Mechanism of Reduced Lung Injury by High-Frequency Nasal Ventilation in a Preterm Lamb Model of Neonatal Chronic Lung Disease

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ABSTRACT: The mechanism underlying the potentially beneficial effects of the “gentler” modes of ventilation on chronic lung disease (CLD) of the premature infant is not known. We have previously demonstrated that alveolar parathyroid hormone-related protein-peroxisome proliferator-activated receptor γ (PTHrP-PPAR γ) signaling is critically important in alveolar formation, and this signaling pathway is disrupted in hyperoxia- and/or volutrauma-induced neonatal rat lung injury. Whether the same paradigm is also applicable to CLD, resulting from prolonged intermittent mandatory ventilation (IMV), and whether differential effects of the mode of ventilation on the PTHrP-PPAR γ signaling pathway explain the potential benefits of the “gentler” modes of ventilation are not known. Using a well-established preterm lamb model of neonatal CLD, we tested the hypothesis that ventilatory support using high-frequency nasal ventilation (HFNV) promotes alveolar PTHrP-PPAR γ signaling, whereas IMV inhibits it. Preterm lambs managed by HFNV or IMV for 21 d following preterm delivery at 132-d gestation were studied by Western hybridization and immunofluorescence labeling for key markers of alveolar homeostasis and injury/repair. In lambs managed by IMV, the abundance of key homeostatic alveolar epithelial-mesenchymal markers was reduced, whereas it was significantly increased in the HFNV group, providing a potential molecular mechanism by which “gentler” modes of ventilation reduce neonatal CLD. (*Pediatr Res* 70: 462–466, 2011)

Despite all the advances, neonatal chronic lung disease (CLD) continues to be a major cause of morbidity and mortality in premature infants (1). Histopathologically, neonatal CLD is characterized by alveolar simplification (2). Molecular pathways that may be dysregulated by preterm birth, and that lead to CLD following prolonged mechanical ventilation with oxygen-rich gas, are not fully understood. Alveolar epithelial-mesenchymal interactions are driven by epithelially derived parathyroid hormone-related protein (PTHrP) and mesenchymally derived peroxisome proliferator-activated receptor γ (PPAR γ). Because PTHrP and PPAR γ are critical for alveolar development (3), we hypothesized that this pathway is disrupted in preterm neonates

that are managed by prolonged mechanical ventilation with oxygen-rich gas.

PTHrP, secreted by alveolar type II (ATII) cells, acts on its receptor on adjoining alveolar interstitial fibroblasts, which are characterized by the expression of the PTHrP receptor (PTHrP-R) and its down-stream target, PPAR γ (3–5). Alveolar interstitial fibroblasts, in turn, secrete leptin, which acts on its receptor on ATII cells (6). This paracrine loop enhances surfactant synthesis and is known to be critically important in maintaining alveolar homeostasis and normal lung development (7).

Using *in vitro* and *in vivo* rat models, we have previously shown that disruption of alveolar PTHrP-PPAR γ paracrine signaling is a central event in alveolar epithelial-mesenchymal interactions (3,8). Whether the same paradigm is relevant to alveolar simplification seen in neonatal CLD is not known. Therefore, we examined whether dysregulation of PTHrP-PPAR γ signaling is also seen in a well-established preterm lamb model of neonatal CLD (9,10).

“Gentler” modes of ventilation such as continuous positive airway pressure or high-frequency ventilation might reduce the occurrence of neonatal CLD (8,11,12). However, the pathways that may contribute to this potentially beneficial effect of the “gentler” modes of ventilation are not known. We also hypothesized that ventilator support using high-frequency nasal ventilation (HFNV) would promote homeostatic PTHrP-PPAR γ alveolar epithelial-mesenchymal signaling, whereas mechanical ventilation would inhibit it. Enhancing alveolar PTHrP-PPAR γ signaling with HFNV may result in less severe neonatal CLD. Therefore, we compared the key molecular markers of PTHrP-PPAR γ signaling in preterm lambs supported on either HFNV or intermittent mandatory ventilation (IMV).

