Positive end-expiratory pressure and surfactant decrease lung injury during initiation of ventilation in fetal sheep

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Submitted 23 May 2011; accepted in final form 14 August 2011

Hillman NH, Nitsos I, Berry C, Pillow JJ, Kallapur SG, Johe AH. Positive end-expiratory pressure and surfactant decrease lung injury during initiation of ventilation in fetal sheep. Am J Physiol Lung Cell Mol Physiol 301: L712–L720, 2011. First published August 19, 2011; doi:10.1152/ajplung.00157.2011.—The initiation of ventilation in preterm, surfactant-deficient sheep without positive end-expiratory pressure (PEEP) causes airway injury and lung inflammation. We hypothesized that PEEP and surfactant treatment would decrease the lung injury from initiation of ventilation with high tidal volumes. Fetal sheep at 128-day gestational age were randomized to ventilation with: 1) no PEEP, no surfactant; 2) 8-cmH2O PEEP, no surfactant; 3) no PEEP + surfactant; 4) 8-cmH2O PEEP + surfactant; or 5) control (2-cmH2O continuous positive airway pressure) (n = 6–7/group). After maternal anesthesia and hysterotomy, the head and chest were exteriorized, and the fetus was intubated. While maintaining placental circulation, the fetus was ventilated for 15 min with a tidal volume escalating to 15 ml/kg using heated, humidified, 100% nitrogen. The fetus then was returned to the uterus, and tissue was collected after 30 min for evaluation of early markers of lung injury. Lambs receiving both surfactant and PEEP had increased dynamic compliance, increased static lung volumes, and decreased total protein and heat shock proteins 70 and 60 in bronchoalveolar lavage fluid compared with other groups. Ventilation, independent of PEEP or surfactant, increased mRNA expression of acute phase response genes and proinflammatory cytokine mRNA in the lung tissue compared with controls. PEEP decreased mRNA for cytokines (2-fold) compared with groups receiving no PEEP. Surfactant administration further decreased some cytokine mRNAs and changed the distribution of early growth response protein-1 expression. The use of PEEP during initiation of ventilation at birth decreased early mediators of lung injury. Surfactant administration changed the distribution of injury and had a moderate additive protective effect.

bronchopulmonary dysplasia; lung inflammation; acute phase response genes; respiratory distress syndrome; prematurity

APPROXIMATELY 10% OF ALL NEWBORNS need some assistance to breathe at birth, and this percentage is much higher in very low birth weight (VLBW) infants (15, 29). Premature infants have immature lungs that are more difficult to ventilate due to surfactant deficiency and are more vulnerable to injury from ventilation (27). Mechanical ventilation is a risk factor for the development of bronchopulmonary dysplasia (BPD) (33), and as few as six large mechanical breaths injure the preterm lung, making it unresponsive to surfactant therapy (3). Clinicians often give large, inconsistent tidal volumes (VT) when resuscitating preterm infants in the delivery room (50). Although the use of positive end-expiratory pressure (PEEP) is beneficial for mechanical ventilation (43), the 2010 International Liaison Committee on Resuscitation guidelines stress that there are no studies specifically examining the use of PEEP during the initiation of ventilation at birth (29). Based on the assumption that PEEP is protective, new devices are now widely used to provide PEEP and control inspiratory pressures in the delivery room.

The fetal lung must transition rapidly at birth from fluid-filled air spaces to a gas exchange organ to sustain life. The movement of fluid can injure the epithelium of the small airways (18, 22), and PEEP should help sustain the movement into the distal lungs and facilitate the development of functional residual capacity (FRC) (53). Surfactant in the fetal lung fluid will lower the pressure required to move fluid down into the small airways and decrease the injury from fluid movement in the airways (22, 23). Thus PEEP and surfactant are two relevant clinical variables that should decrease lung injury from the initiation of ventilation in the surfactant-deficient preterm lung. However, there are no direct demonstrations that PEEP and surfactant use at the initiation of ventilation can modulate early indicators of lung injury in animal models or improve clinical outcomes.

