immunoblot analysis of excised pulmonary arteries from lambs with CLD, compared with control term lambs, showed decreased soluble guanylate cyclase (sGC). These results suggest that loss of pulmonary vascular responsiveness to iNO in preterm lambs with CLD results from impaired signaling, possibly related to deficient or defective activation of sGC, the intermediary enzyme through which iNO induces increased vascular smooth muscle cell cGMP and resultant vasodilation.

neonatal lung injury; bronchopulmonary dysplasia; inhaled nitric oxide; pulmonary circulation; soluble guanylate cyclase; pulmonary vascular resistance; newborn sheep

PULMONARY HYPERTENSION WITH associated right heart failure is a well-documented complication of chronic lung disease (CLD) that often develops in extremely premature infants who require long-term mechanical ventilation to maintain adequate respiratory gas exchange (1, 5, 11). Such infants have repeated episodes of cyanosis that are often attributed to acute constriction of pulmonary arteries, with resultant hypoxemia and bradycardia. In patients who have died with this condition, which is commonly called bronchopulmonary dysplasia (40), histopathology of the lungs typically shows impaired alveolar development with reduced numbers of small pulmonary blood vessels and abundant smooth muscle within the walls of these vessels (9, 14, 16, 24, 26, 36, 54, 58, 60).

We developed an animal model of this disease in which fetal lambs were delivered prematurely by cesarean section and mechanically ventilated for 3 wk with sufficient supplemental oxygen to maintain normal arterial oxygenation. Physiological studies showed persistent elevation of pulmonary vascular resistance (PVR) that was associated with postmortem histological evidence of increased smooth muscle and elastin in the lung circulation, a paucity of alveoli and small pulmonary blood vessels, reduced capillary surface density, and decreased abundance of endothelial nitric oxide synthase (eNOS) compared with the lungs of control lambs that were born at term gestation (2, 7, 35, 43).

Several groups of investigators have demonstrated that inhaled nitric oxide (iNO) reduces PVR and improves arterial oxygenation in newborn infants with persistent pulmonary hypertension (PPHN) (28, 47, 48, 55). Clinical trials of low-dose iNO in newborn infants with PPHN have demonstrated that this treatment improves arterial oxygenation and may reduce the need for invasive forms of treatment,

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such as extracorporeal membrane oxygenation (15, 19).

In newborn lambs with pulmonary hypertension induced by either hypoxia or prenatal closure of the ductus arteriosus, iNO has been shown to reverse pulmonary vasoconstriction without affecting the systemic circulation (45, 63). When nitric oxide (NO) is added to the inspired gas during ventilation, it diffuses into vascular smooth muscle cells in the pulmonary circulation, where it increases the tissue concentration of guanosine 3',5'-cyclic monophosphate (cGMP), thereby inducing vasodilation (21). iNO does not lead to systemic hypotension when it diffuses into the circulation because it is inactivated by tight binding to hemoglobin (23, 41, 44).

Long-term iNO has been shown to reduce the lung vascular remodeling that occurs with chronic pulmonary hypertension (32, 49). In these studies, iNO reduced pulmonary artery wall thickening and neomuscularization of hypoxic newborn and adult rats. These investigators also found that continuous inhalation of NO attenuated turnover of pulmonary artery cells and inhibited neomuscularization of these vessels in rats with monocrotaline-induced lung injury (46). Other studies have shown that iNO can increase arterial blood oxygenation, reduce PVR, and lessen lung neutrophil accumulation in lambs with acute respiratory failure after premature birth (27, 29). At least two reports have indicated that iNO may help to improve oxygenation of arterial blood and shorten the need for ventilatory support in infants with chronic respiratory failure (20, 30). The possible benefit of iNO in human infants with CLD is controversial and awaits the results of ongoing clinical trials.

The purpose of this investigation was to test the potential benefit of iNO in lowering PVR of preterm lambs with CLD. When we discovered that the pulmonary vasodilator effect of iNO was greatly attenuated or lost in lambs with established CLD, we did further studies, which showed that the apparatus for relaxation of lung vascular smooth muscle was intact, but that NO signaling was impaired from deficient soluble guanylate cyclase (sGC) in the pulmonary circulation of lambs with CLD. These observations may have important implications regarding the pathogenesis of pulmonary hypertension in CLD associated with prolonged mechanical ventilation after premature birth, and they also may help to provide the basis for developing novel therapeutic or preventive interventions.

## METHODS

*Preparation of premature lambs for experiments.* We used 12 lambs that were delivered prematurely by cesarean section at  $124 \pm 3$  days gestation (term = 147 days). Their birth weight averaged  $2.52 \pm 0.45$  kg. Detailed descriptions of the operative procedures and early postnatal management of these lambs were published previously (7).

Briefly, time-dated pregnant ewes received spinal anesthesia for a midline hysterotomy, through which we inserted polyvinyl catheters into a carotid artery, a jugular vein, and a hindlimb artery and vein of the fetus using 1% lidocaine

local anesthesia. We withdrew residual lung liquid from the fetal trachea and instilled 10 ml of calf lung surfactant (35 mg/ml Infasurf, gift of Ony, Amherst, NY) into the lung lumen through a 4.5- or 5-mm endotracheal tube just before delivery, followed by 3 wk of mechanical ventilation with a time-cycled, pressure-limited infant respirator (model IV-100B, Sechrist, Anaheim, CA) set at an initial rate of 60–80 breaths/min and an inspiratory time of 0.25 s. These settings were subsequently changed to a rate of 20 breaths/min and an inspiratory time of 0.75 s, which yielded a tidal volume that averaged  $\sim 15$  ml/kg body wt. We kept the end-expiratory pressure at  $\sim 5$ – $6$  cmH<sub>2</sub>O and adjusted the peak-inflation pressure to keep the arterial PCO<sub>2</sub> at  $\sim 35$ – $45$  Torr, with sufficient supplemental oxygen to keep the arterial PO<sub>2</sub> (PaO<sub>2</sub>) at  $\sim 50$ – $90$  Torr. The lambs were managed on a padded platform bed beneath a radiant warmer. Their arterial blood pressure was monitored continuously via a calibrated pressure transducer connected to an electronic recorder, and they received a solution of glucose and saline intravenously (iv) to maintain normal blood glucose and sodium concentrations. The lambs also received 0.01 mg/kg iv buprenorphine (Buprenex, Recrirt and Coleman Pharmaceuticals, Richmond, VA) soon after birth and as often as needed thereafter to prevent agitation.