Abbreviations: ATII, alveolar type II; CCT- α , choline phosphate cytidylyl-transferase- α ; CLD, chronic lung disease; FIO₂, fractional inspired oxygen; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HFNV, high-frequency nasal ventilation; IMV, intermittent mandatory ventilation; PPAR γ , peroxisome proliferator-activated receptor γ ; PTHrP, parathyroid hormone-related protein; PTHrP-R, parathyroid hormone-related protein receptor; SP-B, surfactant protein-B; SP-C, surfactant protein-C; α -SMA, α -smooth muscle actin

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METHODS AND MATERIALS

This study was approved by the Animal Care Committee of The University of Utah School of Medicine.

Animal model. Preterm lambs (~132 d gestation; term ~150 d) were delivered *via* cesarean section and divided into two groups: a group managed by HFNV and a group managed by IMV. The methods for the chronic ventilation model using preterm lambs have been reported (9,10,13–19). Pregnant ewes that carried single or twin fetuses at 130–132 d of gestation (term ~150 d gestation) were used. Pregnant ewes were given an intramuscular injection of dexamethasone phosphate (6 mg; Vedco, Inc., St. Joseph, MO), ~24 h before operative delivery. On the day of delivery, the ewes were given i.m. ketamine hydrochloride (10–20 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA), followed by inhalation anesthesia with 1% isoflurane (Abbott Laboratories, North Chicago, IL). We exposed each fetus by midline hysterotomy, placed catheters in the common carotid artery and external jugular vein, and intubated them with a cuffed endotracheal tube (3.5 to 4.0 French), through which 10 mL of lung liquid was aspirated and replaced with Survant (2.5 mL; NDC 0074-1040-08, Ross Products Division, Abbott Laboratories, Columbus, OH). For the fetuses that were assigned to HFNV (described below), an uncuffed oral/nasal true Murphy tube (3.0 to 4.0 mm ID; 13 cm length) was also inserted through a nostril to reach the middle of the length of the nasal cavity (5–6 cm of the ~10 cm long cavity). Lidocaine (1% solution; Hospira, Inc.) was injected s.c. along the nostril to minimize pain and discomfort. Fetuses were removed from the uterus, and their umbilical cords were tied and cut.

During the initial 2–3 h of resuscitation, all preterm lambs were managed by IMV (model 15215; Bird VIP ventilator, Palm Springs, CA) with warmed and humidified 100% oxygen. Initial ventilator settings were a respiratory rate of 60 breaths/min, inspiratory time of 0.30 s, and a positive end-expiratory pressure of 8 cm H₂O. Peak inspiratory pressure was adjusted to attain a target PaCO₂ between 45 and 60 mm Hg, and a pH between 7.25 and 7.35, and a fractional inspired oxygen (FiO₂) of 1.0. The target expiratory tidal volume, measured by the ventilator, was 5 to 7 mL/kg/breath. A second treatment with Survant (2.5 mL) was given at 5 to 10 min of life. The concentration of inspired O₂ was decreased to maintain the target Pao₂ between 60 and 80 mm Hg. Maintaining the oxygenation target required different FiO₂ between the groups. For the IMV group, the range for FiO₂ was 0.35–0.50, whereas in the HFNV group, the range for FiO₂ was 0.25–0.35 (data not shown; Ref. 9). To stimulate ventilatory drive, all lambs were treated i.v. with a loading dose of caffeine citrate within 30 min of delivery (15 mg/kg, given over 2 h; Mead Johnson & Company, Evansville, IN). Caffeine citrate was given once daily thereafter at a dose of 5 mg/Kg.

For the preterm lambs assigned to the HFNV group, weaning from IMV began at 2–3 h of age. When the lambs breathed spontaneously (3–4 h of age), the ventilatory circuit was changed to a high-frequency flow interrupter ventilator (Percussionaire Corp., Sandpoint, ID). The settings were 20–25 cm H₂O for amplitude, 8–12 cm H₂O for mean pressure, 5–7 cm H₂O for end-expiratory pressure, and 10 Hz for rate.