The initiation of ventilation in preterm sheep with escalating VT values and no PEEP leads to airway epithelial injury, diffuse lung inflammation, and induced lung maturation (18, 20, 21). Conventional mechanical ventilation with PEEP preserved surfactant function in preterm sheep and decreased alveolar protein leak in preterm rabbits relative to ventilation without PEEP (36, 47). The use of PEEP during conventional mechanical ventilation also decreased proinflammatory cytokine mRNA expression compared with lambs ventilated without PEEP (40). Lambs supported with higher levels of PEEP during high-frequency jet ventilation had decreased markers of injury and acute phase responses (39). The use of PEEP during the initiation of ventilation improved recruitment of FRC in preterm rabbits and oxygenation in preterm lambs (46, 53). Our laboratory attempted previously to demonstrate that PEEP use during the initiation of ventilation with 8 or 15 ml/kg would decrease lung injury, but found no effect of PEEP (45). Any effects of PEEP during resuscitation may be obscured by the continued mechanical ventilation, which is needed to allow markers of injury to develop in experimental models (20). Clinical trials to evaluate the initial respiratory management with PEEP in extremely low birth weight (ELBW) infants were also complicated by the effects of neonatal intensive care unit management after the intervention (16, 37). Thus it has been difficult to experimentally demonstrate any beneficial effects of PEEP during the resuscitation of preterm newborns (12).

The role of surfactant therapy during the transition to air breathing has not been fully explored, although recent ultrasound sound studies demonstrated no effect of surfactant treatment on...
fluid clearance in rabbits or newborns (5). Surfactant is an important variable in determining the compliance of the preterm lung, and surfactant treatment will decrease lung injury during mechanical ventilation of preterm rabbits and lambs (23, 56). Even small changes in endogenous surfactant pool sizes can decrease lung injury from mechanical ventilation (17). Surfactant treatment at birth does not lower the rates of BPD relative to treatments given after the initiation of ventilation (30). In recent studies, no benefit was shown for early surfactant administration compared with early use of continuous positive airway pressure (CPAP) for BPD in ELBW infants (16, 37). We have isolated components of resuscitation of the newborn from the confounding effects of continued mechanical ventilation using a fetal sheep model (20). For this study, we evaluated the induction of the very early mediators of lung injury before lung inflammation. Using acute phase response genes previously shown to be differentially responsive to mechanical ventilation (11, 18, 39, 57), we tested the hypothesis that PEEP and/or surfactant treatment during the initiation of ventilation would decrease the mediators that promote lung injury.

METHODS

The investigations were approved by the Animal Ethics Committee of the University of Western Australia and Cincinnati Children’s Hospital Medical Center.

Fetal ventilation procedure. Date-mated Merino ewes at 128 ± 1 days gestational age were premedicated with xylazine (0.5 mg/kg im), ketamine (5 mg/kg iv), and midazolam (0.25 mg/kg iv) before induction of maternal anesthesia with inhaled isoflurane. The fetal head and chest were exteriorized through a midline hysteroatomy, while placental blood flow was maintained (20). The fetus was orally intubated, and lung fluid was passively removed. The lambs were randomized (n = 6–7/group) to ventilation with the following: 1) no PEEP; 2) 8-cmH2O PEEP, no surfactant; 3) no PEEP + surfactant; or 4) 8-cmH2O PEEP + surfactant. In lambs assigned to surfactant groups, surfactant (100 mg/kg, Curosurf, Chiesi Pharma) was given before ventilation. The lambs then received mechanical ventilation (Babylog 8000–4, Dräger, Lübeck, Germany) with VT and PIP 4–10 ml/kg at 5 min, with a peak inspiratory pressure (PIP) limit of 55 cmH2O. The animals receiving only 2-cmH2O CPAP (arterial PCO2, 7 ± 2 mmHg) (5), a CPAP of 2 cmH2O for 15 min. After the intervention, the fetus was returned to the uterus to maintain adequate placental blood flow. The ewe remained under general anesthesia, and the fetal lamb was euthanized 30 min after the end of the ventilation procedure (45 total min after initiation of ventilation).

