Alterations to surfactant precede physiological deterioration during high tidal volume ventilation

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1Lawson Health Research Institute, and Departments of 1Medicine, 2Physiology and Pharmacology, 3Biochemistry, and 4Obstetrics and Gynecology, The University of Western Ontario, London, Ontario, Canada

Submitted 19 December 2007; accepted in final form 9 March 2008

Maruscak AA, Vockeroth DW, Girardi B, Sheikh T, Possmayer F, Lewis JF, Veldhuijen RA. Alterations to surfactant precede physiological deterioration during high tidal volume ventilation. Am J Physiol Lung Cell Mol Physiol 294: L974–L983, 2008. First published March 14, 2008; doi:10.1152/ajplung.00528.2007.—Lung injury due to mechanical ventilation is associated with an impairment of endogenous surfactant. It is unknown whether this impairment is a consequence of or an active contributor to the development and progression of lung injury. To investigate this issue, the present study addressed three questions: Do alterations to surfactant precede physiological lung dysfunction during mechanical ventilation? Which components are responsible for surfactant’s biophysical dysfunction? Does exogenous surfactant supplementation offer a physiological benefit in ventilation-induced lung injury? Adult rats were exposed to either a low-stretch (tidal volume (Vt) = 8 ml/kg, positive end-expiratory pressure (PEEP) = 5 cmH2O, respiratory rate (RR) = 54–56 breaths/min (bp), fractional inspired oxygen (FiO2) = 1.0) or high-stretch (Vt = 30 ml/kg, PEEP = 0 cmH2O, RR = 14–16 bpm, FiO2 = 1.0) ventilation strategy and monitored for either 1 or 2 h. Subsequently, animals were lavaged and the composition and function of surfactant was analyzed. Separate groups of animals received exogenous surfactant after 1 h of high-stretch ventilation and were monitored for an additional 2 h. High stretch induced a significant decrease in blood oxygenation after ventilation. Alterations in surfactant pool sizes and activity were observed at 1 h of high-stretch ventilation and progressed over time. The functional impairment of surfactant appeared to be caused by alterations to the hydrophobic components of surfactant. Exogenous surfactant treatment after a period of high-stretch ventilation mitigated subsequent physiological lung dysfunction. Together, these results suggest that alterations of surfactant are a consequence of the ventilation strategy that impair the biophysical activity of this material and thereby contribute directly to lung dysfunction over time.

Mechanical ventilation is an essential therapeutic intervention employed in all patients with acute lung injury (3). However, it has been well established that mechanical ventilation can also propagate the existing lung injury (5, 30, 37). This damaging effect is caused, in part, by collapse and overdistention of lung regions that occur during ventilation of a heterogeneously injured lung. This phenomenon can be studied in healthy animals by utilizing extremely high tidal volumes and zero end-expiratory pressure during ventilation of normal lungs (37). The injury in these animal models has been termed ventilation-induced lung injury (VILI). Alterations to the endogenous pulmonary surfactant system are an important component of the pathophysiology of VILI (31, 34, 37).

Surfactant is a mixture of phospholipids (80%), neutral lipids (5–10%), and surfactant-associated proteins (SP-A, SP-B, SP-C, and SP-D) that is secreted into the alveolar air space from type II cells (11). Once secreted, surfactant forms highly organized lipid-protein structures that are capable of forming a surface-tension-reducing surface film (38). These biophysically active structures are called the large aggregates (LA). During respiration an inactive form of surfactant is produced, designated the small aggregates (SA). The main function of surfactant is the reduction of surface tension at the air-liquid interface of the alveoli, which allows the lung to inflate with minimal effort, thereby contributing directly to lung function.

As can be deduced from these important biophysical properties of surfactant, impairment or deficiency of this material would result in impaired lung function. The prototypic clinical example of this latter situation is the neonatal respiratory distress syndrome, in which a developmental deficiency of surfactant causes severe lung dysfunction unless exogenous surfactant is administered (27). Impairments of surfactant function in mature lungs contributes to the lung dysfunction associated with acute lung injury; however, to date, exogenous surfactant therapy has not proven to be consistently beneficial in this setting (20, 21).

Alterations of surfactant due to mechanical ventilation have been studied in a variety of animal models of VILI (31, 34, 36). The specific alterations observed include a reduction in the amount, composition, and structure of the LA subfraction and an impairment in the surface activity of these LA, possibly due to the inhibition by serum proteins that had leaked into the lung (7). Interestingly, these observations have been made at a time point at which the lung injury due to ventilation was severe. As such, it is unknown whether alterations of the surfactant system are the cause or a consequence of the lung dysfunction present in these animals.

On the basis of the above information, the present study utilized an animal model of VILI to address three specific questions: 1) Do alterations to pulmonary surfactant precede physiological dysfunction during the development of VILI? 2) Which components of the surfactant system are responsible for its biophysical dysfunction? and 3) Does surfactant supplementation offer a physiological benefit in VILI?

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METHODS

Animal Experimentation

A total of 82 male Sprague-Dawley rats (350–450 g, Charles River, St. Constant, PQ, Canada) were utilized. These animals were used for three separate animal experiments. All procedures were approved by the animal use subcommittee at the University of Western Ontario under the guidelines of the Canadian Council of Animal Care.

