Utility of large-animal models of BPD: chronically ventilated preterm lambs

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Albertine KH. Utility of large-animal models of BPD: chronically ventilated preterm lambs. Am J Physiol Lung Cell Mol Physiol 308: L983–L1001, 2015. First published March 13, 2015; doi:10.1152/ajplung.00178.2014.—This paper is focused on unique insights provided by the preterm lamb physiological model of bronchopulmonary dysplasia (BPD). Connections are also made to insights provided by the former preterm baboon model of BPD, as well as to rodent models of lung injury to the immature, postnatal lung. The preterm lamb and baboon models recapitulate the clinical setting of preterm birth and respiratory failure that require prolonged ventilation support for days or weeks with oxygen-rich gas. An advantage of the preterm lamb model is the large size of preterm lambs, which facilitates physiological studies for days or weeks during the evolution of neonatal chronic lung disease (CLD). To this advantage is linked an integrated array of morphological, biochemical, and molecular analyses that are identifying the role of individual genes in the pathogenesis of neonatal CLD. Results indicate that the mode of ventilation, invasive mechanical ventilation vs. less invasive high-frequency nasal ventilation, is related to outcomes. Our approach also includes pharmacological interventions that test causality of specific molecular players, such as vitamin A supplementation in the pathogenesis of neonatal CLD. The new insights that are being gained from our preterm lamb model may have important translational implications about the pathogenesis and treatment of BPD in preterm human infants.

neonatal chronic lung disease; alveolarization; alveolar simplification; pulmonary hypertension; airway expiratory resistance; nitric oxide; vitamin A; endothelial nitric oxide synthase; nasal CPAP; insulin-like growth factor-1; epigenetics

ACUTE RESPIRATORY FAILURE of preterm human infants reflects immaturity of the lung following preterm birth, disrupted developmental processes, and dysregulated repair responses. This reflection occurs in the neonatal intensive care setting of continuous invasive mechanical ventilation support with oxygen-rich gas that is necessary to keep many preterm human infants alive. Among survivors of preterm birth and initial days of ventilation support with oxygen-rich gas, human infants who require prolonged invasive ventilation support with oxygen-rich gas frequently develop bronchopulmonary dysplasia (BPD). BPD is a significant pediatric health problem because, almost four decades after its initial description (134), this disease of preterm infants remains the most common cause of long-term poor outcomes, including postnatal growth failure, recurrent hospitalization for respiratory tract infections, and intraventricular hemorrhage (53, 178). In the United States, since 2006, the overall rate of prematurity is 1 in 8 births, or ~12% (74, 118). However, the underlying pathogenic mechanisms are incompletely understood.

Gaps in knowledge about the underlying pathogenic mechanisms that contribute to BPD are related, in part, to the challenge of establishing suitable animal models that include preterm birth and respiratory failure requiring prolonged invasive mechanical ventilation with oxygen-rich gas for days, weeks, or months. Two types of animal models have been developed: large-animal models and small-animal models. Large-animal models provide unique opportunity for physiological studies of evolving disease pathogenesis in preterm neonates that are supported in a neonatal intensive care setting for days or weeks, akin to preterm human infants. Small-animal models provide elegant opportunities to manipulate the genome or molecular pathways to reveal mechanisms that impact the immature lung of postnatal pups (113). Insights provided by both types of models are the focus of this paper.

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Species-Specific Lung Anatomy

Species variations are evident in lung anatomy. For context for this review, comparison of lung anatomy is made among humans, monkeys, sheep, rats, and mice. Focus on baboons and sheep is because they are the large-animal models of BPD, rats and mice because they are the small-animal models. Key features of lung comparative anatomy are summarized in Table 1. This table exposes developmental and anatomic similarities and differences among the five species that may be helpful to keep in mind.

At term gestation, the developmental stage is different among the five species (Table 1) (191). The developmental stage at term gestation of the lung of humans, monkeys, and sheep is at the beginning of the alveolar stage. By comparison, the developmental stage of the lung of rats and mice is at the saccular stage. This difference will be returned to later in this review.

A number of gross structural features are species-specific in the adult lung (Table 1). Gross structural differences include lobation, airway generations, airway branching pattern, diameter of the main bronchus, and cartilage in the upper airways (bronchi). The human lung has fewer lobes, more airway generations, and the largest diameter of the main bronchus. More airway generations in the lung of humans translates to the bronchi). The human lung has fewer lobes, more airway generations, and the largest diameter of the main bronchus. More airway generations in the lung of humans translates to the bronchi. The human lung has fewer lobes, more airway generations, and the largest diameter of the main bronchus. More airway generations in the lung of humans translates to the bronchi.

The distribution of various cell types also is species related (Table 1). In the lung of humans, monkeys, and sheep, the principal secretory cell types along the length of the upper airways are mucus and serous epithelial cells in submucosal glands, as well as goblet cells. By comparison, the lung of rats and mice has submucosal glands that are limited to the upper third of the initial intrapulmonary airways. So, too, the number of goblet cells is limited in distribution and number in the lung of rats and mice. Instead, club cells (formerly called Clara cells; Ref. 196) are the principal secretory epithelial cell type in

