KL₄-Surfactant (Lucinactant) Protects Human Airway Epithelium from Hyperoxia

YAN ZHU, THOMAS L. MILLER, AARON CHIDEKEL, AND THOMAS H. SHAFFER

Nemours Research Lung Center [Y.Z., T.L.M., A.C., T.H.S.], Department of Pediatrics [A.C.], Alfred I. duPont Hospital for Children, Wilmington, Delaware 19803; Department of Pediatrics [T.L.M., A.C., T.H.S.], Thomas Jefferson University, Philadelphia, Pennsylvania 19107; Departments of Physiology and Pediatrics [T.H.S.], Temple University School of Medicine, Philadelphia, Pennsylvania 19140

ABSTRACT: Exogenous surfactant is critical in the treatment of neonates with respiratory distress syndrome. Lucinactant (Surfaxin; Discovery Laboratories, Inc.) is a surfactant replacement therapy containing sinulpeptide, which may reduce lung inflammation. This study tested whether Lucinactant reduces markers of inflammation, damage and remodeling in human airway epithelial cells exposed to hyperoxia. Calu-3 monolayers cultured at an air-liquid interface were treated apically with 140 μ L of normal saline, Lucinactant or Beractant (Survanta; Abbott Laboratories, Inc.). Treated monolayers were exposed to 60% O₂/5% CO₂ for 24 or 72 h. Transepithelial resistance (TER; $p < 0.001$) and cell viability ($p < 0.05$) were greater in both surfactant groups compared with saline; by 72 h Lucinactant cells had greater TER than Beractant ($p < 0.001$). Surfactant treated groups secreted less IL-8 than saline ($p < 0.001$), whereas Lucinactant cells secreted less IL-6 than saline and Beractant ($p < 0.001$). Matrix metalloproteinase 7, expressed by saline and Beractant treated cells at 24 h, was attenuated by 72 h by Beractant ($p < 0.001$), but was never detected in Lucinactant cells. Histology indicated less injury with Lucinactant relative to Beractant and saline. These data suggest that Lucinactant was protective compared with Beractant and control. (*Pediatr Res* 64: 154–158, 2008)

Preterm birth is complicated by respiratory distress syndrome (RDS), a condition that threatens survival in this population. In the preterm infant, decreased lung compliance and neonatal RDS are related to deficiencies in the lung surfactant pool (1). In addition, deficiencies in antioxidants (2,3) and antiinflammatory modulators (4,5) potentially worsen RDS and lead to development of chronic lung disease, most notably bronchopulmonary dysplasia (BPD).

RDS necessitates life-sustaining treatment with supplemental oxygen and mechanical ventilation. These therapies independently have been shown to injure the lung and are implicated in the pathogenesis of BPD (6). Surfactant replacement therapy has become standard of care for preventing and treating RDS (7,8). Although effective at acutely improving lung mechanics, oxygenation, and reducing mortality, surfactant replacement therapy has been linked to exaggerated inflammatory responses (9,10) and has not significantly impacted the incidence of BPD.

Lung inflammation in RDS is central to the pathogenesis of BPD (11), and is a complex process involving upregulation of proinflammatory cytokines, including IL-6 and IL-8, and influx of inflammatory cells to the airways (7). Neonatal deficiencies in antioxidants and antiinflammatory modulators favor a proinflammatory process that furthers epithelial injury and pulmonary remodeling by activated matrix modeling proteins such as matrix metalloproteinases (MMPs). MMPs released by injured cells contribute to greater microvascular permeability and release matrix components that function as proinflammatory mediators or growth factors to further inflammation and remodeling (12). MMP activity in the lung and airways has been associated with fibrotic lung diseases, including BPD (13,14).