Each lamb had surgery within 2 days of birth to allow subsequent repeated measurements of PVR. We gave general anesthesia with iv fentanyl (Abbott Laboratories, North Chicago, IL), at an initial dose of 15–20  $\mu$ g/kg, followed by supplemental doses of 10  $\mu$ g/kg as often as needed to prevent intraoperative tachycardia or hypertension. As previously described (7), through a left thoracotomy we surgically ligated the ductus arteriosus and placed catheters in the pulmonary artery and left atrium, and a thermistor wire (model SP 5003, Gould, Statham Instruments, Oakland, CA) directly into the pulmonary artery for subsequent measurement of cardiac output by thermodilution. We also placed an 8-Fr catheter in the left pleural space for postoperative drainage of air and liquid.

The lambs received buprenorphine, 0.01 mg/kg iv every 4–6 h for postoperative analgesia, and they subsequently received both buprenorphine and sedation with either 3–5 mg/kg iv pentobarbital sodium (Vet Lab, Lenexa, KS) or 10 mg/kg iv phenobarbital (Wyeth Laboratories, Philadelphia, PA) as needed for agitation. For the duration of all 3-wk studies, we sampled arterial blood hourly for measurement of pH, PaO<sub>2</sub>, and arterial PCO<sub>2</sub>, and adjusted ventilator settings accordingly. We also measured hematocrit by blood centrifugation at least twice daily and gave transfusions of filtered maternal blood if the hematocrit was  $<35\%$ . We gave iv infusions of maternal plasma or isotonic saline if the mean arterial blood pressure was  $<40$  mmHg.

The lambs received iv 100 mg/kg sodium penicillin every 12 h, and iv 2.5 mg/kg gentamicin every 24 h for at least a week after birth. When signs and symptoms of sepsis developed thereafter, alternative broad-spectrum antibiotics were given. We provided nutrition with iv solutions containing glucose, protein (trophamine), electrolytes, trace metals, and vitamins, and with ewe's milk that was given through an orogastric tube. The lambs were weighed daily to monitor their fluid balance and nutritional status. Serum electrolytes were measured at least once each day with ion-selective electrodes (Na/K/Cl Stat Analyzer, model 644, Ciba Corning Diagnostics, Medfield, MA) as a guide to help adjust fluid and electrolyte management, and blood glucose concentrations were monitored with a rapid detection device (Exactech, Medisense, Waltham, MA). Urine output was determined from diaper weights before and after each voiding.

*Experimental protocol for PVR studies in premature lambs.* During the second week of mechanical ventilation, when the lambs were between 7 and 12 days old, we determined PVR from measurements of pulmonary arterial and left atrial pressures and cardiac output (lung blood flow) during alternating 1-h control periods, each of which was followed by a 1-h experimental period of 1) iNO at a concentration of 15 parts/million (ppm), 2) continuous iv infusion of acetylcholine that was given at a rate of 1.5  $\mu\text{g}/\text{min}$ , and 3) 1 h of 100%  $\text{O}_2$  breathing. Each experimental period was preceded by a 1-h steady-state control period. We repeated this sequence of studies in 8 of the 12 lambs during the last week of mechanical ventilation when the lambs were between 18 and 21 days old. With four of these lambs at the end of *week 3*, we also determined PVR for 1 h before (control period) and during a 30-min iv infusion of 8-bromo-cGMP (Sigma Chemical, St. Louis, MO) delivered at a rate of 150  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ .

During each study, we measured vascular pressures (pulmonary arterial, left atrial, carotid arterial, and jugular venous) with calibrated pressure transducers (BT3DC, Statham Instruments, Oxnard, CA) connected to an eight-channel amplifier recorder (model 7D, Grass Instruments, Quincy, MA), and we measured cardiac output in triplicate by thermodilution (model SP1425 cardiac output computer, Gould, Statham Instruments) using cold, isotonic saline during each 1-h steady-state control period and the subsequent experimental period. We sampled arterial blood hourly for measurement of pH,  $\text{PaO}_2$ , and arterial  $\text{PCO}_2$ , and we also sampled the inspired gas for measurement of its oxygen fraction ( $\text{FiO}_2$ ). In addition, during each baseline control period and experimental period, we obtained plasma by centrifugation of blood samples taken from the left atrium and froze the plasma for subsequent measurement of nitrates and nitrites by chemiluminescence (3, 31).

All of the premature lambs that are included in this report survived for at least 3 wk, after which they received 35 mg/kg of iv pentobarbital for anesthesia to open the chest and remove the lungs for subsequent histopathology, immunolocalization, and quantitative assessment of eNOS (35) and sGC in the pulmonary circulation.