Table 1. Comparative anatomy of human, monkey, sheep, rat, and mouse lungs

<table>
<thead>
<tr>
<th>Lung Anatomical Feature</th>
<th>Humans</th>
<th>Monkeys</th>
<th>Sheep</th>
<th>Rats</th>
<th>Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Developmental stage at full term</td>
<td>Alveolar</td>
<td>Alveolar</td>
<td>Alveolar</td>
<td>Sacculat</td>
<td>Sacculat</td>
</tr>
<tr>
<td>Lobes</td>
<td>3 right; 2 left</td>
<td>4 right; 3 left</td>
<td>4 right; 2 left</td>
<td>4 right; 1 left</td>
<td>4 right; 1 left</td>
</tr>
<tr>
<td>Airway branching pattern</td>
<td>Symmetric</td>
<td>Symmetric</td>
<td>Symmetric</td>
<td>Asymmetric</td>
<td>Asymmetric</td>
</tr>
<tr>
<td>Trachea</td>
<td>Epithelial thickness</td>
<td>50–100 μm</td>
<td>20–30 μm</td>
<td>65–70 μm</td>
<td>ND</td>
</tr>
<tr>
<td>Submucosal glands</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Proximal 1/3rd</td>
<td>Proximal 1/3rd</td>
</tr>
<tr>
<td>Goblet cells</td>
<td>9%</td>
<td>17%</td>
<td>5%</td>
<td>1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Club cells</td>
<td>None</td>
<td>&lt;1%</td>
<td>None</td>
<td>45%</td>
<td>−50%</td>
</tr>
<tr>
<td>Main bronchus diameter 10–15 mm ND</td>
<td>ND</td>
<td>2–3 mm</td>
<td>−1 mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intrapulmonary airways</td>
<td>Cartilage</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Epithelial thickness</td>
<td>40–50 μm</td>
<td>~30 μm</td>
<td>50–60 μm</td>
<td>10–16 μm</td>
<td>8–16 μm</td>
</tr>
<tr>
<td>Submucosal glands</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Goblet cells</td>
<td>10%</td>
<td>15%</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Club cells</td>
<td>3%</td>
<td>5%</td>
<td>Rare</td>
<td>45%</td>
<td>−60%</td>
</tr>
<tr>
<td>Terminal bronchioles</td>
<td>Diameter</td>
<td>~600 μm</td>
<td>~500 μm</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Epithelial thickness</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>~11 μm</td>
<td>~8 μm</td>
</tr>
<tr>
<td>Submucosal glands</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Goblet cells</td>
<td>2%</td>
<td>20%</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Club cells</td>
<td>11–41%</td>
<td>Absent</td>
<td>~60%</td>
<td>~75%</td>
<td>60–80%</td>
</tr>
<tr>
<td>Respiratory bronchioles</td>
<td>Number</td>
<td>Many (~150,000)</td>
<td>Many</td>
<td>Many</td>
<td>Absent (or one)</td>
</tr>
<tr>
<td>Club cells</td>
<td>~20%</td>
<td>&gt;90%</td>
<td>~60%</td>
<td>&gt;50%</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Ratio of lung parenchyma/total lung volume</td>
<td>~12%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>~18%</td>
</tr>
<tr>
<td>Alveolar diameter</td>
<td>100–200 μm</td>
<td>100–200 μm</td>
<td>100–200 μm</td>
<td>50–90 μm</td>
<td>40–80 μm</td>
</tr>
<tr>
<td>Blood-air barrier thickness</td>
<td>~0.70 μm</td>
<td>~0.50 μm</td>
<td>~0.50 μm</td>
<td>~0.40 μm</td>
<td>~0.30 μm</td>
</tr>
<tr>
<td>Pulmonary venule location</td>
<td>Interlobular septa</td>
<td>Interlobular septa</td>
<td>Interlobular septa</td>
<td>Next to bronchioles</td>
<td>Next to bronchioles</td>
</tr>
</tbody>
</table>

ND, not determined. Compiled from Refs. 27, 51, 55, 67, 70, 79, 86, 88, 93, 97, 116, 121, 126, 142–145, 149, 169, 170, 172, 184, 185, 190–192 (Humans and Monkeys); Refs. 8, 117, 146 (Sheep); Refs. 32, 34, 40, 71, 80, 92, 98, 112, 125, 127, 128, 137, 138, 141, 144, 158, 180, 187, 194, 198, 201 (Rats and Mice).
the upper airways of the lung of rats and mice. Club cells are located in bronchioles of all five species. These species-specific distributions of cell types may be associated with different amount and quality of airway secretions, as well as responses to exposure to xenobiotics.

Finally, the first row in Table 1 reminds us that at full-term gestation, lung development is at the transition to the alveolar stage in humans, monkeys, and sheep, whereas lung development is at the transition to the saccular stage in rats and mice. However, bronchopulmonary dysplasia occurs in human infants who are born preterm. Their lungs are at the canicular stage or saccular stage. The structural and functional immaturity of their lungs frequently leads to respiratory distress and failure that necessitates intubation, mechanical ventilation, and surfactant replacement to keep the preterm human infants alive. Herein lays an important distinction from rats and mice because while their lungs are at the saccular stage of development at term gestation they thrive without need for respiratory support. Nonetheless, their immature lungs are susceptible to injury induced by high inspired oxygen, mechanical ventilation, and infection/inflammation. Thus the large-animal preterm models and the small-animal term models are valuable for identifying pathogenic mechanisms and testing treatment approaches.

**Preterm Birth in Humans and the Adverse Outcome of Bronchopulmonary Dysplasia**

The definition of preterm birth in humans is any birth, regardless of weight at birth, that occurs before 37 completed wk of gestation from the first day of menstrual cycle (11a). Pregnancies that end before 20 completed wk of gestation are referred to as miscarriages. Therefore, another definition of preterm birth is any delivery that occurs between 20 and 37 wk of gestation in humans. The rate of preterm birth in the United States has steadily declined to the most recent rate of 12% for 2012 (119). Although the rate of preterm birth has steadily declined, the number of preterm births remains ~500,000/year (74).

When a human infant is born preterm, its lungs are structurally immature. The immature lung’s developmental stage may be at the canicular stage (17–28 wk gestation; term is ~40 wk) or the saccular stage (24–36 wk gestation) of development (33). The canicular stage of lung development is characterized architecturally by thick partitions of mesenchyme that separate the air canals (10). Because the mesenchyme forms thick partitions, the growing capillary bed remains physically distant from the air canals. Also, the air canals are lined by tall (columnar or cuboidal) epithelial cells. These architectural features mean that the diffusion distance from the air spaces to flowing red blood cells is large, which impedes efficient respiratory gas exchange. Furthermore, the preterm human lung at the canicular stage of lung development is deficient in surfactant and surfactant apoproteins, creating high surface tension forces and air space instability (151). Consequently, air space collapse (atelectasis) occurs. Atelectasis contributes to poor oxygenation because of ventilation-perfusion mismatch and intrapulmonary shunt (12).

A similar scenario unfolds when a human infant is born preterm at the saccular stage of lung development. This stage’s architecture is characterized by transformation of air canals into air sacs (10). The structural transformations create larger air space surface area, thinner mesenchymal partitions, shorter epithelial cells, and more capillaries that are closer to the air sacs. Therefore, these developmental transformations reduce the diffusion distance between the expanding surface areas of the gas-exchange air spaces and capillaries. However, intimate structural association between the transforming respiratory gas exchange air spaces and capillaries is not accomplished during the saccular stage.

Opening the collapsed air canals or sacs requires recruitment of collapsed air spaces, as well as air spaces yet to be recruited. Preterm human infants who do not recruit air spaces and do not adequately exchange respiratory gases during spontaneous breathing develop respiratory failure that requires endotracheal intubation and ventilation support to sustain preterm life. When high inspiratory pressures are used, however, the danger is that only the ventilated air spaces will fill with each provided breath, leading to air space distension (overinflation). Preterm human infants who have these characteristics usually develop acute lung injury (respiratory distress syndrome, RDS). Histopathologically, during the initial hours of invasive ventilation support, uneven inflation may be evident, owing to surfactant deficiency and residual, uncleared fetal lung liquid in the air spaces and mesenchyme. By 12–24 h of invasive ventilation support, necrosis of epithelial cells occurs and plasma proteins leak from the underlying blood vessels. Consequently, blankets of extravasated protein accumulate along the air space surfaces, forming hyaline membranes (10, 177). Hyaline membranes are the histopathological basis of the term hyaline membrane disease.