Lung processing and bronchoalveolar lavage analysis. At autopsy, an air deflation pressure-volume curve was measured from an inflation to 40-cmH2O pressure (26). Bronchoalveolar lavage fluid (BALF) of the left lung was collected by repeated saline lavage. BALF was used for measurement of total protein (34) and sandwich ELISA for heat shock protein (HSP) 70 and HSP90 (R&D Systems), and monocyte chemotactic protein-1 (MCP-1) ELISA (in-house) (51). Tissues from the right lower lung and from a segment of right middle bronchus (airway) were snap frozen for RNA isolation. Right upper lobe was inflation fixed with 10% formalin at 30 cmH2O and then paraffin embedded (32).

Quantitative RT-PCR. mRNA was extracted from right lung tissue and right middle lobe bronchus with TRIzol (Invitrogen, USA). cDNA was produced from 1 mg mRNA using Verso cDNA kit (Thermoscientific). Custom Taqman gene primers (Applied Biosystems) were designed from ovine sequences for IL-1β, IL-6, IL-8, MCP-1, early growth response protein 1 (Egr-1), HSP70, nuclear receptor family 77 (Nur77), cysteine rich 61 (Cyr61), and connective tissue growth factor (CTGF). Quantitative RT-PCR was performed with 20 ng cDNA using Tagman Master mix in a 20-µl reaction on a 7300 RT-PCR machine and software (Applied Biosystems). 18s primers (Applied Biosystems) were used for internal loading control, and results are reported as fold increase over mean for control animals.

Immunohistochemistry/in situ hybridization. Immunostaining protocols used paraffin sections (5 µm) of formalin-fixed tissues that were pretreated with 3% hydrogen peroxide to inactivate endogenous peroxidases (28, 31). The sections were incubated with anti-human Egr-1 1:250 dilution (Santa Cruz, USA), Nur77 1:200 (Santa Cruz, USA) in 4% normal goat serum overnight, followed by biotin-labeled secondary antibody. Immunostaining was visualized by Vectastain ABC Peroxidase Elite kit to detect the antigen-antibody complexes (Vector Laboratories). The antigen detection was enhanced with nickel-diaminobenzidine, followed by Tris-cobalt, and the nuclei was counterstained with nuclear fast red or eosin (Egr-1) (31).

In situ localization of mRNA was performed with digoxigenin-labeled anti-sense sheep riboprobes for HSP70, Nur77, Cyr61, and CTGF (Roche). Briefly, digoxigenin-labeled riboprobes (sense and anti-sense) were synthesized from cDNA templates using DIG RNA labeling kits (Roche) and diluted in hybridization buffer to a final concentration of 1 µg/ml. The sections were pretreated with 4% paraformaldehyde, proteinase K treated, and hybridized with the probe overnight at 49–62°C, based on GC content of probe. Sections were washed with formamide, RNase A (100 µg/ml) treated, and then blocked with 10% horse serum. Following incubation overnight at 4°C with anti-digoxigenin antibody (Roche), the slides were developed with nitro blue tetrazolium- 5-bromo-4-chloro-3-indolyl phosphate (Roche) in dark cases. The slides were monitored for color development, then stopped with Tris-EDTA buffer. Controls for specificity of ribo-probe binding included use of the homologous (sense) probe.

Western blot analysis. Protein concentration of nuclear extracts from lung tissue was determined using Bio-Rad Protein Assay (8). Protein (10 µg) was denatured in β-mercaptoethanol at 90 degrees for 5 min, then run on Tris-glycine 10–20% (Invitrogen) gel, and transferred to 0.45-µm nitrocellulose cells (Bio-Rad). Membranes were blocked with 2.5% normal milk fat and then incubated with Egr-1 (sc-189) 1:1,000 (Santa Cruz) or mouse monoclonal TATA binding protein (TBP) 1:300 (Abcam) overnight at 4°C. Appropriate IgG-horseradish peroxidase secondary antibodies were applied at 1:2,000 to 1:5,000 dilutions. Membranes were developed with enhanced chemiluminescence (Pierce/ThermoFisher) and then imaged on ImageQuant Las4000 Imager (GE) and quantified (Multigauge 2.0 software). Egr-1 activity and TBP activity were analyzed on the same gels without membrane stripping and reported as Egr-1/TBP, with results reported as fold increase over control.