Surfactant-associated proteins play important roles in maintaining pulmonary homeostasis. Deficiency of surfactant-associated proteins causes severe respiratory distress, and in the case of surfactant protein B (SP-B), is incompatible with life (15). SP-B is a hydrophobic protein that lowers alveolar surface tension (16), and may function in host defense as well. Lucinactant (Discovery Laboratories, Inc., Warrington, PA) is a synthetic surfactant preparation containing sinulpeptide (KL₄) in a concentration similar to SP-B found in human surfactant. KL₄ is a 21 amino acid peptide comprised of one lysine and four leucines (KLLLLKLLLLKLLLLKLLLLK) (17) that functionally mimics SP-B (18). Because surfactant proteins are present in relatively low amounts in animal-derived surfactant preparations, Lucinactant may offer antiinflammatory protection over these surfactant preparations.

The goal of the current study was to evaluate the potential benefit of Lucinactant in a well-characterized *in vitro* model of hyperoxic lung injury. We hypothesized that Lucinactant would have a favorable effect on indices of inflammation, cell damage, and tissue modeling, compared with an animal-derived surfactant preparation, Beractant (Survanta; Abbott Laboratories, Abbott Park, IL), and normal saline in the presence of hyperoxia.

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Correspondence: Yan Zhu, M.D., Nemours Research Lung Center, Alfred I. duPont Hospital for Children, AR-282, 1600 Rockland Road, Wilmington, DE 19803; e-mail: yzhu@nemours.org

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Abbreviations: ASF, apical surface wash fluid; BPD, bronchopulmonary dysplasia; KL₄ (sinulpeptide), synthetic peptide that mimics; MMP, matrix metalloproteinase SP-B; SP-B, surfactant protein B; TER, transepithelial resistance

MATERIALS AND METHODS

We evaluated the response of Calu-3 monolayers treated with 140 μL of normal saline, Lucinactant or Beractant to 60% O_2 /5% CO_2 for 24 or 72 h. Morphologic indicators of cellular condition including the loss of differentiated cell features, monolayer integrity, cellular swelling, or vacuole formation were determined by hematoxylin and eosin staining of intact monolayers and individual cells. Functional indices of cell injury included the maintenance of monolayer transepithelial resistance (TER), measurement of paracellular permeability, and assessment of cell viability by Trypan blue exclusion. Apical surface wash fluid (ASF) was assayed for IL-6/IL-8 and MMPs 2, 7, and 9 as indicators of inflammation and tissue remodeling.

Calu-3 cell culture. Reagents were purchased from Invitrogen (Carlsbad, CA) and transwell permeable supports from Corning Incorporated Life Sciences (Acton, MA). Calu-3 cells were cultured at 37°C and 5% CO_2 in a 50%/50% mixture of Dulbecco's Modified Eagle's Medium/ Ham's F-12 supplemented with 15% fetal calf serum, 500 u/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin. Cells were grown in 75 cm^2 tissue culture flasks and split when 90–95% confluent.

Calu-3 cell air-liquid interface culture was performed as previously described (19). Calu-3 cells were plated at 2×10^6 cells/ cm^2 onto Costar transwell inserts (0.4 μm pore size, 12-mm diameter, clear polycarbonate membrane; costar plate; Corning Inc., Acton, MA) treated with human Type I collagen (Southern Biotech, Birmingham, AL). Apical culture medium was removed 2 d after plating, and monolayers were grown at an air-liquid interface and fed basolaterally with DMEM/F12 with 15% fetal calf serum. The basolateral medium was changed on alternate days.

After 11 d, confluent Calu-3 monolayers exhibiting TER values of at least 800–1,000 $\Omega \cdot \text{cm}^2$ were treated with 140 μL of saline, Lucinactant, or Beractant, and exposed to hyperoxia ($\text{O}_2 = 60\%$, $\text{CO}_2 = 5\%$, balance nitrogen) in an airtight chamber (MIC-101; Billups-Rothenberg, Del Mar, CA). The volume of 140 μL was used since this is the minimal amount of liquid that enables complete coverage of each Calu-3 monolayer (20). To achieve the desired oxygen concentration, gas was blended using a Servo O_2 -air 960 mixer (Siemens-Elma, Sweden). Gas was warmed and humidified with a MR 730 respiratory humidifier (Fisher & Paykel Healthcare, Laguna Hills, CA). The chamber was flushed for 5 min at a flow rate of 20 lpm. Chamber oxygen level was monitored with an oxygen analyzer (MAXO₂; OM-25AE; Maxtec, Inc., Salt Lake City, UT). After purging, the chamber was sealed and placed in an incubator at 37°C. Gas in the chamber and medium in the plates were changed every 24 h (21).