Each group was managed with the assigned ventilatory strategy for 21 d. Two preterm lambs were studied simultaneously. The order of ventilation mode was alternated so that consecutive preterm lambs were not managed by the same ventilation mode. Preterm lambs were kept prone in a veterinary sling mounted on a heated bed. Saline and dextrose solutions were administered i.v., as were antibiotics and sedatives (pentobarbital sodium; Abbott Laboratories; North Chicago, IL; and buprenorphine hydrochloride, 5 µg/kg every 3 h; Reckitt & Colman Pharmaceuticals, Richmond, VA). The preterm lambs assigned to IMV received 3–5 mg/kg of pentobarbital as needed to minimize discomfort associated with endotracheal intubation. This dosage prevented spontaneous breathing. The preterm lambs assigned to HFNV were given 1–2 mg/kg of pentobarbital so that they breathed spontaneously. Vascular pressures and heart rate were continuously recorded (V6400; SurgVet, Inc.; Waukesha, WI). Arterial blood gases and pH and plasma concentrations of glucose and electrolytes were measured hourly. Plasma concentrations of total protein and hematocrit were measured at 6-h intervals. An orogastric feeding tube was used for enteral feedings, using fresh colostrum from the ewes, beginning at ~4 h of life (3–5 mL/kg every 2 h). The feeding tube was withdrawn between feedings. The volume of colostrum was increased gradually by 5 mL increments, as tolerated, to attain a goal of 60 kcal/kg/d of total energy substrate. We monitored total fluid intake (saline, dextrose, and milk) and output (urine and stool), and made adjustments to maintain fluid homeostasis, as indicated by urine output (>1–2 mL/kg/h) and blood pressure (>45 mm Hg). Chest radiographs were taken daily to assess lung inflation volume and to identify atelectasis. None of the preterm lambs developed air leaks. Indices of infection were monitored by daily leukocyte total and differential cell counts and by core body temperature. None of the preterm lambs required treatment with pressors.

At the end of the 21-d study, the lambs were given heparin (1000 U, i.v.). The HFNV group was reintubated and managed for <1 min with the same ventilator settings during HFNV. All lambs were killed by overdosing them with pentobarbital (60 mg/kg pentobarbital sodium solution; i.v.; Ovation Pharmaceuticals, Inc., Deerfield, IL). The chest was opened, the trachea was ligated at end inspiration to minimize atelectasis, and the lungs and heart were removed as a block. The whole left lung was insufflated with 10% buffered neutral formalin to a static pressure of 25 cm H₂O. Fixed-lung displacement volume was measured by suspension in formalin before the lung was stored in fixative at 4°C for 24 h. Paraffin-embedded tissue blocks were prepared and cut for routine histologic observation (9,10). The right lung was cut into small pieces (~0.5 gm) that were frozen in liquid nitrogen and stored at –80°C. The frozen lung tissue was kept for analysis by Western hybridization and immunofluorescence labeling for key functional markers of alveolar homeostasis and injury/repair.

Western hybridization. Protein extraction and Western hybridization were performed using standard methods, as described by us previously (20). The specific markers analyzed by Western hybridization included surfactant protein (SP)-B, SP-C, choline-phosphate cytidylyltransferase- α (CCT- α), leptin receptor (leptin R), PTHrP, PTHrP-R, PPAR γ , α -smooth muscle actin (α -SMA), and calponin. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize the Western data as we have found it to be a nonvariable control marker in this (data not shown) and other studies of the developing lung (4,5,20–22).

Immunofluorescence. Immunofluorescence double labeling was performed for PPAR γ /Pro-SP-C, PPAR γ /vimentin, PTHrP-R/pro-SP-C, and PTHrP-R/vimentin, as described previously (9,10).

RESULTS

Initially, we profiled the ontogeny of functional markers for alveolar mesenchymal and epithelial cells at 132 d and 145 d gestation (term ~150 d) and at term (Fig. 1). The PTHrP-R and α -SMA are functional markers of lipofibroblasts and myofibroblasts, respectively. Developmentally, PTHrP-R abundance increased progressively between gestational d 132 and term (240% increase). α -SMA also increased during this time frame (40% increase). PTHrP, SP-B, and SP-C are functional markers of alveolar epithelial type II (ATII) cells. These functional markers also increased between gestational d 132 and term by 120, 15, and 100% for PTHrP, SP-B, and SP-C, respectively.