Data analysis and statistics. Results are shown as means ± SE. Statistics were analyzed using InStat (GraphPad) using Student’s t-test and Mann-Whitney nonparametric tests as appropriate. Significance was accepted as P < 0.05.

RESULTS

All of the fetal lambs (n = 5–7 per group) tolerated the escalating VT ventilation and 30 min recovery in utero without complications. There were no differences in blood-gas values between ventilated groups at the end of 15-min ventilation period or at tissue collection. There was a mild hypercarbia in the animals receiving only 2-cmH2O CPAP (arterial Pco2, 74 ± 2 mmHg), which was decreased in all ventilated lambs (average arterial Pco2 of 56 ± 2 mmHg) (P < 0.05). These
differences had resolved by the end of 30-min recovery period. The escalating \( V_t/kg \) were similar between groups at 5, 10, and 15 min (Table 1). PIP was limited to 55 cm\( \text{H}_2\text{O} \), and this pressure was reached in 10/13 animals without surfactant and 6/14 animals receiving surfactant. Dynamic compliance after 15-min ventilation was higher in the surfactant- and PEEP-exposed lambs compared with other ventilated animals (Table 1). The surfactant plus PEEP animals also had higher lung gas volumes at 40 cm\( \text{H}_2\text{O} \) (Table 1).

**Responses to lung overdistention.** The initiation of ventilation at birth caused increased total protein in BALF and the release of HSP70 and HSP60 into the air spaces in all groups relative to the unventilated controls (Fig. 1). Surfactant increased the release of total protein into the BALF, but the addition of PEEP decreased this effect. HSPs are highly expressed by airway epithelium of fetal sheep, and airway stretch releases these proteins into the air spaces (18). The animals who received both surfactant and PEEP had decreased levels of both HSP70 and HSP60 in the BALF compared with animals receiving no PEEP or surfactant, demonstrating less release from airway stretch. In situ localization of HSP70 mRNA demonstrated loss of signal from airway epithelium and increased signal in the smooth muscle surrounding the large airways in all ventilated groups.

**Acute phase-response genes.** Ventilation increased Nur77, CYR61, and CTGF mRNA in the lung and large airways compared with controls (Fig. 2). Increases in mRNA were larger in the lung parenchyma than the mRNA from a segment of right middle bronchus. Nur77 was localized to the smooth muscle surrounding the large airways and sporadic cells in the peripheral lung (Fig. 2C) compared with controls (Fig. 2B). Cyr61 mRNA increased in the peripheral lung tissue with ventilation (Fig. 2D), and animals receiving PEEP alone had lower increases. Cyr61 mRNA was localized to cells within the lung mesenchyme after ventilation (Fig. 2F, *inset*). CTGF was increased in the airways and parenchyma and was localized to the mesenchyme directly adjacent to the larger airways and to distinct cells in the proximity to alveolar crests (Fig. 2I, *inset*).

Egr-1 mRNA was increased in all ventilated lambs compared with control (Fig. 3A). Nuclear Egr-1 protein expression was found throughout the small airways in the lambs ventilated without surfactant (Fig. 3, C and D) compared with no signal in control lambs (Fig. 3B). Surfactant changed the Egr-1 to a nonuniform distribution primarily around the small airways (Fig. 3, E and F). The use of both PEEP and surfactant decreased Egr-1 protein expression by 43% in lung homogenates compared with lambs ventilated without PEEP or surfactant (Fig. 3, G and H).

