

Airway Injury From Initiating Ventilation in Preterm Sheep

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ABSTRACT: Premature infants exposed to ventilation are at risk of developing bronchopulmonary dysplasia and persistent lung disease in childhood. We report where injury occurred within the lung after brief ventilation at birth. Preterm sheep (129 d gestation) were ventilated with an escalating tidal volume to 15 mL/kg by 15 min to injure the lungs, with the placental circulation intact (fetal) or after delivery (newborn). Fetal lambs were returned to the uterus for 2 h 45 min, whereas newborn lambs were maintained with gentle ventilatory support for the same period. The control group was not ventilated. Bronchoalveolar lavage fluid (BALF) and lung tissue were analyzed. In both fetal and newborn lambs, ventilation caused bronchial epithelial disruption in medium-sized airways. Early growth response protein 1 (Egr-1), monocyte chemotactic protein 1 (MCP-1), IL-6, and IL-1 β mRNA increased in the lung tissue from fetal and newborn lambs. Egr-1, MCP-1, and IL-6 mRNA were induced in mesenchymal cells surrounding small airways, whereas IL-1 β mRNA localized to the epithelium of medium/small airways. Ventilation caused loss of heat shock protein 70 (HSP70) mRNA from the bronchial epithelium, but induced mRNA in the smooth muscle surrounding large airways. HSP70 protein decreased in the lung tissue and increased in BALF with ventilation. Initiation of ventilation induced a stress response and inflammatory cytokines in small and medium-sized airways. (*Pediatr Res* 67: 60–65, 2010)

The majority of extremely low-birth weight (ELBW) infants (<1000 g) are intubated and receive ventilatory support after delivery (1), but little is known about the effects of mechanical ventilation on the preterm lung. Ventilation from birth has been associated with an increased incidence of bronchopulmonary dysplasia (BPD) (2), and clinical strategies to transition ELBW infants using continuous positive airway pressure (CPAP) in the delivery room may modestly decrease the incidence of BPD (3,4). Although alveolar simplification is the hallmark of BPD in the postsurfactant treatment era, small airway injury continues to be prominent in older children with a history of BPD (5–8). School-age children diagnosed with moderate to severe BPD have decreased forced expiratory volume in 1 sec (FEV₁), increased respiratory symptoms, and decreased peak flow measurements at 6 y of

life (5). Children born extremely premature also have decreased exercise performance (9).

The transition from a fetus to a newborn requires the initiation of breathing, clearance of fluid from airways, and ventilation of the distal airspaces. Very preterm infants may be more vulnerable to airway injury, because they are born with the lung at the saccular stage of development, before the formation of alveoli (6). In contrast to the adult lung, airways in the preterm lung stretch with normal ventilation and disruptions of airway epithelium are prominent in the lungs of infants who have died of RDS (10,11). The decreased surfactant pools found in premature infants also contribute to non-uniform expansion of the lung by creating areas of focal overdistension and atelectasis (12). We showed previously that regardless of the tidal volume or positive end expiratory pressure (PEEP) used, initiation of ventilation in fluid-filled, surfactant deficient preterm lambs is injurious (13). The preterm lung is likely at risk for small and large airway injury from initiation of ventilation during resuscitation.

We reported previously that fetal ventilation followed by return of the fetus to the uterus for 3 h can be used to evaluate the initial lung injury response (14). Ventilation of fetal lambs with an escalating tidal volume (V_T) to 15 mL/kg by 15 min caused lung injury and inflammation, and indicators of systemic inflammation increased within 3 h (14). Ventilation increased the proinflammatory cytokine mRNA for monocyte chemotactic protein 1 (MCP-1), IL-6 (IL-6), and IL-1 β (IL-1 β) mRNA in the lungs (14). The nuclear transcription factor early growth response protein 1 (Egr-1) promotes expression of proinflammatory cytokines and is inducible by hypoxia and stretch injury within 30 min (15,16). Heat shock protein 70 (HSP70) is a chaperone protein present in the lung epithelium that is responsive to cellular stresses and may influence inflammatory processes (17). Because fetal sheep, unlike adults, do not have alveolar macrophages to initiate the inflammatory response (18), the epithelium or other tissue components may initiate inflammation. We tested the hypothesis that high tidal volume stretch injury would damage the airways of the preterm and increase the expression of the early response genes Egr-1 and HSP70, and the subsequent expression of early response cytokines.