*Experimental protocol for PVR studies in term lambs.* To compare lung vascular responses to iNO and to 8-bromo-cGMP in chronically ventilated preterm lambs vs. control lambs born at term gestation, we used similar surgical methods to ligate the ductus arteriosus and place catheters in the pulmonary artery and left atrium, in addition to the aorta and vena cava, of eight healthy, mature lambs that were born spontaneously at >140 days gestation. In contrast to the preterm lambs, however, these lambs had a 12-mm ultrasonic flow probe (Transonic Systems, Ithaca, NY) implanted around the main pulmonary artery, rather than a thermistor wire within the pulmonary artery, for subsequent measurement of cardiac output using a flow-meter (model T106, Transonic Systems) that was connected to the implanted ultrasonic volume flow sensor. The eight term lambs breathed spontaneously and remained with their ewes between experiments.

During experiments, the term lambs were sedated with 5–10 mg/kg pentobarbital, given iv as often as needed, and they were mechanically ventilated at 20 breaths/min with an inspiratory time of 0.75 s. In four term lambs that were 2 wk old, we studied the pulmonary vascular response to iNO during steady-state hypoxia. In these studies, the lambs initially breathed air for 30–60 min, followed by iNO at 15 ppm for 60–90 min; they then breathed a hypoxic gas mixture (15–18% oxygen) of air and nitrogen to induce steady-state hypoxia for 60–90 min, followed by iNO at 15 ppm for

90 min in the presence of continuing hypoxemia. In four other term lambs that were 3–4 wk old, we used a similar experimental design to test the effects on PVR of a continuous 1-h iv infusion of 8-bromo-cGMP (150  $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) during mechanical ventilation with sustained hypoxemia.

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

*Localization of sGC in pulmonary arterioles.* Immunohistochemistry was used to localize sGC protein expression in the vasculature of lung tissue obtained from preterm lambs that were mechanically ventilated for 3 wk, as previously described, and control lambs born at term. To do these studies, we double clamped a large piece of the ventral portion of the caudal lobe of the left lung at the prevailing peak inflation pressure. This procedure retained the gas and blood volume of the lobe and thereby preserved the three-dimensional configuration of the lung in its *in vivo* state. The clamped lobe was immersed in 10% neutral buffered formalin at 4°C for 24 h, after which the lung tissue was cut into 3-mm-thick slices along parasagittal planes. Large tissue blocks (2–4  $\text{cm}^2$ ; 2–3 per lamb) were dehydrated in a graded ethanol series, embedded in paraffin, and sectioned at 4- $\mu\text{m}$  thickness. To improve antigen detection, the heat-induced epitope retrieval (antigen retrieval) method was used, with microwave irradiation in citrate buffer (catalog no. HK086-9K, BioGenex, San Ramon, CA). Deparaffinized and rehydrated tissue sections were treated with methanol- $\text{H}_2\text{O}_2$  to quench endogenous peroxidase activity and with blocking serum to block nonspecific binding sites. We used a polyclonal antibody (rabbit polyclonal antiserum diluted in buffer solution; catalog no. 160890, Cayman Chemical, Ann Arbor, MI) that was directed against the  $\alpha_1$  and  $\beta_1$  subunits of sGC. The optimal dilution was 1:200. The sections were treated with blocking serum that was appropriately matched for the host species in which the primary antibody was produced before application of the primary antibody. A standard peroxidase method (Elite ABC kit; catalog no. PK-6101, Vector Laboratories, Burlingame, CA) was used for antigen detection. The staining signal was visualized by diaminobenzidine (catalog no. D4293, Sigma Chemical). The tissue sections were counterstained with Gill's no. 3 hematoxylin solution (catalog no. GHS-3-32, Sigma Chemical) diluted 1:10 with water. Immunohistochemical staining controls included substitution of the primary antibody with an irrelevant, species-matched, immunoglobulin isotype-matched primary antibody (anti-insulin), omission of the primary antibody (replaced with blocking buffer), and omission of the secondary antibody (replaced with blocking buffer).

To assess the immunostaining density of sGC protein in lung vascular smooth muscle, we used a computer-assisted true-color imaging system (Bioquant True-Color Windows; R&M Biometrics, Nashville, TN) as previously described (35). Briefly, an observer without knowledge of the group from which each tissue section was obtained placed five uniform-sized, enclosed rectangular frames over the full height (lumen to basement membrane) of the vascular smooth muscle cell layer. The relative density of the brown peroxidase reaction product within each enclosed frame was determined automatically by computer. For each tissue section (1 random section per lamb), 75 framed areas were analyzed for pulmonary arterioles adjacent to terminal bronchioles. Terminal bronchioles were used as independent landmarks so that similar generations of pulmonary arterioles were analyzed.

Minimum relative density was established from the tissue sections that were not treated with primary antibody. Maximum relative density was determined from the most intensely immunostained smooth muscle cells in duplicate tissue sections from the term newborn (1 day old) control lambs. These duplicate tissue sections were processed and imaged with the other sections. We used tissue sections from the newborn control lambs to determine maximum relative density because they demonstrated the most intense staining among the groups. The observer who set the minimum and maximum density levels did not perform the densitometry measurements on the randomized digital images for the three groups of lambs.

**Immunoblot analysis of sGC protein in pulmonary arterioles.** We used immunoblot analysis to measure sGC protein abundance in third- to fifth-generation intrapulmonary arteries that were dissected from the lungs of the chronically ventilated preterm lambs and from two groups of control lambs that were born at term gestation and killed either 1 day or 3 wk after birth. Intrapulmonary arteries were dissected at 4°C from the right caudal lung lobe and immersed immediately in liquid nitrogen, followed by storage at -80°C until subsequent processing as described below.