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**Table 1. Ventilation, compliance, and lung volumes**

<table>
<thead>
<tr>
<th>Group</th>
<th>( N )</th>
<th>Birth Weight, kg</th>
<th>( V_t/kg, \text{ml/kg} )</th>
<th>PIP 15 min, cm( \text{H}_2\text{O} )</th>
<th>Compliance 15 min, ( \text{ml} \cdot \text{kg}^{-1} \cdot \text{cm}\text{H}_2\text{O}^{-1} )</th>
<th>Lung Volume at 40 cm( \text{H}_2\text{O} ), ( \text{ml/kg} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Surf/no PEEP</td>
<td>7</td>
<td>3.3 ± 0.1</td>
<td>5.1 ± 0.2</td>
<td>0.27 ± 0.02</td>
<td>29.6 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>No Surf/PEEP 8 cm( \text{H}_2\text{O} )</td>
<td>6</td>
<td>3.7 ± 0.2</td>
<td>4.8 ± 0.3</td>
<td>52.8 ± 1.6</td>
<td>27.0 ± 5.7</td>
<td></td>
</tr>
<tr>
<td>Surf/no PEEP</td>
<td>7</td>
<td>3.3 ± 0.1</td>
<td>5.4 ± 0.2</td>
<td>54.7 ± 0.3</td>
<td>30.1 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>Surf/PEEP 8 cm( \text{H}_2\text{O} )</td>
<td>7</td>
<td>3.3 ± 0.1</td>
<td>5.4 ± 0.3</td>
<td>54.0 ± 0.5</td>
<td>31.0 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>3.5 ± 0.2</td>
<td>5.4 ± 0.3</td>
<td>51.0 ± 1.5</td>
<td>31.8 ± 2.1†</td>
<td></td>
</tr>
</tbody>
</table>

| No Surf/no PEEP     | 7      | 3.3 ± 0.1       | 5.1 ± 0.2       | 0.27 ± 0.02      | 29.6 ± 3.4               |
| No Surf/PEEP 8 cm\( \text{H}_2\text{O} \) | 6      | 3.7 ± 0.2       | 4.8 ± 0.3       | 52.8 ± 1.6       | 27.0 ± 5.7               |
| Surf/no PEEP        | 7      | 3.3 ± 0.1       | 5.4 ± 0.2       | 54.7 ± 0.3       | 30.1 ± 3.1               |
| Surf/PEEP 8 cm\( \text{H}_2\text{O} \) | 7      | 3.3 ± 0.1       | 5.4 ± 0.3       | 54.0 ± 0.5       | 31.0 ± 3.1               |
| Control             | 5      | 3.5 ± 0.2       | 5.4 ± 0.3       | 51.0 ± 1.5       | 31.8 ± 2.1†              |

Values are means ± SE; \( N \), no. of animals; Surf, surfactant; PEEP, positive end-expiratory pressure; \( V_t \), tidal volume; PIP, peak inspiratory pressure. *\( P < 0.05 \) vs. no surfactant, no PEEP. †\( P < 0.05 \) vs. all other ventilated groups.

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**Fig. 1. Total protein, heat shock protein (HSP) 70, and HSP60 in bronchoalveolar lavage increased in all groups with ventilation [−/−: no positive end-expiratory pressure (PEEP), no surfactant (Surf); +/+: PEEP, no surfactant; −/+: no PEEP, surfactant; +/+: PEEP, surfactant]. A: total protein was increased in lambs receiving surfactant and no PEEP and decreased with the addition of PEEP. B: surfactant and PEEP administration decreased HSP70 excretion into bronchoalveolar lavage fluid (BALF) with ventilation. C: HSP60 was also increased in BALF with ventilation and decreased with PEEP use. C, control. Values are means ± SE. *\( P < 0.05 \) vs. animals receiving no PEEP, no surfactant. †\( P < 0.05 \) vs. all other ventilated groups. ‡\( P < 0.05 \) vs. no PEEP, surfactant group.
Cytokine responses in lung parenchyma. Ventilation increased mRNA expression for MCP-1, IL-1β, IL-8, and IL-6 in all groups compared with controls (Fig. 4A). Similar mRNA expression patterns were seen for all cytokines. The use of PEEP decreased the proinflammatory cytokine mRNA expression levels relative to groups not treated with PEEP (Fig. 4B). When the interventions were grouped based on PEEP (n = 13) or no PEEP (n = 14), proinflammatory cytokine mRNA expression decreased twofold with the use of PEEP (P < 0.05). When surfactant treatment was added to PEEP, cytokine expression decreased further (Fig. 4C). When animals were analyzed based on surfactant administration (surfactant, n = 14 vs. no surfactant, n = 13), there were no differences in cytokine profiles. MCP-1 protein was measured in the BALF of all ventilated animals and followed trends similar to the mRNA expression (no PEEP, no surfactant = 0.14 ± 0.07 ng/kg vs. PEEP + surfactant = 0.03 ± 0.01 ng/kg; P > 0.05).