Abbreviations: BAL, bronchoalveolar lavage; BPD, bronchopulmonary dysplasia; Egr-1, early growth response protein 1; HSP70, heat shock protein 70; MCP-1, monocyte chemotactic protein 1

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METHODS

The animal studies were performed in Perth, Western Australia; Cincinnati Children's Hospital and the Western Australia Department of Agriculture approved the animal use procedures. Some indices of global lung injury were previously reported for these animals (14).

Animals were assigned to three groups ($n = 6-8/\text{group}$): 1) fetal ventilation with placental support (fetal), 2) newborn resuscitation and ventilation (newborn), or 3) controls. After maternal hysterotomy, fetal lambs had their head and chest exteriorized. The fetus was intubated, and lung fluid was removed with modest suction on endotracheal tube (10–15 mL per animal). Placental circulation remained intact throughout the procedure. Newborn lambs were surgically delivered, intubated, and lung fluid was removed before 15-min intervention.

Initial 15-min intervention. The fetal and newborn lambs were ventilated (rate 30 breaths/min, inspiratory time 1 s, FiO_2 0.4) with a Bourns BP200 time-cycled, pressure-limited infant ventilator using 12 L/min flow with heated and humidified air and no PEEP. Tidal volumes were increased throughout the ventilation period to achieve 5 mL/kg by 5 min, 10 mL/kg by 10 min, and 15 mL/kg by 15 min of ventilation (14). An inline flow sensor and monitor (Florian Infant Graphics Monitor, Acutronic Medical Systems, Switzerland) continuously measured V_T . Each lamb was treated with 100 mg/kg bovine surfactant at 15 min of age (Survanta, Ross Laboratories, OH).

Following 2 h 45-min procedure. Fetal lambs were returned to the uterus, and the ewes were allowed to recover. Each fetal lamb was delivered for plasma and tissue collection 2 h and 45 min after the 15-min fetal ventilation period. After the initial 15-min ventilation period with escalating tidal volumes, newborn lambs were ventilated (40 breaths/min, PEEP 5 cm H_2O , inspiratory time 0.7 s, FiO_2 0.40) with a heated and humidified oxygen and air mixture for 2 h 45 min. The subsequent ventilation targeted tidal volumes were 7 mL/kg, similar to those achieved by preterm lambs spontaneously breathing on CPAP (19). Peak inspiratory pressures were adjusted to maintain a tidal volume of 7 mL/kg with a targeted PaCO_2 of 50 mm Hg. Samples were collected 2 h 45 min after the end of the initial resuscitation period.

Controls. Nonmanipulated twins of lambs of fetal group had tissue collected after delivery of the twin.

Lung processing and BALF analysis. The lungs were weighed, and the left lung was lavaged with saline three times for bronchoalveolar lavage (BAL) (20). BAL was used for measurement of HSP70. Tissues from the right lung were snap frozen for RNA analysis. The right upper lobe of the lung was inflation fixed with 10% formalin (21). Injury was scored on blinded hematoxylin-eosin-stained tissue. Ten random high power fields were scored on a 0 to 2 scale for septation thickness (0 = thin distal airway walls, 1 = mild thickening of airway walls with thin alveolar regions, and 2 = thickening of all airspaces), hemorrhage (0 = no hemorrhage, 1 = hemorrhage into parenchyma, small airway hemorrhages, and 2 = large airway hemorrhage), inflammation (0 = no inflammatory cells, 1 = 1–5 cells/hpf, and 2 = >5 inflammatory cells/hpf), and epithelial sloughing (0 = no sloughing, 1 = epithelial wall disruption, and 2 = denuded epithelium in airspace) (total 8 points). Airways with smooth muscle, glands, and cartilage were identified as large airways; airways with only smooth muscle were considered medium-sized airways; and distal bronchioles without smooth muscle often with branching airspaces were identified as small airways.