Survival of preterm human infants with RDS has increased since the era of antenatal glucocorticoid treatment and postnatal surfactant replacement, as well as gentler ventilation modes (83, 105, 106, 129). Survival rates at some centers are better than 90% for preterm human infants weighing 1,500 g or less at birth (36, 171). Nonetheless, more than 40% (10,000–15,000 per year) of these preterm human infants required prolonged intubation and ventilation support and therefore progress to BPD (66, 95). BPD is associated most with very low birth weight, antenatal infection, and need for sustained mechanical ventilation with oxygen-rich gas (96, 101).

The histopathology of contemporary BPD is characterized by alveolar simplification (10). Alveolar simplification is evident as distended distal air spaces that have few secondary septa, air space walls that are thick and cellular, and few capillaries that also are distant from the air spaces. A physiological consequence of reduced development of air space and capillary surface area is continued inadequate exchange of respiratory gases (hypoxemia and hypercarbia, with low blood pH).

**Animal Models as Tools To Identify Pathogenic Mechanisms Leading to BPD**

The first large-animal model of BPD used preterm baboons. That model, developed by Coalson and colleagues and first reported in the 1980s (46–49, 63), managed preterm baboons with invasive mechanical ventilation and oxygen-rich gas for days or weeks in the neonatal intensive care setting. Prolonged hyperoxia (100% inspired oxygen) was used in the early years of that unique model. Subsequent iterations of the preterm
baboon model limited oxygen exposure to maintain adequate arterial oxygenation (60–90 mmHg).

Our group’s first report using the preterm lamb model of BPD was 1997 (139). The preterm lamb model also limits oxygen exposure (<50% inspired oxygen) to maintain adequate arterial oxygenation of 60–90 mmHg. An advantage of preterm lambs is their larger size (~3 kg at 131-day gestation) compared with preterm baboons (~1/10th the birth weight). Larger size makes preterm lambs more amenable to frequent blood sampling across weeks of neonatal intensive care support. Another advantage related to the larger size of preterm lambs is a large caliber of blood vessels and lymphatics for chronic catheters. We use the blood vessel catheters for continuous measurements of pulmonary and systemic hemodynamic parameters and the lymphatic catheter for continuous assessment of lung liquid filtration (20). A third size advantage of preterm lambs is that their organs are larger, which is valuable for postmortem sampling.

Two notable differences remain in the physiological conditions between chronically ventilated preterm lambs with evolving neonatal chronic lung disease (CLD) and chronically ventilated preterm human infants who develop BPD. One difference is that fetal lambs are delivered prematurely by Cesarean section, without prior labor and without intentional intratruterine infection. Many preterm human infants who develop BPD were born after the onset of preterm labor, often complicated by intratruterine infection (59, 159, 160). Another difference, at least during the initial years using the preterm lamb model, is that the ductus arteriosus was surgically ligated within a few days of preterm birth (6, 20, 139). The protocol was subsequently changed to let the ductus close spontaneously (5, 135, 150). Rationale for eliminating ductal ligation was to minimize surgical trauma to the lung and postoperative edema, inflammation, infection, and atelectasis. Pharmacological closure, using indomethacin (42) or ibuprofen (122), has not been used in this model. Our experience is that the ductus is physiologically closed during the first days of life.

The obvious phylogenetic advantage of baboons is their closeness to humans compared with sheep (and rats and mice). Another advantage of preterm baboons is that they are more immature at the time of operative delivery (~134–147 days of gestation; 65–75% of gestation) than preterm lambs (125–131 days of gestation; 80–85% gestation). However, the lungs of both species are within the canalicular or saccular stage of lung development, depending on gestation age at the time of operative delivery. Both gestational ranges are at the lower limit of viability of preterm baboons and preterm lambs in the neonatal intensive care setting. The preterm baboon program ended a decade ago, leaving the preterm lamb model as the only large-animal model of BPD.

The uniqueness of both large-animal models is that they reproduce the clinical setting of preterm birth and respiratory failure requiring prolonged invasive mechanical ventilation with oxygen-rich gas for days or weeks. Both models also recapitulate the pathophysiology and pathology of evolving neonatal CLD in preterm baboons (46, 49, 50, 58, 63, 124, 199) and preterm lambs (5, 6, 20, 24, 25, 111, 139, 150). We use the term “evolving neonatal CLD” for the preterm baboon and preterm lamb models and reserve the term “BPD” for preterm human infants, to avoid confusion between large-animal models vs. the human disease.

The preterm baboon and preterm lamb models are challenging because they require intensive and lengthy life-support care 24 h per day for days or weeks. The arduous nature of these physiological studies is costly. Also, sample size is small (typically 4–6/group), in part because of cost and in part to comply with necessary guidelines for care and use of animals for biomedical research (to reduce, refine, and replace). Of these three Rs, replace is not an option because the uniqueness of both large-animal models is they recapitulate the neonatal intensive care setting because BPD occurs in preterm human infants who develop respiratory failure that requires prolonged invasive mechanical ventilation with oxygen-rich gas for days, weeks, or months. Counterbalancing the guideline to reduce the number of animals used is the expectation that study design be sufficiently powered to detect statistical differences between groups. The sample size and counterbalance to detect statistical differences also apply to models that use rats or mice.

Pathophysiology and Histopathology of Respiratory Failure

A worrisome outcome among former preterm human infants is that carbon monoxide diffusing capacity is lower than across the lung of control, healthy infants who are born at term gestation (14, 19, 132, 133). Why is diffusing capacity worse? Clues come from understanding the normal process by which the developing walls of the premature lung undergo morphogenesis to create a thin barrier across which oxygen and carbon dioxide must rapidly diffuse postnatally. Alveolar walls of the human adult lung are thin (~1.0 μm) (4, 54, 193). Thinness facilitates rapid diffusion of oxygen and carbon dioxide, in part because water is the principal impediment to molecular diffusion, particularly oxygen diffusion (4). The structural elements of the adult alveolar wall are the alveolar epithelium, the interstitium that makes up the alveolar wall, and the alveolar endothelium.

Developing air space walls at the time of preterm birth are thick and cellular, and blood vessels are distant from the air space lining epithelial cells (7, 33, 43, 45). Thickness, cellularity, and blood vessel distance reflect the developmental stage at which preterm birth occurs. All three structural features are greater when preterm birth occurs during canalicular stage than during saccular stage. Likewise, primary or secondary septation is dependent on the developmental stage at which preterm birth occurs. Primary septation is necessary for subdivision of the air capillaries into air sacules. Secondary septation subdivides the air sacules into anatomic alveoli. If preterm birth occurs during the canalicular stage, and prolonged invasive mechanical ventilation is required, then primary septation is disrupted. If preterm birth happens during the saccular stage, secondary septation is disrupted by prolonged invasive mechanical ventilation. Without sequential subdivision, air space surface area is inadequate for gas exchange to support postnatal life. Presence of distal air space walls that are thick and cellular, and blood vessels that are distant from the air space-lining epithelial cells are characteristic histopathological features of alveolar simplification in preterm human infants who die with BPD (45, 90), as well as preterm baboons and preterm lambs (Fig. 1) that have evolving neonatal CLD.