DISCUSSION

The 2010 guidelines for newborn resuscitation suggested that the use of PEEP in the delivery room should be beneficial, but stressed that studies to directly address this issue were lacking (29). This fetal lamb model, which isolated the lung injury and avoided oxygen exposure by ventilation with nitrogen, allowed us to demonstrate the beneficial effects of PEEP (20, 29). Our laboratory’s previous inability to show a benefit with PEEP during resuscitation of lambs using VT values of 8 or 15 ml/kg was likely...
due to injury caused by a subsequent period of ventilation (45). Although newborn infants requiring resuscitation often are maintained on mechanical ventilation for a period of time, any reduction in the initial injury at birth should be beneficial. Mechanical ventilation at birth triggers multiple, overlapping inflammatory and developmental pathways (21), and these pathways likely contribute to the initial injury that can progress to BPD (33).

The use of PEEP during the initiation of ventilation decreased markers of lung injury and activation of the proinflammatory cascade. The benefits of PEEP were seen mainly in the peripheral lung tissue as decreased cytokine mRNA expression compared with lambs ventilated without PEEP. The use of PEEP alone did not alter the indicators of airway stretch (HSP70 protein release, or mRNA upregulation, or increased acute phase mRNA in the airways). Phase-contrast X-ray imaging of preterm rabbits at birth showed PEEP decreased the influx of fluid into the airways during exhalation and helped recruit FRC (53). Although the PEEP may have maintained the lung fluid in the more distal lung, the airways were likely stretched, as they are highly compliant and distensible compared with the low compliance of the uninflated lung immediately at birth (18). The Egr-1 signal was intense around the small airways and bronchioles in lambs ventilated without PEEP or surfactant, demonstrating widespread activation in the peripheral lungs. There were consistent trends for decreased Egr-1 mRNA and protein for animals treated with PEEP. Cyr61 mRNA, an acute phase response gene primarily localized to the mesenchyme surrounding the alveolar ridges, decreased with the use of PEEP. The immature, surfactant-deficient lung is more prone to alveolar collapse, and PEEP decreases injury from collapse, by reducing atelectasis (40). PEEP decreases areas of atelectasis on computerized tomography scans of mechanically ventilated sheep (54). Recruitment maneuvers to decrease atelectasis before or during mechanical ventilation are being explored, but debate exists as to whether these maneuvers and the overdistention they may cause is helpful or harmful (48). Our laboratory showed previously that 7-cmH2O PEEP during prolonged ventilation improved oxygenation and surfactant pool size relative to no PEEP or a PEEP of 4 cmH2O (36), but the 7-cmH2O PEEP group had increased alveolar protein leak, lung inflammation, and alveolar distention (40). We used a higher PEEP of 8 cmH2O than in our laboratory’s previous studies in newborns.
lams (45) because ventilation with 8-cmH2O PEEP improved oxygenation during the initiation of ventilation in preterm lambs without significantly affecting arterial pressure (46). A PEEP of 8 cmH2O was the initial level of CPAP used in a trial to evaluate CPAP vs. intubation and ventilation in the delivery room (37).