Immunohistochemistry. Immunostaining protocols were used as reported (20). Paraffin sections (5 μm) of formalin-fixed tissue were pretreated with 3% hydrogen peroxide to destroy endogenous peroxidases. The sections were incubated with the primary antibody [HSP70 (Biogenex, CA), Egr-1 (Santa Cruz, CA), inducible nitric oxide synthase (iNOS; Transduction, USA), VEGFR2 (Cell Signaling, USA), and IL-1 β (internally produced)] with a blocking agent, followed by biotin labeled secondary antibody. Immunostaining was visualized by Vectastain ABC peroxidase Elite kit to detect the antigen:antibody complexes (Vector Laboratories Inc). The antigen detection was enhanced with nickel-DAB, followed by Tris-cobalt and the nuclei counterstained with nuclear fast red.

Western blots. The lung tissue was homogenized in 50 mM Tris-HCl buffer, pH 7.5, and 0.1 mM EDTA. Homogenates were centrifuged and supernatants collected. Forty micrograms of protein was denatured, electrophoresed on a 10 to 20% Tris-glycine gel (Invitrogen, CA), and transferred to a nitrocellulose membrane. The membrane was incubated overnight at 4°C with HSP70 antibody diluted 1:1000 in TBS-tween and 5% milk. The membrane was incubated for 1 h in room temperature with secondary antibody conjugated to horseradish peroxidase diluted at 1:5000 in 5% milk. Detection by chemiluminescence was performed using ECL reagents (GE Healthcare, NJ). Beta-actin (C4) (mouse MAb against chicken gizzard actin) as a primary antibody diluted at 1:2000 and a secondary antibody, goat anti-mouse IgG conjugated to horseradish peroxidase (1:5000 dilution) (Santa Cruz Biotechnology) was used to re-probe the blots as loading controls (20).

ELISA for HSP70. A HSP70 ELISA for human HSP70 was cross-reactive with sheep (R&D Systems, USA) and was used to measure HSP70 in bronchoalveolar lavage fluid (BALF), lung homogenate, and plasma.

RNase protection assays. Total RNA was isolated using a modified Chomczynski method (22), and 10 μg of total lung RNA was used for RNase protection assays with sheep-specific riboprobes for IL-1 β , IL-6, MCP-1, HSP70, Egr-1, VEGF, and L32 (23). Solution hybridization was performed with a molar excess of [α - ^{32}P]UTP-labeled probes for 16 h at 56°C. Single-stranded RNA was digested with RNase A/T1 (Pharmingen, San Diego, CA). RNase was inactivated, and the protected RNA was precipitated using RNase inactivation buffer (Ambion, Austin, TX). L32 (ribosomal protein mRNA) was used as an internal control for loading (23). The protected fragments were resolved on 6% polyacrylamide 8 M urea gels, visualized by autoradiography, and quantified on a Phospho Imager using ImageQuant version 1.2 software (Molecular Dynamics, Sunnyvale, CA).

In situ hybridization. *In situ* localization of mRNA was performed with digoxigenin-labeled antisense sheep riboprobes for IL-1 β , IL-6, MCP-1, Egr-1, and HSP70 (Roche, IN). Briefly, digoxigenin-labeled riboprobes (sense and antisense) were synthesized from cDNA templates using DIG RNA labeling kits (Roche) and diluted in hybridization buffer to a final concentration of 1 $\mu\text{g}/\text{mL}$. The sections were pretreated with 4% paraformaldehyde, proteinase K treated, and hybridized with the probe overnight at 49 to 62°C, based on GC content of probe. Sections were washed with formamide, RNase A (100 $\mu\text{g}/\text{mL}$) treated, and then blocked with 10% horse serum. After incubation overnight at 4°C with anti-digoxigenin antibody (Roche), the slides were developed with NBT-BCIP (Roche) in dark cases. The slides were monitored for color development and then stopped with TE buffer. Controls for specificity of ribo-probe binding included use of the homologous (sense) probe.