Another disrupted process is capillary growth. Normally during lung development during the second half of gestation, the number and surface area of blood vessels increases in the
thick, cellular mesenchymal walls that separate air canals and the subsequent air saccules (31, 163). Accompanying this growth is extension of capillaries into the mesenchymal core of secondary septa as the secondary septa sprout from the saccular walls. Preterm birth and prolonged invasive mechanical ventilation with oxygen-rich gas reduce capillary growth in the lung of preterm lambs (5, 6, 20) and preterm baboons (114). The lungs of the preterm lambs also have stunted secondary septa that lack capillaries (5). Without capillaries in the developing air space walls and stunted secondary septa, the simplified parenchyma cannot meet the need for efficient exchange of respiratory gases. This deficiency of capillary vascular surface area is compounded by fewer pre- and postcapillary arterioles and venules (6, 20). The breadth of diminished growth of pulmonary capillaries and microvessels contributes to the respiratory failure that necessitates continued invasive mechanical ventilation with oxygen-rich gas (6, 135).

A third histopathological characteristic of alveolar simplification is disrupted expression and accumulation of elastin. Appropriate expression of elastin and accumulation of elastic fibers are necessary for normal alveolar secondary septal formation, as demonstrated by two studies that used knockout mice constructs (108, 195). One study used mice that lacked platelet-derived growth factor-A (108). The lungs of these mice lack both myofibroblasts and elastin. The mice reach term gestation; however, they develop respiratory failure in the initial postnatal days of life. Their lungs have alveolar simplification. The other study used elastin knockout mice (195). The pups survive through the initial postnatal days of life but they are cyanotic. Their lungs have distal air spaces that are arrested at the saccular stage of lung development. Coincidently, their lungs also have fewer vascular and airway generations. The latter coincident histopathological feature suggests that elastin synthesis and deposition also regulate vascular and airway growth in the immature lung.

The lung of preterm lambs with evolving neonatal CLD has aberrant and continuously excessive expression of elastin and accumulation of elastic fibers (139). Upregulated expression of elastin mRNA occurs swiftly, by the third day of invasive mechanical ventilation. Excess and aberrant accumulation occurs in the walls lining the saccules and simplified alveoli (6, 139). Particularly conspicuous is accumulation of disorganized bundles of elastic fibers at the sites of stunted secondary septa. Expression and accumulation of elastin persists in established neonatal CLD 3–4 wk later. Persistence suggests that dysregulation of elastin gene expression and elastic fiber assembly is driven by invasive mechanical ventilation. The mechanisms driving this outcome are not known.

Less invasive ventilation leads to better lung outcomes. Encouraging observations are emerging when less invasive modes of ventilation are used, such as nasal continuous positive airway pressure (nasal CPAP). Encouragement stems from association between less invasive modes of ventilation with lower incidence and/or severity of BPD in some preterm human infants (189). Evidence from preterm baboon and preterm lamb studies is consistent because less invasive modes...
of ventilation lead to less inflammation (91), more production of surfactant (131), and better alveolarization and capillary growth compared with invasive mechanical ventilation (91, 135, 150, 182).

Two recent physiological reports by our group shows that less invasive high-frequency nasal ventilation (HFNV) in spontaneously breathing preterm lambs is associated with appropriate respiratory gas exchange for 3 days or 21 days (135, 150). We use a high-frequency, flow-interruption ventilator that also provides background conventional breaths (model VDR4, Percussionaire, Sand Point, ID). This ventilator is flow regulated and time cycled and delivers high-frequency, subphysiological tidal volumes in the background of which are controlled-pressure, low-frequency breathing cycles (conventional breaths) (110). We found that significantly lower applied oxygen and lower respiratory system pressures are required to maintain physiologically targeted values of arterial partial pressure of oxygen (60–90 mmHg) and carbon dioxide (45–55 mmHg) compared with invasive mechanical ventilation.

Another physiological result in our most recent report is that intratracheal pressure is very low during less invasive HFNV (135). We measured intratracheal pressure in situ while the preterm lambs breathed spontaneously. We found that mean intratracheal pressure is ~1/10th that during invasive mechanical ventilation. Our in situ measurements show that less invasive HFNV maintains positive mean intratracheal pressure of 1–2 cmH2O, which also means that positive end-expiratory pressure is low.

An important morphological result is that alveoli form in the lung of preterm lambs that are supported by less invasive HFNV (Fig. 1). The distal air space walls are thin and have few mesenchymal cells within them, and secondary septa are plentiful and thin and have capillaries. Each of these structural features of alveolarization is missing from the simplified architecture in the lung of preterm lambs that are supported by invasive mechanical ventilation (Fig. 1).

Why did we use a ventilator that provides pressure-limited, time-cycled conventional breaths at low frequency to deliver physiological tidal volume in the background behind high-frequency, small percussive pressures that deliver subphysiological tidal volumes (22)? Because bubble nasal CPAP did not sustain acceptable respiratory gas exchange longer than 3–4 h of postnatal life despite treating the preterm lambs antenatally with corticosteroids to promote lung development, perinatally with exogenous surfactant to increase lung compliance, and postnatally with caffeine citrate to stimulate breathing. Instead, they gradually accumulated carbon dioxide and their pH progressively decreased to unphysiological levels.

An important advantage of using less invasive HFNV for our studies is that this noninvasive mode of gentle ventilation provides positive gold standards for physiological, morphological, and molecular outcomes in the lung. Prior to using less invasive HFNV, we relied exclusively on reference lambs for normal development. The value of reference lambs is that they provide context for the stage of lung development at the time of delivering fetal lambs for preterm studies (same postconceptional age) and at the time when the preterm lamb studies end (same postconceptional age). Other reference groups provide context for postnatal age at 3 days or 21 days of life (same postnatal age), with or without invasive mechanical ventilation (25). The purpose of the latter reference groups is to differentiate the impact of invasive mechanical ventilation on the preterm lung vs. the term lung. However, none of these reference groups meet the clinical setting of preterm delivery, respiratory failure, and invasive mechanical ventilation with oxygen-rich gas. Creating the less invasive HFNV mode meets the clinical setting, using preterm lambs, with the distinction being that the outcomes are good (135, 150).