Surfactant treatment before the initiation of ventilation had modest additive protective effects in lambs receiving PEEP. PEEP was necessary for optimal response to surfactant in premature rabbits (41, 47), and PEEP has been shown to be more important than surfactant in the recruitment of FRC (52). The lambs given surfactant and PEEP had higher lung compliance during the ventilation period and had larger lung volumes on postmortem pressure-volume curves. Although the Vt values were not significantly different between lambs receiving PEEP alone and those with both PEEP and surfactant, the number of lambs achieving the 15 ml/kg VT was higher in PEEP and surfactant animals, and fewer required the maximum PIP of 55 cmH2O. It is likely that the combination of PEEP and surfactant resulted in ventilation with a higher FRC. The decreased release of HSPs (HSP70 and HSP60) in the BALF suggests less airway stretch in the lambs receiving PEEP and surfactant (18). Our laboratory demonstrated previously that airway epithelial HSP70 was released into the BALF with ventilation, and its mRNA increased in the smooth muscles (18). The release of HSP70 and HSP60 into the air spaces may have implications for lung inflammation, as both are endogenous ligands for toll-like receptors (7). The distribution of Egr-1 staining was more heterogeneous in the lambs receiving surfactant, with patchy areas of signal surrounded by areas of staining in all lambs receiving surfactant (Fig. 4). In regions with nuclear Egr-1 staining, the intensity of Egr-1 signal was similar between groups, which suggests the differences in Egr-1 protein on Western blots were due to areas without Egr-1 activation. Our laboratory demonstrated previously that preterm lambs with endogenous surfactant pool sizes of >5–8 mg/kg have less Egr-1 staining than more surfactant-deficient lambs (17). It should be noted that surfactant treatment before ventilation caused increased total protein levels in the BALF, which corrected with PEEP, and caused a loss of the beneficial effects of PEEP on the expression of Cyr61 and CTGF in the lung. These effects could be due to overdistention of some of the alveoli during ventilation. The changes in Egr-1 distribution, along with increased compliance, suggest the lambs that received both surfactant and PEEP had higher FRC and a more uniform distribution of gas into the lung.

We explored the response and location of Egr-1, Cyr61, CTGF, and Nur77 because many acute phase response genes quickly change with mechanical ventilation or in response to factors that stimulate lung growth (57). Egr-1 activation occurs early in ventilation-induced injury, with expression in the distal airways and alveolar regions of premature sheep (18). Both Cyr61 and CTGF are secreted proteins of similar structure with a wide range of cellular functions (4). Lung fibroblasts when mechanically stressed have rapid upregulation of CTGF and Cyr61 mRNA levels that rapidly return to baseline once the cells are relaxed (49). Although CTGF and Cyr61 may have different effects on lung pathology, their coexpression occurs with multiple stimuli and in multiple tissue types (6). In preterm lambs, Cyr61, CTGF, and Egr-1 increased within 30 min of mechanical ventilation with Vt values of 5 ml/kg and can distinguish injury with ventilation with Vt values of 5–10 ml/kg (57). These markers decreased in lambs ventilated with high-frequency jet ventilation and time-adjusted PEEP levels (39). Cyr61 modulates angiogenesis through interaction with proangiogenic growth factors, and mice lacking Cyr61 have embryonic lethality due to placental insufficiency and compromised vessel integrity (6). Increased levels of Cyr61 in breast cancer leads to increased angiogenesis within the tumor and promotes resistance to anti-angiogenic chemotherapies (35). Cyr61 also protected lung epithelial cells exposed to hyperoxia and slowed the growth of small-cell lung cancer cells (25, 55). CTGF directly binds vascular endothelial growth factor and inhibits angiogenesis (24). Overexpression of CTGF in mice disrupted alveolarization and increased pulmonary hypertension (9) effects, similar to the pathophysiology of BPD in premature infants (1). The location of the CTGF mRNA to alveolar crests and in very distinct cells (Fig. 2i) is consistent with possible paracrine effects on alveolarization (33). A limitation of our study is our inability to determine the exact cell types expressing mRNA. Mechanical ventilation at birth

Fig. 4. Lung cytokine mRNA expression. Mechanical ventilation increased the mRNA for monocyte chemotactic protein-1 (MCP-1), IL-1β, IL-6, and IL-8 compared with controls. Animals ventilated with PEEP had less increase in mRNA, and the addition of surfactant and PEEP had less cytokine mRNA. Values are means ± SE. *P < 0.05 vs. animals receiving no PEEP, no surfactant. **P < 0.05 vs. PEEP 8 cmH2O, no surfactant. ‡P < 0.05 vs. no PEEP, surfactant.
alters the balance of angiogenic growth factors and likely contributes to the phenotype seen in infants with BPD.