At each experimental time-point, monolayers were used for measurement of TER ($n = 6$ per condition) or ASF collection ($n = 6$ per condition). Monolayers were then harvested by trypsinization for assessment of cell viability ($n = 6$ per condition) and cytomorphometry ($n = 3$ per condition), or fixed in 4% paraformaldehyde for histology ($n = 3$ per condition).

ASF samples were collected as previously described (22). Monolayers were washed twice with 140 μL of normal saline. Washes from each insert were combined for a total volume of 280 μL and centrifuged for 15 min at 13,000 rcf and 4°C to remove debris. Supernatants were stored in aliquots at –70°C for subsequent assays.

Measurement of cell viability. Cell viability was assessed using the Trypan blue-exclusion assay. Single cell suspensions were created and aliquots of each (20 μL) were mixed with 20 μL of 0.4% Trypan blue solution (Sigma Chemical Co.-Aldrich, St. Louis, MO). Cells were counted with a hemocytometer and cell viability (%) determined by the ratio of total viable cells (unstained) to total cells (stained and unstained).

Measurement of TER. TER measurements were made using STX2 electrodes and an EVOM meter (World Precision Instruments, Sarasota, FL). At baseline and after treatment, 0.5 mL of medium was added apically and 1 mL of medium was added basolaterally and electrodes inserted into each pool of medium to measure TER.

To validate TER as a measurement of monolayer integrity, we measured changes in permeability of Calu-3 monolayers with FITC-dextran (23). Monolayers with TER values between 150 and 1250 $\Omega \cdot \text{cm}^2$ were washed and FITC-dextran, 10 kD molecular weight; 1.0 mg in 0.2 mL phosphate-buffered saline, was added apically, and 0.7 mL phosphate-buffered saline without FITC-dextran was added basolaterally. The solution on each side of the monolayer was sampled (10 μL aliquots) at 10, 15, 25, and 30 min. The fluorescence of paired samples was analyzed at 496 nm with a fluorescent plate reader (CytoFluor Series 4000, PerSeptive Biosystems, Stafford, TX). The rate of FITC-dextran flux was determined by plotting the ratio of basal/apical fluorescent units against time relative to monolayer TER.

Measurement of IL-6 and IL-8 levels in Calu-3 ASF. ASF levels of IL-6 and IL-8 were measured in duplicate by quantitative ELISA using human IL-6 and IL-8 Quantikine ELISA kits (R & D Systems, Minneapolis, MN). The test sensitivity for respective immunoassays was as follows: IL-6 (0.039 pg/mL)

and IL-8 (10 pg/mL). Interassay and intraassay coefficients of variance are <10%.

MMP analysis by substrate zymography. Substrate zymography quantified the relative abundance of active and latent forms of MMP-2, MMP-7, and MMP-9. Loading buffer was added to ASF samples, and equal volumes of each sample were loaded into gels for electrophoresis. Protein was separated in 10% acrylamide gels containing 1 mg/mL gelatin (MMP-2 and MMP-9; Sigma Chemical Co., St. Louis, MO) or casein substrate (MMP-7; Sigma Chemical Co.) as previously described (24). After electrophoresis, SDS was eluted by washing the gels in 2.5% Triton-X-100. Gels were incubated for 18 (gelatin) or 48 h (casein) at 37°C in buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM CaCl_2 , and 0.01 mM ZnCl_2 . Gels were stained with Coomassie brilliant blue R250 and digitized for analysis.

Densitometric image analysis was performed using Scion Image software (Scion Corp., Frederick, MD) to determine pixel values for the expression of each sample. A ratio of the quantity of active to latent forms for each relevant MMP (A:L ratio) was calculated for each sample as the ratio of pixel values.