Segments of the pulmonary arteries were placed in a tissue homogenizer that contained an ice-cold solution of Tris·HCl buffer (50 mM, pH 7.5, 4°C) with 20 mM 3-[(3-cholamidopropyl) dimethylammonio]-L-propane sulfonate, 3 mM dithiothreitol, 20 μM tetrahydrobiopterin, and protease inhibitors (2 μg/ml pepstatin A, 20 μg/ml leupeptin, 40 μg/ml N-α-p-tosyl-L-lysine chloromethylketone, and 20 μg/ml aprotinin) (Roche Molecular Biochem, Indianapolis, IN). Tissue homogenization was performed at 4°C. The homogenates were centrifuged at 16,000 g for 30 min, and aliquots of the supernatants were frozen at -80°C. Protein content was determined by bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL) using bovine serum albumin as the standard (53).

SDS-PAGE was performed on 25 μg of total protein per sample by using the method of Laemmli (34). Proteins were electrophoretically transferred to nitrocellulose membranes and immersed for 1 h in buffer containing 150 mM NaCl, 50 mM Tris·HCl, 0.1% Tween 20, and 5% instant nonfat dry milk. The membranes were incubated for 2 h at room temperature in the presence of a primary antibody directed against the α<sub>1</sub> and β<sub>1</sub> subunits of sGC (rabbit polyclonal antiserum diluted with blocking buffer at 1:1,000; catalog no. 160890, Cayman Chemical). After incubation with the primary antibody, the nitrocellulose membranes were washed with 150 mM NaCl buffer containing 50 mM Tris·HCl and

0.1% Tween 20, followed by incubation for 1 h with a secondary antibody that was bound to horseradish peroxidase (catalog no. 401393, Calbiochem, San Diego, CA). The membranes were washed and the bands were analyzed by chemiluminescence (ECL Western blotting detection system, catalog no. RPN2209, Amersham Pharmacia Biotech, Piscataway, NJ) and quantified by densitometry (National Institutes of Health Image software).

**Statistics.** Data in the text, tables, and figures are expressed as means ± SD. We used Student's paired *t*-test to test for significant differences between physiological data that were obtained during the 1-h control period and the subsequent experimental period (iNO, acetylcholine infusion, 100% oxygen breathing or 8-bromo-cGMP infusion) at the end of weeks 1 and 3 of continuous mechanical ventilation. We used ANOVA and the Student-Neuman-Keuls comparison test to identify significant differences in baseline PVR at the end of weeks 1 and 3 in chronically ventilated preterm vs. term lambs. For comparison of immunoblot measurements of sGC protein abundance between chronically ventilated preterm lambs and control lambs born at term, statistical significance was assessed by ANOVA with the Student-Neuman-Keuls multiple-comparison test to identify differences between experimental and control groups (62). We considered values significantly different if the *P* value was <0.05.

## RESULTS

Table 1 shows summary data for the 12 studies of iNO done in preterm lambs at the end of week 1. Their body weight was 2.65 ± 0.52 kg, and they were 10 ± 2 days old. F<sub>I</sub>O<sub>2</sub> and ventilator settings were kept constant during control and experimental periods. PVR consistently decreased by an average of 20 ± 7%, and Pa<sub>O</sub><sub>2</sub> increased from 63 ± 15 to 74 ± 24 Torr when iNO was given at 15 ppm for 1 h. This pulmonary vascular response to iNO was similar in magnitude to the response seen in healthy term lambs that received iNO during normoxia, but it was less than the response that occurred in the more mature lambs when they received iNO in the presence of sustained hypoxia (Table 2).

When the chronically ventilated preterm lambs received an iv infusion of acetylcholine at the end of week 1, systemic and pulmonary arterial blood pressures decreased acutely (data not shown), as expected. During the steady-state 1-h infusion of acetylcholine, how-

Table 1. Summary of pulmonary circulation studies before and during 1 h of iNO at 15 ppm in chronically ventilated preterm lambs

Experimental Period	Fraction of Inspired O <sub>2</sub>	Pa <sub>O</sub> <sub>2</sub> , Torr	Pa <sub>C</sub> O <sub>2</sub> , Torr	Arterial pH	Vascular Pressures, mmHg		Cardiac Output, l/min	Pulmonary Vascular Resistance, mmHg·l <sup>-1</sup> ·min
					Pulmonary artery	Left atrium		
<i>Studies at the end of week 1, n = 12</i>								
Baseline control	0.38 ± 0.17	63 ± 15	42 ± 16	7.35 ± 0.10	27 ± 11	5 ± 1	0.76 ± 0.23	31.3 ± 17.1
iNO	0.37 ± 0.17	74 ± 24*	41 ± 16	7.35 ± 0.10	23 ± 7	5 ± 2	0.82 ± 0.28	25.1 ± 13.7*
<i>Studies at the end of week 3, n = 8</i>								
Baseline control	0.43 ± 0.35	61 ± 13	41 ± 10	7.36 ± 0.10	25 ± 12	5 ± 2	0.98 ± 0.49	26.0 ± 16.6
iNO	0.43 ± 0.35	71 ± 22	39 ± 24	7.36 ± 0.12	23 ± 11	5 ± 2	0.98 ± 0.52	24.6 ± 17.7

Values are means ± SD. iNO, inhaled nitric oxide; ppm, parts/million; Pa<sub>O</sub><sub>2</sub>, arterial PO<sub>2</sub>; Pa<sub>C</sub>O<sub>2</sub>, arterial PCO<sub>2</sub>. \*Significant difference between baseline control and iNO, *P* < 0.05.