Nur77, also known as NR4A1 or NGFI-B, is an orphan nuclear receptor that has no known ligand, but affects multiple metabolic pathways (42). Nur77 is induced by stress and norepinephrine release, and exercise will induce Nur77 in skeletal muscle (42). It increased 12-fold with high Vt ventilation in rats (11) and upregulation during overventilation was not due to inflammation alone (14). Nur77 mRNA was localized to the smooth muscles surrounding the larger airways in these lambs in a distribution similar to HSP70 following airway stretch. We also found increased signal in scattered cells throughout the peripheral lung. The role of Nur77 in lung injury and mechanical ventilation may involve multiple pathways. Nur77 contributes to vascular remodeling (62) through regulation of VEGF-A and angiogenesis (60). Nur77 also interacts with VEGF-A to increase vascular permeability, partially through regulation of endothelial nitric oxide synthase (61). In vivo, Nur77 appears to regulate genes involved with glucose transport and metabolism (42). Nur77 activation also plays a role in apoptosis in some cancers and cardiomyocytes (10, 58, 59). VLBW infants with BPD display both alveolar and capillary simplification, and mechanical ventilation alters transcription factors important for angiogenesis, such as VEGF (1). Analysis of postmortem lungs of ELBW infants ventilated from birth for a short period of time also demonstrates alterations in genes important for angiogenesis, such as VEGF (13). The early activation of Nur77, CTGF, or Cyr61 may contribute to the complex pathways that interact to cause BPD.

An advantage of a fetal model of resuscitation is our ability to isolate events during the initiation of ventilation from those that develop during continued ventilation (20). Although all ventilated lambs demonstrated activation of acute phase response genes, we are now able to demonstrate a benefit to PEEP that was not apparent in our laboratory’s initial studies in newborn lambs (45). It is likely that newborn lambs, when ventilated for 2 h, reached a threshold stimulus level that increased multiple inflammatory and noninflammatory pathways. The threshold levels for injury likely differed between lambs, since the animals were delivered at a gestational age at which large variations in surfactant pool sizes exist (17, 45). A similar differential effect of surfactant levels was seen in lambs breathing on CPAP (38), and lambs with surfactant deficiency had inflammation when maintained only on CPAP (44). In our study, we avoided the confounding effects of differential lung maturation by studying fetal lambs at a stage at which all lambs were surfactant deficient. High Vt ventilation at birth did not severely injure the lungs of term lambs (2); thus CPAP/PEEP may have less benefit for the mature lung. Variations in initial injury response must invariably exist within the ventilation trials in VLBW, which makes demonstrating a difference between inventions difficult (16, 37). Clinical studies of PEEP during resuscitation of VLBW infant would require very large patient numbers to demonstrate a benefit, if the primary outcome were BPD. There are many injury pathways, including the acute phase genes studied here, which are initiated by ventilation and may lead to BPD. A better understanding of the pathways involved and mechanisms of injury may lead to alternative approaches for decreasing BPD.

Conclusions. The initiation of ventilation in fetal lambs causes lung injury, acute phase response gene activation, and activation of proinflammatory cascades. The use of PEEP during this transition period from fetus to newborn decreases the early markers of lung injury, and surfactant administration at birth further decreases these markers of injury. Surfactant administration before ventilation at birth has an additive effect to PEEP, although this is impractical in the delivery room. We have demonstrated a benefit of PEEP during the initiation of ventilation, which provides experimental evidence for the 2010 International Liaison Committee on Resuscitation recommendation for the use of devices that reliably provide PEEP for resuscitation of newborn infants.

ACKNOWLEDGMENTS

Chiesi Pharma, Italy, kindly provided surfactant, and Fisher and Paykel Helathcare, New Zealand, kindly provided ventilator circuits. We thank Talia Sanders and Gabrielle Musk for help with animal anesthesia, Matt Kemp for animal breeding, and Megan McCalUliffe for work in the laboratory.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