Histology and cytomorphology of Calu-3 cells. Fixed polycarbonate filters with adherent monolayers were separated from transwell inserts and embedded in paraffin. Cross-sections were obtained (5 μm thick) and stained with hematoxylin and eosin ($n = 3$ per condition).

Cytospin slides were prepared using 100 μL of single cell suspensions and stained for cell differentiation using KwikDiff solutions (Thermo Electron Corporation, Pittsburgh, PA). Cytomorphology was evaluated in a semiquantitative manner as the ratio of abnormal cells to total cells. Abnormal cells were defined by the following characteristics: 1) abnormal cellular appearance, 2) swollen nuclei, 3) intracellular and nuclear vacuoles, and/or 4) diffuse cytoplasm and cellular debris. Two hundred cells for each condition were evaluated in a blinded fashion.

Statistical analysis. All data are expressed as mean $\pm$ SEM. Differences between surfactant treatments and control for each outcome and the time of treatment was analyzed using two-way ANOVA. Where applicable, one-way ANOVA was run across different treatments at each time interval (*i.e.*, 24 and 72 h) and *post hoc* analyses were done using Bonferroni comparisons. Significance was accepted at $p < 0.05$.

RESULTS

Calu-3 monolayers treated with either Lucinactant or Beractant demonstrated greater cell viability ($p < 0.05$) than monolayers treated with normal saline at both 24 and 72 h time points (Fig. 1A). There was no difference in viability as a function of surfactant preparation at either time point. Treatment with either surfactant resulted in an increase in TER compared with normal saline at 24 and 72 h (Fig. 1B; $p < 0.001$). However, by 72 h, Lucinactant-treated cells had greater TER than those treated with Beractant ($p < 0.001$).

Data for FITC-dextran flux assays are shown in Fig. 2. At lower TER values (189, 272, and 883 $\Omega \cdot \text{cm}^2$), there was an inverse relationship between FITC-dextran flux and TER. At TER values above 883 $\Omega \cdot \text{cm}^2$, there was no detectable flux of FITC-dextran in the 30-min time period.

Figure 1. Cell survival and function for hyperoxic exposed Calu-3 monolayers treated with normal saline (■), Lucinactant (□) or Beractant (▨). Surfactant-treated monolayers (both surfactant preparations) demonstrated greater cell viability (A; $p < 0.05$) and transepithelial resistance (TER; B; $p < 0.001$) compared with saline. By 72 h, Lucinactant-treated cells had greater TER than Beractant-treated cells ($p < 0.001$). Data are mean $\pm$ SEM and $n = 6$ for each condition; *greater than saline; §greater than saline and Beractant.

Figure 2. FITC-dextran flux across Calu-3 monolayers with incremental values for transepithelial resistance (TER) to electrical current. These TER validation studies showed an inverse relationship between FITC-dextran flux and TER, which plateau with no detectable flux at or above a TER of $883 \Omega \cdot \text{cm}^2$. Data are a ratio of FITC-dextran concentrations in basolateral to apical cell culture medium at each time interval for respective monolayers of incremental TER. —TER = 189; ---TER = 272; ...TER = 883; -·-TER = 1239.

Figure 3. Proinflammatory mediators in apical surface wash fluid (ASF) from hyperoxic exposed Calu-3 monolayers treated with normal saline (■), Lucinactant (□), or Beractant (▨). Monolayers treated with Lucinactant secreted less IL-6 than those treated with Beractant or normal saline, independent of time (A; $p < 0.001$). Both surfactant preparations reduced IL-8 secretion compared with normal saline at 24 h (B; $p < 0.001$), where IL-8 secretion was also less for Lucinactant compared with Beractant ($p < 0.001$). Data are mean $\pm$ SEM and $n = 6$ for each condition; *less than saline; §less than saline and Beractant.

ASF samples from Lucinactant-treated monolayers contained less IL-6 (Fig. 3A) than those treated with either normal saline or Beractant at both 24 and 72 h ($p < 0.001$). Secretion of IL-6 by Beractant-treated monolayers was not different from normal saline. At 24 h, IL-8 (Fig. 3B) concentrations in ASF were less for both surfactant-treated groups than for normal saline ($p < 0.001$), and Lucinactant-treated monolayers produced less IL-8 than Beractant at 24 h ($p < 0.001$). By 72 h, IL-8 concentrations in ASF were reduced in all monolayers with no difference between groups.