Statistics. All values are expressed as mean \pm SD, and comparisons between intervention groups were made with two-tailed Mann-Whitney nonparametric tests or Welch *t* tests. Significance was accepted at $p < 0.05$. Figures show individual values and means, and were log transformed when appropriate.

RESULTS

The lambs had similar birth weights and had achieved similar tidal volumes and lung compliance values at the end of the 15-min resuscitation period. In the newborn ventilation group, the mean FiO_2 was 0.41 ± 0.16 and Pao_2 was 33.9 ± 10.8 mm Hg. The total injury scoring was similar between ventilated groups with both fetal (2.8 ± 1.0) and newborn (3.2 ± 0.8) groups increased over controls (0.1 ± 0.2). There were bronchial epithelial disruptions in medium-sized airways without signs of hemorrhage for both ventilated groups. Lambs in the newborn group had increased alveolar wall thickness compared with the fetal group. Inflammatory cells were occasionally seen within the airways of the ventilated groups, but not in the controls.

Early growth response protein 1. Egr-1 mRNA was increased 3.5 ± 2.1 -fold in the fetal group and increased 15.8 ± 3.8 -fold with stretch injury and continued ventilation in the newborn group. After 3 hours, the mRNA was expressed in the inflammatory cells in the fetal ventilation group and throughout the small airways and inflammatory cells in the newborn group (Fig. 1A–C). Egr-1 protein was localized in the fetal group to areas surrounding the small airways compared with the unventilated controls (Fig. 1E). More extensive staining of the smooth muscle and distal airspaces was seen in animals ventilated for 3 hours (Fig. 1F).

Heat shock protein 70. HSP70 mRNA was localized to the bronchial epithelial cells in the nonventilated controls (Fig. 2A). HSP70 mRNA expression was lower than controls in airway epithelial cells of the fetal group and was virtually absent from the bronchial epithelial cells and glands in the newborn group (Fig. 2B and C). HSP70 mRNA decreased in

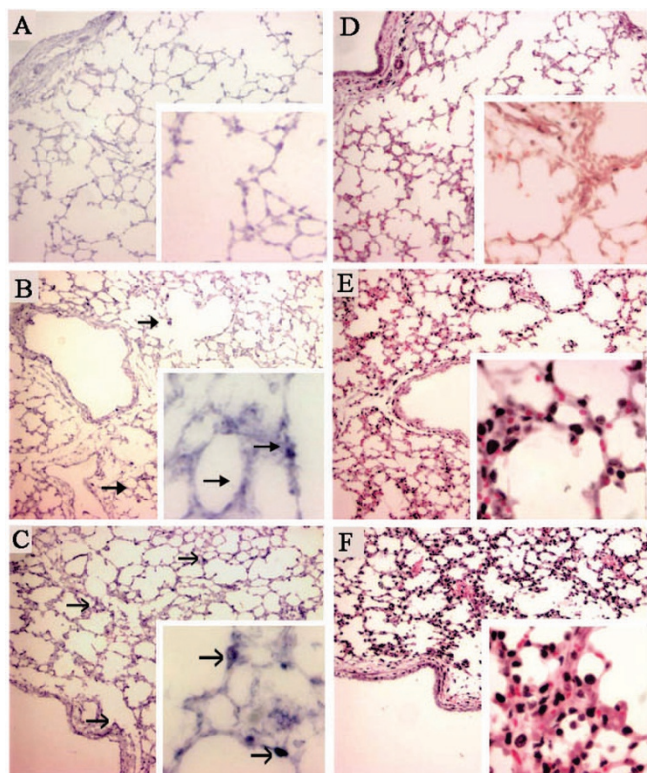


Figure 1. Egr-1 mRNA and protein induced in small airways. A–C, Digoxigenin-labeled *in situ* hybridization of Egr-1 demonstrated induction of mRNA around small airways [arrows, insert (40 \times)] with fetal ventilation (B) and with newborn ventilation (C). Egr-1 protein (D–E) is increased in small airways of the fetal group [E, insert (40 \times)] and throughout the lung parenchyma of the newborn group (F) compared with controls (D).