Having two ventilation modes that provide either bad outcome (invasive mechanical ventilation) vs. good outcome (less invasive HFNV) in the lung is proving to be important for identifying mechanisms that contribute to the pathogenesis of neonatal CLD. For normal alveolarization, the thick and cellular walls of the distal air spaces need to thin, in part by reducing the number of mesenchymal cells in the distal air space walls. Invasive mechanical ventilation sustains the thick and cellular mesenchymal walls of the distal air spaces (Fig. 1), in part by decreasing apoptosis and increasing proliferation of mesenchymal cells in the distal air space walls (135, 150). That is, invasive mechanical ventilation shifts the balance in favor of persistence and proliferation of mesenchymal cells, leading to persistently thick and cellular distal air space walls (135, 150). Less invasive HFNV, by comparison, leads to thin and less cellular distal air space walls (Fig. 1). These structural benefits coincide with shift toward more apoptosis, and less proliferation, of mesenchymal cells in the distal air space walls. These results may provide an explanation for why sustained inflation at birth does not protect preterm lambs from lung injury (81).

The two ventilation modes also differentially impact growth of capillaries in the lung. For normal alveolarization, capillaries grow along the thinning distal air space walls and into the sprouting secondary septa. Invasive mechanical ventilation decreases both processes (135, 150). The downregulated molecular players are vascular endothelial growth factor (VEGF), its functional receptor 2 in the lung (VEGF-R2), and midkine (150). Similar molecular downregulation occurs in the lung of preterm baboons that are supported by invasive mechanical ventilation (114). In this regard, the two physiological models are consistent. Less invasive HFNV of preterm lambs, by comparison, preserves these vascular growth factors in the thinning distal air space walls and sprouting secondary septa. Physiologically, our studies also show that less invasive HFNV is accompanied by acceptable respiratory gas exchange across the developing lung.

We conclude from these recent studies that less invasive HFNV for 3 days (150) or 21 days (135) provides acceptable respiratory gas exchange accompanied by appropriate alveolarization. Neither of these good outcomes is associated with invasive mechanical ventilation. Whether these results are translatable will require using the same type of ventilator to support preterm human infants.

Vitamin A therapy improves alveolarization in chronically ventilated preterm lambs. A treatment approach that has potential to improve outcomes among some chronically ventilated preterm human infants is vitamin A supplementation. The rationale for supplementation is that preterm human infants are deficient in retinol (29, 85, 166, 167). However, inconsistent results have been obtained from large multicenter studies, with some studies showing that vitamin A supplementation reduces the need for oxygen at 36 wk postmenstrual age in subgroups of preterm human infants at risk of BPD, whereas other studies...
have not shown benefit (11, 186). Whether vitamin A supplementation affected alveolarization in the lung of the treated preterm human infants is not known because autopsy studies have not been reported.

We tested the hypothesis that vitamin A supplementation promotes alveolarization in our preterm lamb model of neonatal CLD. Supplemental vitamin A (Aquasol; 5,000 U·kg\(^{-1}\)·day\(^{-1}\)) given to mechanically ventilated preterm lambs for 21 days increased plasma retinol level compared with preterm lambs supported by invasive mechanical ventilation alone. Daily vitamin A supplementation improved alveolarization and alveolar capillary growth (Fig. 2) (5). Vitamin A supplementation also increased mRNA and protein levels of VEGF, its functional receptor VEGF-R2, and midkine in the same lungs. Vitamin A supplementation reduced elastin mRNA expression and elastic fiber accumulation in the developing lung parenchyma. Accompanying these beneficial structural and molecular outcomes was a trend for better respiratory gas exchange. These results are different from studies in fetal lambs that were treated with all-trans retinoic acid 3 h before intra-amniotic injection of endotoxin (10). Rationale for prior treating with all-trans retinoic acid is that chorioamnionitis reduces retinoic acid concentration. However, all-trans retinoic acid did not prevent inflammation-induced alveolar simplification. This outcome may contribute to lack of benefit of vitamin A supplementation to preterm human infants.

Supplemental vitamin A (Aquasol; 5,000 U·kg\(^{-1}\)·day\(^{-1}\)) given to mechanically ventilated preterm baboons for 2 wk did not lead to alveolarization or alveolar capillary growth, or expression of vascular growth factors (140). Nor did vitamin A treatment reduce the excessive, aberrant expression of elastin and accumulation of elastic fibers in the lung of the preterm baboons. A potential explanation for these negative outcomes is that the treatment period was short: 2 wk. Perhaps the treatment period was insufficient for downstream signaling and subsequent impact on alveolar secondary septation, which also may account for lack of improved respiratory gas exchange in the chronically ventilated preterm baboons.

Pathophysiology and Pathology of Pulmonary Hypertension

Pulmonary hypertension with associated right heart failure is a frequent complication of BPD in preterm human infants (2, 16, 35). A recent clinical study showed that ~18% of 145 preterm human infants at 26 wk gestational age had pulmonary hypertension at any time during their hospitalization (17). Such preterm human infants have recurring episodes of cyanosis that are attributed to acute constriction of pulmonary arteries, leading to hypoxemia and bradycardia. The molecular basis of pulmonary hypertension remains incompletely understood, which may limit development of novel therapies to minimize...
the severity or duration of pulmonary hypertension in chronically ventilated preterm human infants.

We used our preterm lamb model to investigate the pathophysiological, histopathological, and molecular basis of pulmonary hypertension during the evolution of neonatal CLD. We ventilated the preterm lambs with either slow, deep inflations (20 breaths/min; ~15 ml/kg tidal volume) or fast, shallow inflations (60 breaths/min; ~5 ml/kg tidal volume) for 3–4 wk (20). The purpose of using two tidal volumes was to identify the impact of lung overdistension on outcomes. However, for the purpose of this paper, results are reported for the 60 breaths/min (~5 ml/kg tidal volume) group because this level of targeted lung volume support is more consistent with clinical management of invasively ventilated preterm human infants. None of these preterm lambs received antenatal steroids. All of them received surfactant at birth. They subsequently had surgical ligation of their ductus arteriosus, as well as placement of vascular catheters and thermistor wire in the pulmonary artery to serially calculate pulmonary vascular resistance. Both groups of preterm lambs developed neonatal CLD based on oxygenation and ventilation parameters, serial chest radiographs, and postmortem pathology. Pathophysiological results using this study design are summarized in the next four subsections.

Evolving neonatal CLD leads to pulmonary hypertension and vascular pathology. Normally, pulmonary vascular resistance decreases during the first postnatal month of life after birth at term gestation (2, 16). In preterm infants who develop BPD, however, a frequent occurrence is development of pulmonary hypertension (15). Insights into the pathogenesis of pulmonary hypertension are provided by our preterm lamb model of evolving neonatal CLD.