Table 2. Summary of pulmonary circulation studies before and during 1 h of iNO at 15 ppm, first during normoxia, and then during hypoxia, in four 2-wk-old term lambs

Experimental Period	Fraction of Inspired O <sub>2</sub>	P <sub>aO<sub>2</sub></sub> , Torr	Vascular Pressures, mmHg		Cardiac Output, l/min	Pulmonary Vascular Resistance, mmHg·l <sup>-1</sup> ·min
			Pulmonary artery	Left atrium		
Studies during normoxia						
Baseline control	0.22 ± 0.01	62 ± 5	23 ± 3	4 ± 1	2.85 ± 0.46	6.84 ± 1.94
Inhaled NO	0.22 ± 0.01	68 ± 11	19 ± 2*	4 ± 2	2.76 ± 0.52	5.68 ± 1.70*
Studies during hypoxia						
Baseline control	0.22 ± 0.01	63 ± 10	25 ± 3	5 ± 2	2.67 ± 0.43	7.64 ± 1.97
Hypoxia	0.17 ± 0.02*	34 ± 2*	34 ± 4*	5 ± 2	2.97 ± 0.61*	10.34 ± 2.58*
Hypoxic and inhaled NO	0.16 ± 0.02	36 ± 5	25 ± 3*	4 ± 2	2.95 ± 0.42	7.27 ± 2.13*

Values are means ± SD. \*Significant difference compared with preceding period,  $P < 0.05$ .

ever, PVR did not change significantly (Fig. 1). Likewise, 100% oxygen breathing for 1 h after a 1-h control period at the prevailing F<sub>I</sub>O<sub>2</sub> yielded no significant change in PVR (Fig. 1), despite the fact that P<sub>aO<sub>2</sub></sub> increased from 67 ± 18 to 225 ± 71 Torr.

When we repeated this sequence of studies in 8 of the 12 lambs during the final week of mechanical ventilation (age 19 ± 1 days), iNO at 15 ppm for 1 h had no significant effect on PVR (Table 1; Fig. 2). Plasma concentrations of nitrates and nitrites in samples of blood taken from the left atrium of lambs before and during iNO showed similar increases when iNO was given at the end of *week 1* (plasma nitrate/nitrite concentration in μmol/l, 25.6 ± 13.1 during baseline period, 38.1 ± 19.9 during iNO; significant increase,  $P < 0.05$ ) and at the end of *week 3* (plasma nitrate/nitrite concentration in μmol/l, 19.0 ± 8.6 during baseline period, 28.1 during iNO; significant increase,  $P < 0.05$ ). As in the previous studies done at the end of *week 1*, neither acetylcholine infusion nor 100% oxygen breathing had a significant effect on PVR at the end of *week 3* (data not shown).

To see whether the loss of iNO effect on PVR during development of CLD might reflect pulmonary vascular

smooth muscle dysfunction, we infused 8-bromo-cGMP iv at 150 μg·kg<sup>-1</sup>·min<sup>-1</sup> for 15–30 min in four preterm lambs that had been mechanically ventilated for 3 wk. Cardiac output increased by ~20% during infusion of 8-bromo-cGMP, and PVR consistently decreased by 30–35% (Table 3). In similar studies performed with healthy term lambs, iv infusion of 8-bromo-cGMP during steady-state hypoxia did not alter cardiac output but did reduce PVR by an average of 39%, similar to the decrease that occurred in the chronically ventilated premature lambs (Table 4).

We wondered whether deficient pulmonary expression of sGC after premature birth and prolonged mechanical ventilation might account for the failure of the lung circulation to dilate in response to iNO. To test this hypothesis, we performed immunohistochemistry by using a polyclonal antibody directed against the α<sub>1</sub> and β<sub>1</sub> subunits of sGC, and we compared immunostaining of lungs obtained from chronically ventilated preterm lambs with lungs from two groups of control term lambs that were 1 day old (same postconceptional age as the preterm lambs were at death) or 3 wk old (same postnatal age as the preterm lambs were at death). Using computer-assisted image analysis densitometry to quantify immunostaining for the α<sub>1</sub> and β<sub>1</sub>

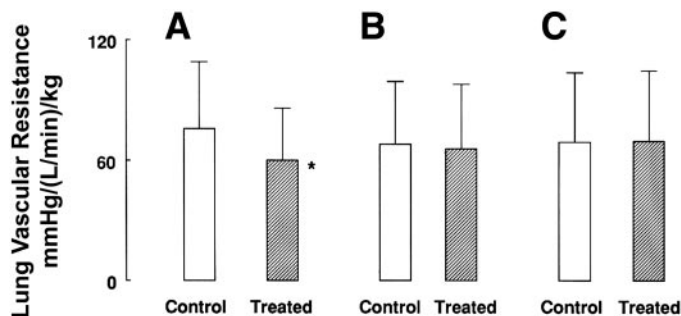


Fig. 1. Summary data (means ± SD) for lung vascular resistance expressed per kilogram body weight, measured at the end of *week 1* (7–12 days after birth) in 12 chronically ventilated preterm lambs. Open bars are values obtained during a 1-h control period, and hatched bars are values obtained during a 1-h experimental period. A: results of studies in which the lambs received inhaled nitric oxide (iNO) at 15 parts/million (ppm) for 1 h. B: results of studies in which the lambs received intravenous (iv) acetylcholine at 1.5 μg/min for 1 h. C: results of studies in which the lambs breathed 100% oxygen for 1 h. \*Significant difference during the experimental period compared with the control period,  $P < 0.05$ .

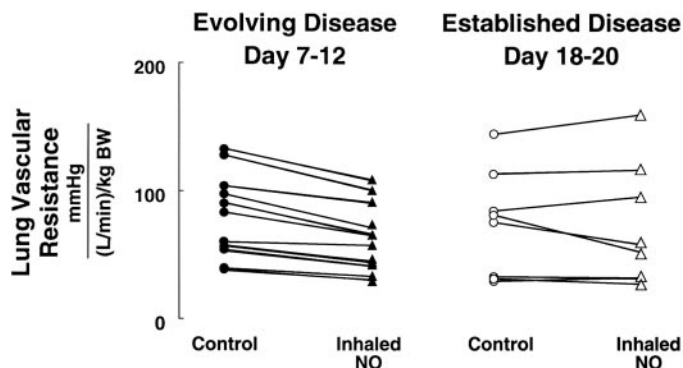


Fig. 2. Lung vascular response to iNO at 15 ppm in chronically ventilated preterm lambs early ( $n = 12$ ) and late ( $n = 8$ ) in the course of evolving chronic lung disease (CLD). Lung vascular resistance is calculated from the pressure difference across the pulmonary circulation divided by blood flow/kg body wt. Pulmonary vascular resistance decreased with iNO in all lambs at the end of *week 1* but did not change significantly at the end of *week 3*.