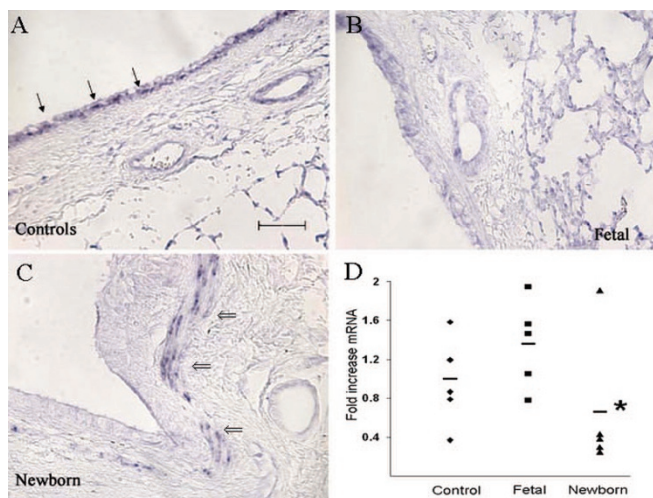


Figure 2. HSP70 mRNA. (A) HSP70 mRNA was localized to the bronchial epithelium (arrows) of nonventilated controls. B, Fetal ventilation decreased HSP70 mRNA in airway epithelial cells. C, Newborn ventilation induced HSP70 mRNA in the smooth muscle (open arrows) surrounding large airways. D, RNase protection assay for HSP70 mRNA in the lung. * $p < 0.05$ vs controls. Scale bar = 25 μ m.

lung homogenates from the newborn lambs (Fig. 2D). In contrast, HSP70 mRNA appeared in the smooth muscle of the large airways after ventilation (Fig. 2C). HSP70 protein was expressed in the ciliated cells of the bronchial epithelium in the nonventilated controls (Fig. 3A); consistent with the

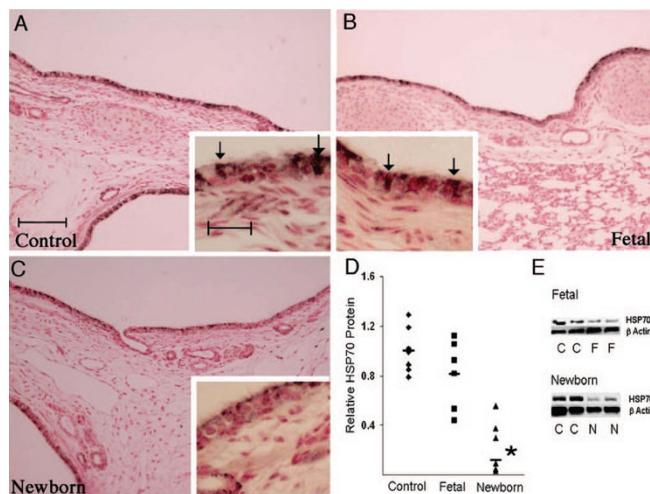


Figure 3. HSP70 immunohistochemistry. HSP70 protein was located in the ciliated cells (upper insert arrows) of the bronchial epithelium of the large airways in control lambs (A). HSP70 staining was decreased with fetal (B) and newborn (C) ventilation. D, Western blot with lung homogenates demonstrated decreased protein levels in fetal (F) and newborn (N) lambs. (E) Representative western blots. * $p < 0.01$ vs controls.