Pulmonary vascular resistance decreased postnatally in normal reference lambs that were born at term gestation and lived for 3 wk (Fig. 3). By comparison, pulmonary vascular resistance did not decrease from the first to the last (third) week of invasive mechanical ventilation (Fig. 3) (20). Accompanying development of pulmonary hypertension is persistent muscularization of pulmonary arterioles adjacent to terminal bronchioles (Fig. 3). We used terminal bronchioles as the independent structural landmark to identify the same generation of pulmonary arterioles, regardless of their diameter. Persistence was revealed by comparison with unventilated fetal and postnatal reference lambs, which provided normal developmental perspective (6, 20, 139). This comparative quantitative histological analysis revealed that greater thickness of the smooth muscle wall of pulmonary arterioles at the end of 3 wk of invasive mechanical ventilation is equivalent to that of reference fetal lambs that were matched for the gestational age when the preterm lambs were delivered. We also showed that the number of lung microvessels (Fig. 4) and surface density of capillaries (refer to Fig. 2) are significantly smaller compared with lungs of reference term lambs (20). Fewer pulmonary microvessels, including capillaries, could contribute to pulmonary hypertension through reduced total vascular surface area in the lung. These structural results are important because they demonstrate that prolonged invasive mechanical ventilation of preterm lambs decreases development of the pulmonary microcirculation, as occurs in the lung of preterm human infants who died with BPD (28, 39, 44, 82, 84, 115, 176, 183, 188).

Evolving neonatal CLD leads to edema and pulmonary hypertension. The pathophysiological and histopathological results in chronically ventilated preterm lambs led us to ask several pathophysiological questions. The first question is whether increased pulmonary vascular resistance led to pulmonary edema.

Postmortem extravascular lung water was significantly greater in chronically ventilated preterm lambs than in reference lambs that were born at term gestation (henceforth called reference term lambs) (Fig. 5) (20). Therefore, pulmonary edema formed in the lungs of chronically ventilated preterm lambs. Pulmonary edema was evident as cuffs of interstitial pulmonary edema liquid surrounding pulmonary blood vessels and airways, as well as within distended interlobular connective tissue septa. Lung lymphatics in both interstitial compartments were dilated. Whereas air space edema liquid was unusual in regions of hyperinflated distal air spaces, air space edema was present in regions that contained collapsed air spaces. The features were not present in the lung of reference term lambs. These results led us to ask whether the pulmonary edema is caused by increased hydrostatic pressure or increased permeability.
Evolving neonatal CLD leads to increased hydrostatic pulmonary edema. A feature of acute lung injury, as occurs in RDS, is pulmonary edema (2, 16, 22, 82). The pulmonary edema liquid is protein rich in both the lung interstitium and air spaces (23, 37, 89). Whether neonatal CLD is associated with protein-rich edema liquid was not known at the time that we used lung lymph to investigate the pathogenesis of pulmonary edema during the evolution of neonatal CLD in chronically ventilated preterm lambs.

We addressed the uncertainty by cannulating the efferent duct of the caudal mediastinal lymph node to collect lung lymph (20). The unique lymphatic anatomy of sheep (9, 161, 174, 175) allows continuous physiological collection of lung lymph, which is used to differentiate increased hydrostatic pulmonary edema from increased permeability pulmonary edema (30, 173). Briefly, we measured lung lymph and plasma protein concentrations to calculate the lymph-to-plasma (L/P) ratio and lymph protein clearance. Decreasing or low L/P ratio is consistent with increased hydrostatic pulmonary edema. Increasing or constant L/P ratio, by comparison, is consistent with increased permeability pulmonary edema (173). We found that the L/P ratio decreased over the 3 wk of invasive mechanical ventilation of preterm lambs, although the difference from week 1 to week 3 was not statistically significant. Lymph protein clearance did not change over the same time period, owing to increased lymph flow. Thus these data are consistent with increased hydrostatic pulmonary edema in chronically ventilated preterm lambs (173).

These physiological results are unique between the two preterm large-animal models of neonatal CLD because comparable physiological measurements of cardiovascular and pulmonary lymphatic function were not made in preterm baboons. Our physiological results are informative because they demonstrate that prolonged invasive mechanical ventilation leads to pulmonary edema from increased hydrostatic pressure. Therefore, the preterm lamb model filled two important gaps in knowledge about pulmonary vascular and lymphatic function during the evolution of neonatal CLD.

Evolving neonatal CLD dysregulates the nitric oxide signaling pathway. What molecular factors might contribute to increased pulmonary vascular resistance, persistent muscularization of pulmonary arterioles, smaller surface area of pulmonary microvessels and capillaries, and increased hydrostatic pulmonary edema in chronically ventilated preterm lambs? A pathway of interest at the time was dysregulation of the nitric oxide (NO) signaling pathway.

Several lines of evidence led us to test the NO signaling pathway. One line of evidence is that NO is important for regulating smooth muscle tone in pulmonary blood vessels and airways of newborn animals. NO diffusion into the cytoplasm of smooth muscle cells increases the concentration of guanosine 3',5'-cyclic monophosphate (cGMP), which induces smooth muscle relaxation, and therefore leads to vasodilation.

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**Fig. 4.** Extra-alveolar microvessel number (A) and capillary surface density (B) increased in term reference lambs that were not ventilated (means ± SD; n = 5/group). Extra-alveolar microvessel number and capillary surface density were significantly lower in preterm lambs that were supported by invasive mechanical ventilation (IMV) for 3 wk compared with both newborn references (*P < 0.05). Adapted from Ref. 20.

**Fig. 5.** Extravascular lung water, an indicator of pulmonary edema, is significantly greater in the lung of preterm lambs supported by invasive mechanical ventilation (IMV) compared with both groups of newborn references (*P < 0.05; means ± SD; n = 6/group). Data from Ref. 20.
(26). Diffusion of physiological doses (5–15 parts/million, ppm) of inhaled NO (iNO) into blood does not lead to systemic hypotension because NO is inactivated by tight binding to hemoglobin (73, 136, 152). The role of NO on airway smooth muscle tone will be discussed separately, after this discussion on pulmonary blood vessels.

Another line of evidence is that inhibition of NO production disrupts vascular smooth muscle tone in pulmonary blood vessels. Inhibition attenuates ~50% of the normal postnatal decrease of pulmonary vascular resistance in newborn lambs (1, 65) and newborn piglets (147). Conversely, iNO abruptly and profoundly decreases pulmonary vascular resistance, with associated increase in arterial oxygenation, in newborn animals and human infants with pulmonary hypertension (41, 52, 57, 68, 94, 109, 131a, 153–155, 157, 203, 204).

NO also acts as a signaling molecule to inhibit growth of vascular muscle cells in vitro (69, 77, 181). In vivo studies provide supportive evidence for the latter action of NO. For example, inhibiting endogenous production of NO enhances smooth muscle growth in response to injury (162). By comparison, exogenously supplied NO, either by an NO donor or inhalation, inhibits smooth muscle growth after mechanical injury (76, 104, 164).