Table 3. Summary of pulmonary circulation studies before and during 30-min intravenous infusion of 8-bromo-cGMP in 4 chronically ventilated preterm lambs

Experimental Period	Fraction of Inspired O <sub>2</sub>	PaO <sub>2</sub> , Torr	Vascular Pressures, mmHg		Cardiac Output, l/min	Pulmonary Vascular Resistance, mmHg·l <sup>-1</sup> ·min <sup>-1</sup>
			Pulmonary artery	Left atrium		
Baseline control	0.25 ± 0.05	57 ± 11	22 ± 1	6 ± 2	1.31 ± 0.49	14.6 ± 7.8
8-Bromo-cGMP	0.25 ± 0.05	54 ± 14	20 ± 1	6 ± 2	1.58 ± 0.64*	10.1 ± 5.1*

Values are means ± SD. cGMP, guanosine 3',5'-cyclic monophosphate. 8-Bromo-cGMP (150 μg·kg<sup>-1</sup>·min<sup>-1</sup>) was infused intravenously at the end of *week 3*. \*Significant difference compared with preceding period, *P* < 0.05.

subunits of sGC in the lung tissue of these lambs, we found that immunostaining was less in pulmonary vascular smooth muscle of lambs with chronic lung injury than it was in the relevant control lambs (Fig. 3, A and B). We then did immunoblot (Western) analysis using the same polyclonal antibody to the α<sub>1</sub> and β<sub>1</sub> subunits of sGC to assess the abundance of sGC protein in excised intrapulmonary arteries obtained from lungs of chronically ventilated preterm lambs and control term lambs. The abundance of sGC was significantly less in the isolated pulmonary arteries of the preterm lambs with lung injury than it was in control lambs killed either 1 day or 3 wk after birth at term (Fig. 4).

## DISCUSSION

Chronic lung injury from prolonged mechanical ventilation after premature birth inhibits the normal post-natal decrease in PVR (7) and reduces pulmonary expression of eNOS in newborn sheep (35). Because iNO reduces PVR in both human infants and newborn lambs with PPHN, we studied the effect of iNO on PVR in preterm lambs that had been mechanically ventilated for 7 to 12 days, such that their baseline PVR was at least two- to threefold greater than the PVR of control term lambs that we studied. PVR consistently decreased by an average of ~20%, and PaO<sub>2</sub> increased by ~15–20% in response to iNO. On the same day that iNO decreased PVR in these lambs, iv infusion of acetylcholine, an endothelial-dependent vasodilator, had no significant effect on steady-state PVR. This blunted or absent pulmonary vascular response to acetylcholine raises the possibility that the lung endothelium may have been damaged by the end of *week 1*.

After an additional 2 wk of mechanical ventilation, PVR remained high compared with the PVR of lambs born at term gestation. Baseline PVR was not significantly

different than it was at the end of *week 1*, and yet administration of iNO for 1 h had no significant effect on PVR at the end of *week 3*. The finding that plasma concentrations of NO metabolites increased during delivery of iNO at the end of *week 3*, as they did at the end of *week 1*, indicated that NO reached the pulmonary circulation in these lambs despite the presence of chronic lung injury (2, 7, 43).

We speculated that this loss of iNO effect on PVR during development of CLD might have been due to abnormal function of lung vascular smooth muscle. To test this possibility, we infused iv 8-bromo-cGMP in four preterm lambs that had not responded to iNO at the end of *week 3*. PVR consistently decreased by 30–35%, comparable to the response that occurred in healthy 3-wk-old term lambs with acute pulmonary hypertension, induced by steady-state alveolar hypoxia, when they received the cGMP analog. These findings suggest that the mechanism regulating smooth muscle relaxation in the pulmonary circulation was operative in the preterm lambs despite prolonged mechanical ventilation.