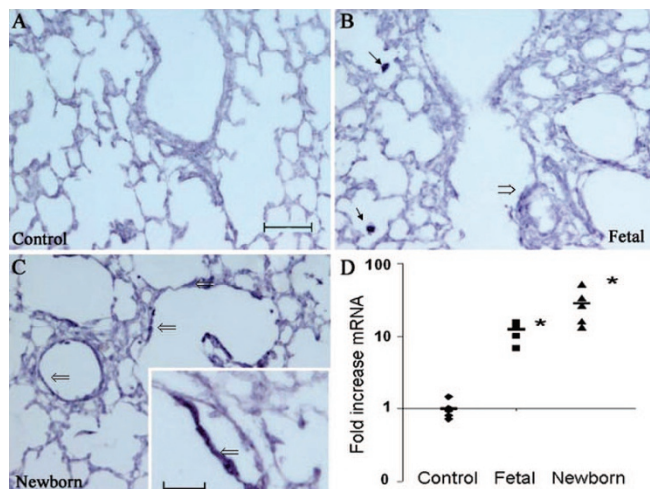


Figure 4. IL-1 β mRNA localization with ventilation. Compared with controls (A), fetal ventilation increased IL-1 β mRNA in (B) the inflammatory cells (arrows) and epithelial cells (open arrows). C, Induction of IL-1 β mRNA was more evident in the epithelial cells along small airways with continued ventilation, especially in sloughed epithelium (insert). D, RNase protection assay for the lung tissue demonstrated increased IL-1 β with fetal ventilation with further increase with ventilation. * $p < 0.05$ vs controls. Scale bar = 25 μ m (insert 10 μ m).

mRNA data, the protein levels were lower in fetal and newborn lambs (Fig. 3B and C). Unlike the mRNA, HSP70 protein was not increased in airway smooth muscle at 3 h. HSP70 protein in lung homogenates was 20% lower in the fetal group and 80% lower in the newborn group (Fig. 3D). HSP70 in BAL fluid was 4 ± 0.8 -fold higher in the fetal group and 5 ± 1.3 -fold higher in newborn group than in the unventilated controls. HSP70 was detected in the plasma of the unventilated controls, and levels did not change with ventilation.

IL-1 β , MCP-1 and IL-6 expression. IL-1 β mRNA was 12-fold higher in the fetal group and 28-fold higher in the newborn lambs (Fig. 4D) than for controls. IL-1 β mRNA was

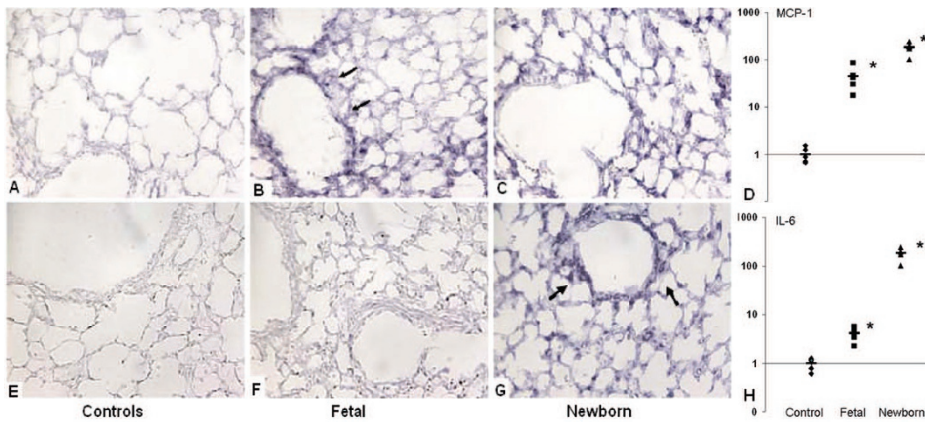


Figure 5. MCP-1 and IL-6 mRNA localization. Compared with controls (A), MCP-1 mRNA was increased in mesenchyme surrounding small airway (arrows) with fetal ventilation (B) and more diffusely with continued newborn ventilation (C). IL-6 mRNA increased slightly in fetal group (F), relative to controls (E), and with newborn ventilation (G) leading to increased mRNA in mesenchyme surrounding the small airways (arrows). RNase protection assays (D, H) correlated with the expression data with MCP-1 increasing more dramatically with fetal ventilation. * $p < 0.05$ vs controls.

localized to inflammatory cells (Fig. 4B) and to the bronchial epithelium of small airways, particularly in areas with bronchial epithelial sloughing (Fig. 4C). Control lungs (Fig. 4A) had no IL-1 β mRNA. IL-1 β protein was localized to inflammatory cells lining the small airways (data not shown).