Clinical trials published before 2000, when our first physiological report on dysregulation of the pulmonary circulation in chronically ventilated preterm lambs was published, showed that iNO decreases pulmonary vascular resistance and improves arterial oxygenation in human newborn infants with persistent pulmonary hypertension (94, 131a, 155, 157). Other clinical trials showed that low-dose iNO given to human newborn infants with persistent pulmonary hypertension reduces the need for extracorporeal membrane oxygenation (41, 56). Likewise, experimental animal studies showed that iNO reverses pulmonary vasoconstriction following either hypoxia or prenatal closure of the ductus arteriosus (154, 203). The systemic circulation was not dilated in those studies.

Based on the aforementioned roles of NO in regulation of pulmonary vascular tone, reactivity, and smooth muscle growth, we performed three physiological studies. We 1) measured nitric oxide synthases (NOSs) and soluble guanylate cyclase (sGC) in the lung of chronically ventilated preterm lambs, 2) provided iNO for 1 h at weekly intervals to chronically ventilated preterm lambs, and 3) gave iNO continuously for 3 wk to chronically ventilated preterm lambs.

For the first assessment, we quantified protein abundance of endothelial nitric oxide synthase (eNOS), inducible NO synthase (iNOS), and sGC (24, 111). We used quantitative immunoblot analysis of third to fifth generation intrapulmonary arterioles and semiquantitative immunohistochemistry of pulmonary arterioles adjacent to terminal bronchioles. Our results showed less eNOS protein in endothelial cells of intrapulmonary arteries (Fig. 6) and less immunolocalization of eNOS in pulmonary arterioles next to terminal bronchioles in the lung of the chronically ventilated preterm lambs compared with reference term lambs at 1 day of age (same postconceptional age) (24, 111). For iNOS, neither protein abundance nor localization was different compared with the reference term lambs (24, 111). We used the same analytical methods to determine abundance and localization of sGC. The latter results showed that sGC protein abundance is less in the lung of chronically ventilated preterm lambs than in reference term lambs (Fig. 6) (24, 111). Together, these results show that the normal developmental pattern of pulmonary expression of eNOS and sGC is perturbed by prolonged invasive mechanical ventilation. This perturbation also may contribute to the increased pulmonary vascular resistance that occurs in chronically ventilated preterm lambs.

Our second assessment used preterm lambs that had implanted catheters for measurement of pulmonary vascular pressure and blood flow to calculate pulmonary vascular resistance. During weeks 2 and 3 of invasive mechanical ventilation, we treated the preterm lambs with 5–15 parts/million (ppm) of
iNO for 1 h. At 1 to 2 wk of continuous invasive mechanical ventilation, iNO for 1 h decreased pulmonary vascular resistance by ~20% (Fig. 7). However, when we repeated the same treatment at the end of week 3 of continuous mechanical ventilation, iNO did not decrease pulmonary vascular resistance (Fig. 7). These results could be explained, however, by dysfunction of pulmonary vascular smooth muscle cells. That the pulmonary vascular smooth cells retained capacity to relax was proven by infusing 8-bromo-guanosine 3',5'-cyclic monophosphate (8-bromo-cGMP; 150 μg·kg⁻¹·min⁻¹, intravenously) for 30 min in four of the same preterm lambs at the end of week 3 of mechanical ventilation (Table 2). Pulmonary vascular resistance consistently decreased by ~30%. Besides being unique physiological results among the preterm models of neonatal CLD, these results may provide a basis for developing novel therapeutic or preventive interventions for pulmonary hypertension in chronically ventilated preterm human infants. For example, analogs of cGMP may merit investigation as potentially useful therapeutic tools in conditions where lung injury is associated with pulmonary hypertension. Further investigation would be based on our physiological results indicating that prolonged invasive mechanical ventilation of preterm lambs does not disrupt the normal apparatus for relaxation of pulmonary vascular smooth muscle, whereby cGMP activates cGMP-dependent protein kinases (38).

Our third assessment tested whether continuous low-dose iNO from birth reduces pulmonary vascular resistance and attenuates persistent muscularization of pulmonary arterial vessels in preterm lambs with evolving neonatal CLD. Rationale for this assessment stemmed at the time from studies that used chronically hypoxic rats with pulmonary hypertension (99, 156). Continuous iNO reduced the anticipated pulmonary vascular remodeling that is associated with chronic hypoxia in rats. We hypothesized that continuous iNO starting at preterm birth would inhibit pulmonary vascular smooth muscle growth and thereby reduce pulmonary vascular resistance. To test this hypothesis, we mechanically ventilated eight preterm lambs for 3 wk, four of them with continuous iNO at 5–15 ppm, beginning at preterm birth. Contrary to our hypothesis, pulmonary vascular resistance was not significantly different between iNO-treated and untreated preterm lambs after 3 wk of continuous iNO administration during mechanical ventilation (Fig. 8). Nor did vascular smooth muscle thickness of pulmonary arterioles decrease (Fig. 8).

Another surprise is the observation that eNOS protein abundance in pulmonary endothelial cells did not decrease over time. The latter result surprised us because diminished eNOS is sometimes used to explain the apparent elevation of pulmonary vascular resistance that frequently occurs when iNO therapy is discontinued. On the other hand, our surprising finding is consistent with the results of in vitro studies, which showed that NO donors increase NOS expression in cultured pulmonary artery endothelial cells derived from fetal sheep (202).

An overall conclusion from the three physiological studies is that prolonged invasive mechanical ventilation disrupts the NO signaling pathway. Among the consequences of prolonged invasive mechanical ventilation is that it decreases the normal developmental pattern of pulmonary vascular abundance of eNOS and sGC. Accompanying the decreases is persistent muscularization of pulmonary arterial vessels. We suggest that these alterations may contribute to attenuated or absent lung vascular response to continuous iNO in chronically ventilated preterm human infants.

Pathophysiology and Pathology of Excess Airway Expiratory Resistance

Preterm human infants with BPD have increased inspiratory and expiratory airway resistance, low specific compliance, and low functional residual capacity (72, 130). The mechanisms leading to reduced lung mechanics remain incompletely understood. Our initial studies using preterm lambs that we mechanically ventilated for 3–4 wk revealed that airway resistance is greater than in reference term lambs. Associated with this pathophysiological outcome is greater muscularization of airways and lower abundance of eNOS protein in airway epithelium (111). Protein abundance of sGC also is lower (21). One study at that time showed that all three isoforms of NO synthase (eNOS, iNOS, and nNOS) are expressed in airway epithelial cells of both sheep and baboons during normal fetal development (165, 168). Another contemporary study showed that eNOS protein abundance and total NOS activity are reduced in the lungs of preterm baboons after 2 wk of invasive mechanical ventilation (3). Reduced NOS activity could contribute to the increased airway resistance and...
informative because they reveal that continuous iNO during 3 wk of invasive mechanical ventilation prevents increased airway resistance, muscularization of airways, and loss of airway epithelial cell abundance of eNOS protein compared with the adjacent pulmonary arterial vessels.