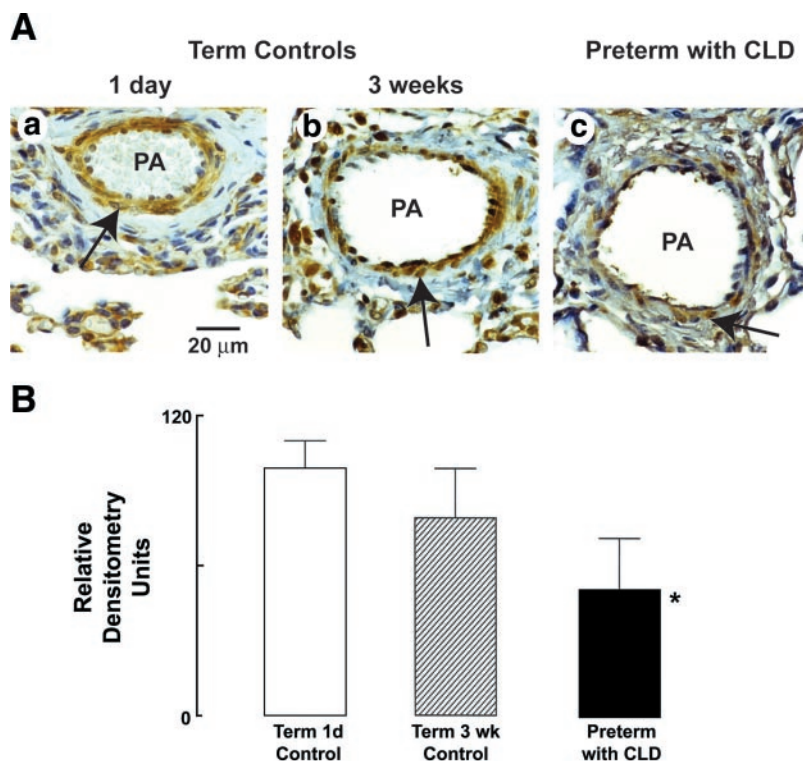
The studies of the pulmonary vascular response to 8-bromo-cGMP in the preterm lambs presented an interesting caveat with respect to the severity of their lung injury. Baseline PVR at the end of *week 3* averaged 15 ± 8 Torr·l<sup>-1</sup>·min<sup>-1</sup> and F<sub>I</sub>O<sub>2</sub> averaged 0.25 ± 0.05 for the four lambs that received 8-bromo-cGMP, whereas baseline PVR averaged 26 ± 17 Torr·l<sup>-1</sup>·min<sup>-1</sup> and F<sub>I</sub>O<sub>2</sub> was 0.43 ± 0.35 for the entire group of eight lambs that were tested with iNO at the end of *week 3*. Although these differences were not statistically significant (*P* > 0.05), they caused us to consider that lung injury may have been less severe in those lambs whose PVR decreased in response to 8-bromo-cGMP. If this were so, we might have expected PVR to decrease in response to iNO in these four lambs, and

Table 4. Summary of pulmonary circulation studies before and during intravenous infusion of 8-bromo-cGMP in the presence of hypoxia in 4 term lambs

Experimental Period	Fraction of Inspired O <sub>2</sub>	PaO <sub>2</sub> , Torr	Vascular Pressures, mmHg		Cardiac Output, l/min	Pulmonary Vascular Resistance, mmHg·l <sup>-1</sup> ·min <sup>-1</sup>
			Pulmonary artery	Left atrium		
Baseline normoxia	0.25 ± 0.03	73 ± 12	25 ± 6	3 ± 1	3.32 ± 0.55	6.81 ± 1.40
Hypoxia	0.17 ± 0.01*	33 ± 5*	39 ± 4*	3 ± 2	3.90 ± 0.91	9.58 ± 2.59*
8-Bromo-cGMP	0.17 ± 0.02	32 ± 5	27 ± 2*	3 ± 2	4.08 ± 0.75	5.86 ± 1.27*

Values are means ± SD. 8-Bromo-cGMP was infused at a rate of 150 μg·kg<sup>-1</sup>·min. \*Significant difference compared with preceding period, *P* < 0.05.

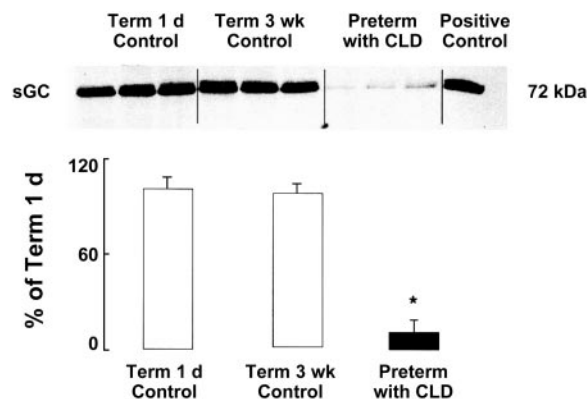
**Fig. 3. A:** sections of lung obtained from 3 lambs: a 1-day-old term control lamb (a), a 3-wk-old term control lamb (b), and a 3-wk-old preterm lamb that had been mechanically ventilated for 3 wk after birth by cesarean section (c). The sections show immunostaining (yellow) of a polyclonal antibody directed against the  $\alpha_1$  and  $\beta_1$  subunits of soluble guanylate cyclase (sGC) in pulmonary arterial (PA) smooth muscle. **B:** summary data (means  $\pm$  SD) for  $\alpha_1/\beta_1$  sGC immunostaining of lungs from 1-day (d)-old and 3-wk-old control lambs born at term and chronically ventilated preterm lambs ( $n = 5$  for all 3 groups) assessed by computerized image analysis. \*Significant difference compared with both control groups,  $P < 0.05$ .



yet we found that PVR did not change ( $16.4 \pm 10.9$  before iNO,  $16.4 \pm 13.1$  during iNO) when these lambs received iNO at the end of *week 3*. Moreover, when we assessed the degree of pulmonary edema by measuring postmortem extravascular lung water as an index of the severity of lung injury in these four lambs, it averaged  $6.6 \pm 1.8$  g/g dry lung tissue, compared with  $6.2 \pm 2.1$  g/g dry lung tissue for the entire group of eight lambs that were given iNO at the end of *week 3*. This observation suggests that pulmonary vascular relaxation in response to 8-bromo-cGMP was not attributable to less severe lung injury in these four lambs.

Based on our laboratory's previous finding of diminished eNOS in the lungs of lambs with CLD (35), we wondered whether loss of the pulmonary vasodilator response to iNO in preterm lambs with CLD might reflect impaired NO signaling, perhaps from deficient pulmonary expression of sGC, the intermediary enzyme through which NO induces an increase in vascular smooth muscle cGMP and resultant vasodilation. Immunohistochemistry performed on lung tissue sections and immunoblot (Western) analysis performed on excised small pulmonary arteries showed that sGC protein was less abundant in lung vascular smooth muscle of lambs with CLD than it was in relevant control lambs. These observations suggest that prolonged mechanical ventilation after premature birth may alter the normal developmental pattern of pulmonary arterial smooth muscle expression of sGC, which in turn could contribute to the increased PVR and attenuated or absent lung vascular response to iNO that we observed in lambs with CLD.