We then determined the effects of HFNV *versus* IMV on SP abundance by ATII cells (Fig. 2). SP-B abundance was significantly stimulated by HFNV (37%); IMV significantly decreased SP-B abundance compared with the HFNV (59%) group. SP-C abundance was significantly increased by HFNV (200%) compared with term and 40% compared with IMV. The beneficial effect of HFNV *versus* IMV was further corroborated by examining the abundance of the ATII cell functional marker CCT- α , the rate-limiting enzyme in surfactant phospholipid synthesis (Fig. 3). Choline phosphate cytidylyltransferase- α abundance was significantly enhanced by HFNV (40%) but was unaffected by IMV. Leptin R abundance, another functional marker of the ATII cell, was also significantly increased by HFNV (~60%), but was unaffected by IMV.

We next examined the effects of HFNV *versus* IMV on the levels of functional markers of alveolar interstitial lipofibroblasts (Fig. 4). PTHrP-R abundance was significantly increased by HFNV (77%), but not by IMV. PPAR γ abundance was significantly increased by HFNV (57%), but was also unaffected by IMV. As for functional markers of myofibroblasts, α -SMA was significantly inhibited by HFNV (30%), but was unaffected by IMV (Fig. 5). In contrast, calponin was

Figure 1. Developmental abundance profile for markers of alveolar homeostasis. The abundance of PTHrP-R and α -SMA, functional markers of lipofibroblasts and myofibroblasts, respectively, increased between gestational d 132 and term. Similarly, abundance of PTHrP, SP-B, and SP-C, functional markers of alveolar epithelial type II cells, also increased between gestational d 132 and term (T; $*p < 0.05$ vs F132 group; values are SEM; $n = 5$).

Figure 2. Effect of ventilatory strategy on epithelial differentiation markers SP-B and -C. SP-B abundance was significantly greater in the HFNV group, whereas it was significantly decreased in the IMV group, compared with the term (T) group. SP-C abundance was increased significantly by both HFNV and IMV, but more so by HFNV ($*p < 0.05$ vs term; $**p < 0.05$ vs HFNV; values are SEM; $n = 5$).

Figure 3. Effect of ventilatory strategy on epithelial differentiation markers choline phosphate cytidyltransferase- α (CCT- α) and leptin rec. Abundance of both CCT- α and leptin rec were increased significantly by HFNV, but were unaffected by IMV ($*p < 0.05$ vs term (T); values are SEM; $n = 5$).

unaffected by HFNV, but was significantly increased by IMV (20%).

Analysis of lung histologic sections by immunofluorescence localized PPAR γ , pro-SP-C, PTHrP, and PTHrP-R based on our Western analysis findings (Fig. 6). Lung tissue sections from the HFNV group showed obvious immunostaining for PPAR γ , pro-SP-C, and PTHrP-R, whereas there was less immunostaining for vimentin. However, lung tissue sections from the IMV group showed increased staining for vimentin, also increasing pro-SP-C staining, while having no effect on either PPAR γ or PTHrP-R staining.

Figure 4. Effect of ventilatory strategy on alveolar interstitial lipofibroblast differentiation markers PTHrP-R and PPAR γ . PTHrP-R abundance was significantly increased by both HFNV and IMV. PPAR γ abundance was significantly increased by HFNV, but was unaffected by IMV ($*p < 0.05$ vs term (T); values are SEM; $n = 5$).

Figure 5. Effect of ventilatory strategy on alveolar interstitial myofibroblast differentiation markers α -SMA and calponin. α -SMA was significantly inhibited by HFNV, but was unaffected by IMV. Calponin was unaffected by HFNV, but was significantly increased by IMV ($*p < 0.05$ vs term (T); values are SEM; $n = 5$).