Our results for airways are supported by results of other studies. One study showed that iNO decreased airway resistance in anesthetized newborn piglets (147). Another study showed that iNO decreased airway resistance in anesthetized adult guinea pigs during induced bronchoconstriction (61). A cellular consequence of NO is that it reduces proliferation of airway smooth muscle cells in vitro (77, 181). In aggregate, these results indicate that NO positively influences airway smooth muscle tone and abundance during postnatal development, as well as in adults.

An overall conclusion is that prolonged invasive mechanical ventilation also disrupts the NO signaling pathway in airways.

abundance of airway smooth muscle, as shown in vitro (69, 77, 181, 197) and in vivo in newborn rats and piglets (120, 147).

When we began our study of the potential beneficial role of continuous iNO therapy in our preterm lamb model of evolving neonatal CLD, we anticipated that the impact of iNO would be greatest on the pulmonary vasculature. To our surprise, continuous iNO had greater impact on the structure and function of the airways. Airway resistance was significantly (~40%) lower in the preterm lambs that received continuous iNO, even at 3 wk of mechanical ventilation (Fig. 9). Postmortem histopathology showed significantly (~50%) less smooth muscle in the wall of distal airways in the preterm lambs that we treated with iNO compared with reference term lambs (Fig. 9). Immunoblot analysis for eNOS and sGC proteins in airways taken from iNO-treated preterm lambs showed comparable abundance to that in airways of reference term lambs. These results are

Fig. 8. Preterm lambs were given inhaled nitric oxide (5–15 ppm) continuously for 3 wk. At the end of 3 wk of continuous inhalation of nitric oxide (iNO), pulmonary vascular resistance (A) and pulmonary arteriole smooth muscle area (B) remained the same as the respective values for preterm lambs that did not inhale nitric oxide (NO iNO) (means ± SD; n = 5/group). Adapted from Ref. 21 with permission of the American Thoracic Society.

Fig. 9. Preterm lambs were given inhaled nitric oxide (5–15 ppm) continuously for 3 wk. At the end of 3 wk of continuous inhalation of nitric oxide (iNO), respiratory tract resistance (A) and terminal bronchiole smooth muscle area (B) were significantly lower than the respective values for preterm lambs that did not inhale nitric oxide (NO iNO) (*P < 0.05; means ± SD; n = 5/group). Adapted from Ref. 21 with permission of the American Thoracic Society.
However, unlike the neighboring pulmonary arteries in the same lungs, continuous iNO improves airway development. Perhaps the lack of effect on the neighboring pulmonary arteries is due to diffusion distance from the lumen of airways, where iNO concentration is high, to pulmonary arterial endothelial and smooth muscle cells, where the iNO concentration would be lower or nil. Nonetheless, the positive influence of continuous iNO on airway development is encouraging because of the potential to improve peripheral distribution of ventilation and reduce airway hyperreactivity in chronically ventilated preterm human infants.

Continuous iNO therapy improves alveolarization in chronically ventilated preterm lambs. Returning to our study that provided continuous iNO for 3 wk, another surprising outcome is improved alveolarization (Fig. 10) (21). We found that radial alveolar count and secondary septal volume density are greater, whereas distal air space walls are thinner, than in the lung of untreated, mechanically ventilated preterm lambs (Fig. 10). Our structural results are consistent with results of studies using rats and mice that had various insults (13, 62, 78, 102, 107, 179, 200).

A potential mechanism for improved alveolarization may be via the role of NO on expression of vascular growth factor signaling molecules. For example, NO induces synthesis of VEGF in vascular smooth muscle cells (60). Relevance of the signaling cascade from NO to VEGF in neonatal CLD is that expression of VEGF and its functional receptor VEGF-R2 are diminished in the lung of preterm human infants who died of BPD (18) and in the preterm baboon model of this disease (114). Both VEGF and VEGF-R2 are important regulators of alveolarization through their impact on capillary formation (87, 103). Therefore, a possibility is that delivery of iNO leads to downstream signaling of VEGF and VEGF-R2 in the preterm lung during prolonged invasive mechanical ventilation. This possibility is consistent with our observation that continuous iNO therapy preserves pulmonary vascular eNOS protein abundance in chronically ventilated preterm lambs (21).

Different outcome followed treating chronically ventilated preterm baboons with continuous iNO (123). The lung of the treated preterm baboons did not have structural evidence of alveolarization. Why might the experiment using preterm baboons have different outcome compared with preterm lambs? One reason may be that the preterm baboons received lower concentration of continuous iNO (5 ppm). Our preterm lambs received 5–15 ppm iNO continuously. Also, the preterm baboons were ventilated and treated for less time (2 wk). Our preterm lambs were ventilated and treated for 3–4 wk. We suggest that the lower concentration of iNO and its shorter duration of treatment may account for the lack of efficacy in chronically ventilated preterm baboons compared with chronically ventilated preterm lambs.

Mechanisms Leading to Long-Term Outcomes Among Survivors of Neonatal CLD

Persistent pulmonary morbidities such as pulmonary hypertension (2, 16, 17, 35), excess airway expiratory resistance and airway hyperreactivity (64, 72, 130), and lower carbon monoxide diffusing capacity (14, 19, 132, 133) later in life are important outcomes for survivors of preterm birth and BPD. Some mechanistic insights into long-term outcomes are provided by the preterm baboon model. The lungs of former preterm baboons that were mechanically ventilated for 14 days and recovered for 33 wk had persistently enlarged air spaces and less parenchymal tissue than control baboons (48). Thirty-three weeks of life for baboons is equivalent to ~2 yr of postnatal age for humans. Much remains to be learned, mechanistically, about persistent morbidities of the pulmonary vasculature and airways in survivors of BPD and ways to minimize the morbidities.

Summary

This review focused on unique insights provided by the preterm lamb physiological model of neonatal CLD. The preterm lamb model and the preterm baboon model recapitulate the clinical setting of preterm birth and respiratory failure
that require prolonged ventilation support with oxygen-rich gas. The impact of both models is that they provide mechanistic insights into the pathogenesis of neonatal CLD. A particular advantage of the preterm lamb model is the large size of preterm lambs, which facilitates pathophysiological studies during the evolution of neonatal CLD. To this pathophysiological advantage is linked an array of morphological, biochemical, and molecular analyses that together provide a bigger picture of the mechanisms leading to neonatal CLD. Our results indicate that the mode of ventilation is related to lung outcomes. Specifically, poor outcomes are the result from invasive mechanical ventilation, whereas better outcomes are the result of less invasive high-frequency nasal ventilation. Our integrative studies also show that daily supplementation with vitamin A during invasive mechanical ventilation partially improves lung outcomes. The new insights that are being gained may have important translational implications about the pathogenesis and treatment of BPD in preterm human infants.

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