Reduced abundance of sGC in the pulmonary circulation of chronically ventilated premature lambs is consistent with our previous observation of diminished pulmonary expression of eNOS in lambs with CLD (35). As NO may regulate vascular smooth muscle cell proliferation (22, 56), as well as vascular tone and reactivity, deficient eNOS and sGC in the lung circulation likely account, at least in part, for the increased abundance of pulmonary arterial smooth muscle and elevated PVR that coexist in lambs with CLD (7). Our studies have not defined the time course of diminished expression of either eNOS or sGC in the pulmonary



**Fig. 4.** Immunoblot (Western) analysis of sGC protein in excised pulmonary arterioles of newborn lambs: 1-day-old term controls, 3-wk-old term controls, and chronically ventilated preterm lambs. Values are means  $\pm$  SD, expressed as % of the 1-day-old term controls;  $n = 3$  for each group. \*Significant difference compared with both control groups,  $P < 0.05$ .

circulation of the chronically ventilated preterm lambs, but our physiological studies suggest that eNOS protein abundance or activity may have been reduced as early as 1 wk after birth because there was no significant decrease in PVR in response to acetylcholine, which has been shown to dilate the pulmonary circulation in late-gestation fetal sheep through release of endogenous NO (57). The fact that iNO caused a significant decrease in PVR after 7–12 days of mechanical ventilation suggests that sGC was present in the pulmonary circulation and responsive to NO at this point in the evolution of CLD, whereas the lack of physiological response to iNO at the end of *week 3* correlated with the markedly diminished abundance of sGC protein in small pulmonary arteries noted after 3 wk of mechanical ventilation, when CLD was well established.

sGC is an obligate heterodimer composed of two subunits and is expressed in the lung predominantly in vascular and airway smooth muscle cells (8, 39). Studies of the ontogeny of the  $\alpha_1$  and  $\beta_1$  isoforms of sGC mRNA and of the sGC protein in rat lung showed that expression increases late in fetal gestation and becomes maximal about a week after birth, with increased postnatal expression in lung vascular endothelium (4, 8). Previous immunohistochemical studies of the pulmonary circulation in fetal sheep showed prominent expression of sGC protein in distal arteries and even greater immunostaining of preacinar veins; these findings were consistent with physiological responses to sodium nitroprusside that were measured in these vessels (18).

Studies by other investigators have shown that pulmonary vascular expression of both eNOS and sGC are reduced in lambs with PPHN induced by prenatal closure of the ductus arteriosus (6, 52, 59). In this condition, pulmonary arterial pressure soon after birth was considerably greater than it was in the 3-wk-old lambs with CLD that we studied, and structural remodeling of the lung circulation was different as well (7, 38, 61). Although there was abundant smooth muscle around small pulmonary arteries in both conditions, the most striking abnormality of the lambs with PPHN was extension of smooth muscle to intraacinar pulmonary arteries, which are normally nonmuscular, and periadventitial fibrosis around these vessels, without a reduction in the number of small intraacinar arteries (61). In contrast, the preterm lambs with CLD had decreased numbers of small blood vessels and diminished capillary surface density in their lungs, with associated alveolar hypoplasia, increased smooth muscle and elastin in pulmonary arterioles, and interstitial pulmonary edema, without an apparent difference in lung collagen or perivascular fibrosis (2, 7, 43).

The differences in pulmonary pathology between these two ovine models of neonatal lung disease probably reflect differences in both the stage of lung vascular development at which the injury was induced and the specific stimulus by which the injury was created. The lamb model of PPHN was created by changing blood pressure and blood flow in the pulmonary circu-

lation of fetal lambs at 123–134 days of gestation and then delivering the lambs at 138–144 days of gestation. A recent report (10) indicates that the pathophysiology of this experimental model of PPHN may be related to increased formation of reactive oxygen species and reduced activity of superoxide dismutase in the pulmonary circulation, which results in lung vascular injury and remodeling. In contrast, the lamb model of CLD was created by delivering the lambs prematurely at ~125 days of gestation and then mechanically ventilating them for 3–4 wk, during which there was prolonged, repetitive lung stretch and exposure to noxious stimuli, including oxygen and various microbial organisms that are known to induce inflammation (12, 17) and proteolytic activity (37, 42) within the lung, which in turn may degrade pivotal proteins within the pulmonary circulation and thereby disrupt both lung vascular structure and function. Bacterial sepsis, treated with broad-spectrum antibiotics, was a frequent complication of prolonged mechanical ventilation in these premature lambs, which may have contributed to their lung vascular dysfunction (7). Several studies have provided evidence that sepsis, or more specifically exposure to *Escherichia coli* lipopolysaccharide, inhibits production of cGMP and blunts the pulmonary vasodilator response to iNO, with associated reduction of sGC mRNA, protein, and enzyme activity (25, 33, 50, 51).

Our observation that iv delivery of a cGMP analog in lambs with CLD caused a decrease in PVR that was comparable to the response that occurred in term lambs with hypoxia-induced pulmonary hypertension indicates that prolonged repetitive stretch of the incompletely developed lung does not disrupt the normal apparatus for relaxation of pulmonary vascular smooth muscle, whereby cGMP activates cGMP-dependent protein kinases (13). Thus, even in the presence of lung vascular dysfunction associated with loss of eNOS and sGC proteins in the pulmonary circulation, analogs of cGMP appear to merit further investigation as a potentially useful therapeutic tool in conditions where lung injury is associated with pulmonary hypertension.

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