Zymography detected MMP-2 and MMP-7 in their pro-forms, whereas no MMP-9 was detected in ASF from any group. Data for MMP-2 and 7 are shown in Table 1. MMP-2 was expressed in all groups at 24 h with no group differences, but by 72 h, MMP-2 expression was undetectable in all groups. MMP-7 was expressed by monolayers from the normal saline and Beractant groups at 24 h, with no difference between these groups. By 72 h, MMP-7 expression by the normal saline group was not different from 24 h, but was reduced to undetectable levels in the Beractant group ($p < 0.001$ compared with normal saline and 24 h Beractant val-

Table 1. Data for matrix metalloproteinases (MMPs) 2 and 7 activity in Calu-3 monolayer apical surface wash fluid

	MMP-2		MMP-7	
	24 h	72 h	24 h	72 h
Normal Saline	912 $\pm$ 200	UD*	988 $\pm$ 215	703 $\pm$ 278
Lucinactant	947 $\pm$ 151	UD*	UD*†	UD*
Beractant	1043 $\pm$ 135	UD*	1386 $\pm$ 358	UD*

Data are expressed as pixels values from image analysis and reported as mean $\pm$ SEM. Undetectable (UD) is shown for conditions where MMP expression was below the assay detection limit.

* $p < 0.001$ compared with 24 h normal saline values.

† $p < 0.001$ compared with 24 h Beractant values.

Figure 4. Histologic analysis of Calu-3 monolayers on transwell membrane filters ($n = 6$ for each condition). Monolayers treated with normal saline (A and D) demonstrate abnormal cellular appearances, a loss of cellular integrity, and swollen nuclei. All images were examined by light microscopy at $40\times$ magnification (calibration bar = $10 \mu\text{m}$).

Figure 5. Cytomorphological examination of Calu-3 cells treated with normal saline (A and D), Lucinactant (B and E), and Beractant (C and F) after 24 and 72 h of hyperoxia. Monolayers treated with Lucinactant showed less swollen nuclei and intracellular vacuoles than those treated with Beractant or normal saline. Cells treated with normal saline demonstrate more abnormal cellular appearances. All cytopins were examined by light microscopy at $40\times$ magnification (calibration bar = $20 \mu\text{m}$).

ues). However, the Lucinactant group never expressed detectable amounts of MMP-7, making these monolayers different from normal saline independent of time ($p < 0.001$), and different from Beractant at 24 h ($p < 0.001$).

Representative histology at 24 and 72 h are presented in Figs. 4 and 5. Calu-3 cells from all groups exhibited abnormal cellular appearances, a loss of cellular integrity, and swollen nuclei. Cells obtained from monolayers treated with Lucinactant showed less swollen nuclei and intracellular vacuoles than those treated with Beractant or normal saline, independent of time. Semiquantitative assessment of cytomorphology (Fig. 6) showed a main effect for group, independent of time, where the ratio of abnormal cells to total cell count was less for the Lucinactant and Beractant groups than for normal saline, and furthermore, Lucinactant was less than Beractant ($p < 0.001$).

Figure 6. Semiquantitative assessment of cytomorphology. A ratio of abnormal cells to total cell count showed that there were less abnormal cells in the Lucinactant (□) and Beractant (▨) groups compared with normal saline (■), and less abnormal cells after Lucinactant treatment compared with Beractant ($p < 0.001$). Data are mean $\pm$ SEM and $n = 6$ for each condition; *less than saline; §less than saline and Beractant.