DISCUSSION

The present data provide important molecular insights to the well-described differential effects of HFNV *versus* IMV on the risk of neonatal CLD in preterm newborns. On the one hand, HFNV promoted the coordinate expression of the epithelial (SP-B, SP-C, CCT- α , and leptin-R) and mesenchymal (PTHrP-R and PPAR γ) signaling components of the PTHrP-PPAR γ paracrine signaling pathway for alveolar homeostasis (3–8). On the other hand, IMV either inhibited such signaling or failed to stimulate this prohomeostatic mechanism during a critical period in alveolarization. The net result of these two

Figure 6. Validation of the effects of ventilatory strategy on alveolar differentiation markers by immunofluorescence. Immunofluorescence double labeling with PPAR γ /pro-SP-C (green/red) (A), PPAR γ /Vimentin (red/green) (B), PTHrP-R/pro-SP-C (green/red) (C), and PTHrP-R/Vimentin (red/green) (D) confirmed that HFNV increased staining for PPAR γ , pro-SP-C, and PTHrP-R, while inhibiting the staining of vimentin. On the other hand, IMV increased the staining for vimentin, while also increasing pro-SP-C staining, and having no effect on PPAR γ and PTHrP staining. Scale bar = 20 μ m.

ventilatory modalities would, as expected, be either pro- or antihomoeostatic causing a decreased or increased incidence of neonatal CLD, respectively.

Physiologically, PTHrP promotes alveolar homeostasis in the developing lung through a paracrine pathway, structurally and functionally linking the maturation of the endoderm and mesoderm (3,7,23). PTHrP is expressed and secreted by ATII cells under the influence of stretch (7,24,25), subsequently binding to its cognate receptor on adipothelial fibroblasts, inducing the lipofibroblast phenotype. The lipofibroblast serves two major purposes in the ontogeny of the alveolar acinus—it is cytoprotective against oxygen-free radicals (26) and produces leptin (5), which stimulates surfactant synthesis by the ATII cell by binding to its receptor on the ATII cell (5–7). The expression of leptin and its receptor are also stimulated by stretching of the alveolar wall, generating additive, or synergistic interactions between PTHrP and leptin for surfactant production (24,25,27). This exquisite interlocking of ligands and receptors, regulated by the stretching of the alveolar wall, functionally integrates the structure and function of the alveolus.

The PTHrP-PPAR γ -mediated epithelial-mesenchymal paracrine signaling mechanism of alveolar acinar development and homeostasis also predicts rational targets for failed signaling in disease. We have shown that in the absence of PTHrP-PPAR γ signaling, due to ATII cell immaturity and/or injury (barotrauma (24,25,27), hyperoxia (20,28,21), infection (22), or nicotine (29–32), the lipofibroblast transdifferentiates

into a myofibroblast because of the down-regulation of PPAR γ , the nuclear transcription factor necessary for adipocyte differentiation (33). Conversely, treatment of any one of these mechanisms of cell-molecular signaling breakdown with a PPAR γ agonist, such as rosiglitazone or prostaglandin J₂, can either prevent (20–22,31,32) or reverse (30) the failure of cell-cell signaling that causes neonatal CLD, providing a possible targeted molecular approach to effectively prevent or treat neonatal CLD based on its pathophysiology.

It is important to note that there are key limitations to our study: 1) because both groups of preterm lambs were resuscitated with IMV for 2 to 3 h before weaning the high-frequency group to HFNV, it is possible that the lung injury initiated even during the brief period of IMV before switching to HFNV might have obscured the differences in the molecular markers examined by us. However, if that were the case, the actual molecular differences would have been even more pronounced than those noted by us; and 2) as noted in our previous study (9), the other potential confounding differences between the two groups of preterm lambs were the amounts of sedatives used and the enteral nutrition delivered. Therefore, we cannot exclude the possibility that some of the differences in the expression of the molecular markers observed by us between the two groups were because of the greater amount of sedatives and decreased amount of enteral feeding in the IMV group *versus* the HFNV group.

In summary, our results show that in a preterm lamb model of CLD, HFNV enhances homeostatic alveolar epithelial-mesenchymal paracrine signaling driven by PTHrP-PPAR γ , whereas IMV inhibits it. We therefore speculate that decreased neonatal CLD in infants supported by HFNV is likely due to its positive effect on PTHrP-PPAR γ -driven alveolar homeostatic epithelial-mesenchymal signaling. Because the inherent immaturity of alveolar structure and function of the preterm lung are physiologic limits to which mechanical ventilatory support can be reduced in the face of the need for adequate amounts of circulating oxygen, we further speculate that combining treatment with PTHrP/PPAR γ agonists with HFNV may further diminish or eliminate the risk of neonatal CLD in the future.

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