MCP-1 was 44-fold higher relative to the control lung in the lungs of lambs in the fetal group and was increased 180-fold in the newborn group (Fig. 5). The MCP-1 was localized primarily to the parenchyma surrounding the small airways in the fetal group. MCP-1 localization was more diffuse with the 3-h newborn ventilation, involving more mesenchyme surrounding the distal airways.

In comparison with MCP-1, IL-6 mRNA increased only 4-fold in the fetal group but increased 300-fold with continued ventilation in the newborn group. *In situ* localization demonstrated increased mRNA in the mesenchyme surrounding the smaller airways with ventilation (Fig. 5), similar to MCP-1. Minimal expression of IL-6 mRNA was evident for the fetal group.

VEGF, VEGFR2, iNOS expression. Immunohistochemistry for iNOS (iNOS/NOSII) was negative for the fetal and newborn lambs (data not shown), suggesting that the inflammatory cells were not activated by the 3-h interventions. VEGF mRNA in the lungs was not different from control in fetal or newborn lambs. VEGFR2 protein levels were also not different.

DISCUSSION

The initiation of ventilation with large tidal volumes caused changes in both the large and small airways of these preterm lambs. Because newborns must clear the airways of fluid before initiation of gas exchange, the fetal lung fluid is forced into the distal airspaces and lung parenchyma by the initial breaths (24). As functional residual capacity is recruited and the lung parenchyma expands, the airways are stretched by ventilation, and this airway deformation will move from the large airways to the smaller airways (25). Although epithelial sloughing was noted in medium-sized airways, extensive tissue damage and hemorrhage were not seen in these lambs. A graduated injury response occurred in the animals maintained on placental support (fetal) relative to animals exposed to continued ventilation (newborn). This study used a unique model to evaluate the stretch injury induced by initiation of

ventilation at birth. Unlike adult animals, where alveolar macrophages play a primary role in the initiation of the inflammatory response to stretch injury (26), fetal sheep do not have mature macrophages in the lungs (18). Negative iNOS staining and the absence of significant numbers of inflammatory cells within areas of intense IL-6 and MCP-1 mRNA production suggest that the cells within the mesenchyme surrounding the airways initially produce these proinflammatory mediators. Therefore, this study is informative about the tissue components responsible for injury responses induced by ventilation of the preterm lung.

The cytokines had variable patterns of expression around the small airways. The IL-1 β localization to the bronchial epithelium, including areas with extensive sloughing, is similar to the IL-1 β injury response in the adult rats (15). Similar epithelial sloughing is seen in models of rapid expansion of a fluid-filled, surfactant-deficient small airways (27). The MCP-1 and IL-6 mRNA were localized to the small airways, but were also found in the surrounding mesenchyme. Although the location of the mRNA induction for IL-6 and MCP-1 was similar, the timing or threshold after injury seems to be different, with MCP-1 increased by 15-min ventilation and IL-6 requiring continued ventilation for high induction. Our results demonstrate that cytokine production from stretch injury in the fetal sheep is primarily produced by the cells of the lung parenchyma.