DISCUSSION

This study used cultured human airway epithelial cells to characterize the effects of two surfactant preparations on cellular responses to moderate hyperoxia. Calu-3 cells are derived from human bronchial submucosal glands and are a well-characterized cell line forming high-resistance monolayers at an air-liquid interface. This model has been used to screen drug candidates delivered to the respiratory epithelium (25,26), and to evaluate oxidant stress in lung disease (27). Furthermore, the basal level apical fluid and protein secretion in Calu-3 cultures (19,21,22) are similar to primary airway cultures (28–30). Thus, Calu-3 cells mimic the characteristics of the bronchiolar epithelium, which *in vivo* serves as the barrier layer between inspired gas and other visceral tissues.

Surfactant treatment improved cell survival and function and reduced airway epithelial cell inflammation compared with treatment with normal saline. Furthermore, Lucinactant improved cell function over an animal-derived surfactant by 72 h. These findings are consistent with the effects of KL₄ peptide that prevents hyperoxic and lipopolysaccharide-induced lung injury in mice (31). There is much clinical interest in the potential differences and possible advantages of natural surfactant preparations compared with synthetic surfactant preparations. In clinical trials, Lucinactant lowered the death rate from RDS at 14 d, but had no impact on BPD or mortality at 36-wk postmenstrual age (32), or at 1 y (33). Meta-analyses of clinical trials comparing natural and synthetic surfactants have also shown little difference (34). Further *in vitro* study may clarify any distinctions and even aid in future study design. Surfactant-treated monolayers demonstrated greater cell viability, enhanced histologic outcomes and TER compared with normal saline. By 72 h, Lucinactant-treated monolayers had improved cellular appearance and greater TER than those treated with Beractant. TER was a surrogate marker for epithelial permeability, representing the integrity of intracellular junctions (23). The relationship between TER and intracellular junctions is supported by the

correlation between TER and rates of FITC-dextran flux whereby the disruption of tight-junctions allows FITC-dextran to pass through the paracellular space and be detected at the basolateral side.

The proinflammatory mediators IL-6 and IL-8 are chemokines causing neutrophil infiltration and exaggerated systemic inflammation (35). It has previously been demonstrated that elevated IL-6 and IL-8 levels *in vivo* can predict chronic lung disease in preterm infants (36,37). In our previous studies, hyperoxia induced Calu-3 cells to secrete high levels of IL-6 and IL-8 in a dose-dependent manner (20): at 24 h, ASF IL-6 and IL-8 from Calu-3 monolayers exposed to 60% FiO₂ was greater than those exposed to 21% FiO₂ (control), whereas by 72 h IL-6 returned toward control level (20). The current data demonstrated that the secretion of IL-6 and IL-8 in ASF of Calu-3 monolayers exposed to 60% FiO₂ and treated with Lucinactant or Beractant surfactant was reduced at 24 and 72 h compared with monolayers treated with normal saline. For the Lucinactant group, IL-6 and IL-8 levels were much lower than both the normal saline group and the Beractant group indicating that Lucinactant reduced inflammation. This may be attributable to KL₄, a conclusion consistent with a study that demonstrated decreased inflammation and enhanced lung function in adult mice with SP-B deficiency treated with SP-B *in vivo* (38).

MMPs were a focus of this study since a growing body of evidence suggests that these tissue-modeling proteases play a role in the structural development of conducting airways. Differences in MMP activity have been associated with BPD (13) and chronic fibrotic lung diseases in other populations (14). Furthermore, our laboratory has demonstrated that MMPs are secreted into the airway lumen during injury (39), and therefore could play a role in epithelial denudation. The decrease in MMP-7 associated with Lucinactant therapy is viewed as protective because MMP-7 is known to facilitate passage of neutrophils across the alveolar epithelium (40,41). Data from the current study indicate that MMP-7 is a factor in airway injury associated with elevated oxygen concentration, and that the expression of this mediator can be attenuated by surfactant therapy.

In summary, this *in vitro* study of airway epithelial cells treated with normal saline, Lucinactant or Beractant demonstrated that the peptide-based synthetic surfactant Lucinactant provides antiinflammatory protection compared with an animal-derived surfactant and saline control over 72 h of moderate hyperoxia. KL₄-surfactant may have an intrinsic benefit over animal-derived surfactants with respect to attenuating the deleterious effects of supplemental oxygen therapy.

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