The large airways demonstrated loss of HSP70 protein from epithelial cells without further induction of mRNA and with soluble HSP70 in the airspace after ventilation. HSP70 is an intracellular chaperone protein that is present in the epithelial cells of the lung (17). Intracellular HSP70 can help stabilize I κ B, can decrease NF- κ B translocation into the nucleus, and can inhibit the production of proinflammatory cytokines (28). HSP70 also can be released as extracellular HSP70 (eHSP70) (29), which can act as an endogenous ligand for Toll-like receptor 4 (TLR4) (30). In these preterm lambs, HSP70 staining was intense in the ciliated cells of the bronchial epithelium of control animals, and this epithelial HSP70 decreased with ventilation. Consistent with the expression data, HSP70 mRNA and protein expression decreased in the lung homogenates. In contrast, HSP70 protein increased in the BALF, suggesting release of HSP70 from the injured epithelium into the airway. Interestingly, HSP70 levels did not

increase in the plasma. The release of HSP70 into the BAL probably resulted from active exocytosis in the form of eHSP70, because the intact epithelium lacked HSP70. We previously showed increased TLR4 mRNA expression in these lungs, which could result from eHSP70 signaling (14). Unlike adult animals, such as the rat (15), there was no increased HSP70 mRNA with ventilation-induced injury. Ventilation of preterm sheep decreased the HSP70 mRNA and protein in the bronchial epithelial cells. This difference in responses was reported for newborn and adult rats (31), suggesting that the difference may result from the stage of lung development.

The smooth muscle surrounding the larger airways responded to the 15 min of mechanical ventilation, as demonstrated by increased HSP70 mRNA. This localization of HSP70 expression to the smooth muscle following airway stretch is a novel observation, and it is consistent with the increase in HSP70 in the skeletal muscles of mice exposed to excessive cyclical stretch (32). The increase in HSP70 mRNA seen in this study may indicate that damage is caused by distending the airways. The airway effects may be magnified by the more compliant airways of preterm animals, which also have decreased contractility of airway smooth muscle (33). The initial expansion of the fluid-filled lung is anatomically location dependent, with newborn rabbit lungs aerating the dependent portions of their lungs more slowly than nondependent regions (24). We only had tissue from the right upper lobe to evaluate, and other areas of the lung may have had differing degrees of injury (34). Our measurements were limited to 3 hours, and changes in the smooth muscle and HSP70 may become more prominent with time. Continuous stretching of the compliant airways of premature infants with ventilation could lead to the smooth muscle hypertrophy or airway malacia, and both abnormalities have been reported for premature infants (35).

Early growth protein 1 (Egr-1) increased with both fetal and newborn ventilation and may be an early initiator of the stretch-induced lung inflammation. Fetal ventilation increased the intensity of staining in the nuclei of cells surrounding the small airways. *In situ* localization for Egr-1 mRNA was similar to protein localization, suggesting the 15 min of fetal ventilation primarily injured the airways. Similar to our findings, increased expression Egr-1 was reported in rats and lambs exposed to high tidal volume ventilation within 30 min (15,16). After 3 hours of ventilation, the majority of the cells in the preterm lungs were Egr-1 positive. In mice, Egr-1 signaling has been linked to ventilator-induced lung injury (VILI), as Egr-1 knock out mice do not develop VILI, and inhibitors of Egr-1, such as peroxisome proliferator-activated receptor- γ agonists, decrease VILI (36). Egr-1 knock out mice also have decreased levels of circulating inflammatory mediators and survive longer after pulmonary ischemia-reperfusion (37). We demonstrate that an early response gene (Egr-1) is induced by 15 min of ventilation, predominantly in cells surrounding the small airways of the preterm lung.

Our studies provide clues to pathogenesis of lung abnormalities in preterm infants. Although alveolar simplification is the hallmark of BPD on autopsy in the postsurfactant era,

residual small airway injury is characteristic of older children with a history of BPD. School-aged children diagnosed with moderate-to-severe BPD demonstrated decreased FEV₁ (5). Children born extremely prematurely also demonstrated decreased exercise performance (9). Alveolar simplification leads to the need for oxygen in infants with BPD, and airway injury and possible airway smooth muscle hypertrophy may contribute to the decreased FEV₁ seen in infants with BPD. The initiation of ventilation induced expression of a stress-response and inflammatory cytokines primarily in small- and medium-sized airways and may be a first step toward persistent airway abnormalities.

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