For all three experiments rats were acclimated for a minimum of 3 days in an animal housing facility, during which time they were group housed with free access to water and standard chow. The mass of each rat was recorded and animals were anesthetized with an intraperitoneal injection of 75 mg/kg ketamine and 5 mg/kg xylazine in sterile 0.15 M NaCl. Following anesthesia, the animals received a subcutaneous injection of analgesic (0.05–0.1 mg/kg buprenorphine). The left carotid artery and both jugular veins were exposed and cannulated with PE-50 tubing. The carotid line was used to obtain arterial blood samples for blood gas measurements (model ABLS500, Radiometer, Copenhagen, Denmark), monitor heart rate and blood pressure, and deliver fluid (sterile NaCl and 100 IU heparin/l) by use of an infusion pump set at 0.5 ml/h. The right jugular line was used to deliver additional anesthetic (0.5–2.0 mg/100 g h−1 propofol), and the left jugular line was used to deliver additional fluid (0.5 ml/100 g h−1). The trachea was exposed and a 14-gauge plastic endotracheal tube was secured with 2-0 surgical silk. The animal was administered a neuromuscular paralytic agent intravenously (2 mg/kg pancuronium bromide) to stop spontaneous breathing and was immediately connected to a volume-cycled rodent ventilator (Harvard Instruments, St. Laurent, PQ, Canada) set at a baseline tidal volume (VT) of 8 ml/kg, a positive-end expiratory pressure (PEEP) of 5 cmH2O, a respiratory rate (RR) of 54–56 breaths/min (bpm), and a fractional inspired oxygen (FIO2) of 1.0. All rats were ventilated by this strategy for 10 min prior to the first blood gas measurement. Airway pressures were measured throughout ventilation via an airway pressure monitor (Sechrist Industries, Anaheim, CA) connected to the inspiratory limb of the ventilator. Inclusion criteria for all experiments required animals to have an arterial partial pressure of oxygen (Pao2) to fractional percentage of inspired oxygen ratio (Pao2/FIO2) >400 mmHg, arterial partial pressure of carbon dioxide (Paco2) between 35–40 mmHg, peak inspiratory pressure (PIP) <15 cmH2O, and mean systolic blood pressure between 80 and 120 mmHg.

Experiment 1

The first experiment utilized 20 animals to examine the effects of two different ventilation strategies employed for two different time periods on physiology, surfactant pool sizes, cholesterol and surfactant protein levels in the LA, total protein concentrations in the lung lavage, and concentrations of IL-6 and TNF-α in the lavage.

Upon reaching inclusion criteria, animals were randomized to receive mechanical ventilation for 1 or 2 h, with either a noninjurious, low-stretch (LS) ventilation strategy with PEEP (VT = 8 ml/kg, PEEP = 5 cmH2O, RR = 54–56 bpm) designated as LS1h and LS2h groups, or an injurious high-stretch (HS) strategy with no PEEP (VT = 30 ml/kg, PEEP = 0 cmH2O, RR = 14–16 bpm) designated as HS1h and HS2h. During randomization, all animals were briefly disconnected from the ventilator prior to initiation of the respective ventilation strategy; whereas animals in the LS groups were reconnected to the ventilator with the baseline settings, HS animals were reconnected to a second ventilator calibrated to a VT of 30 ml/kg. Measures of blood pressure and PIP were recorded every 15 min, with blood gas measurements taken at 15 min and then every half hour following randomization.

After the 1- or 2-h ventilation period, animals were euthanized with an intravenous overdose of pentobarbital sodium (110 mg/kg). A midline sternotomy was performed to expose the lungs and animals were exsanguinated by transection of the descending aorta. Lung compliance was assessed via pressure-volume curve measurements as previously described (7). Briefly, lungs were allowed to deflate passively and connected to a U-tube manometer attached to a pressure gauge. The lung was inflated in stepwise increments of 2 cmH2O pressure to a maximal pressure of 26 cmH2O while the administered volume was recorded at each pressure value. The same stepwise procedure was performed on deflation (7).

Surfactant, total protein, and cytokine analysis. Following pressure-volume curve analyses, lungs were lavaged with 10 ml of sterile 0.15 M NaCl, which was instilled and withdrawn three times (7). One millilitre of the first lavage was aliquoted for cytokine analysis. The remaining lavage was combined with the fluid from four additional 10-ml lavages. Total lavage volume was recorded and centrifuged at 150 g for 10 min to remove cellular debris. The supernatant of this centrifugation was termed total surfactant (TS). A 3-ml aliquot of TS was retained for analysis. Centrifugation at 40,000 g for 15 min separated the remaining TS into the surfactant containing the small aggregate subfraction (SA) and pellet containing the large aggregate subfraction (LA). The LA pellet from each animal was then resuspended in 2 ml of sterile 0.15 M NaCl. All samples were stored at −20°C following separation into the TS, LA, and SA subfractions.

A chloroform-methanol lipid extraction was performed on aliquots of TS, LA, and SA samples, followed by a Duck-Chong phosphorus assay to determine the amount of phospholipids within these subfractions (2, 8). A Micro BCA protein assay was used to determine the amount of total protein in the TS and LA subfractions, by following the manufacturer’s instructions (Pierce Biotechnology, Rockford, IL). Total cholesterol present in the LA subfraction was quantified by an enzymatic colorimetric method (Wako Chemicals, Richmond, VA) as previously described (24).

SP-A measurements. A volume equivalent to 3 μg of phospholipid from LA samples were loaded into a stacking gel and the proteins were separated via 2 h of electrophoresis at 100 V (Bio-Rad Laboratories, Mississauga, ON, Canada) through a 10% polyacrylamide separating gel containing 0.1% SDS. The samples were run alongside 10 μl of human SP-A standard (0.19 μg/μl) and a 10-μl molecular weight standard (Kaleidoscope prestained standards). The proteins were then transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membranes were blocked overnight at 4°C in 5% blocking agent. The membranes were blocked overnight at 4°C in Tris-buffered saline, pH 7.4. The membranes were then incubated for 2 h in rabbit anti-rat SP-A IgG primary antibody with a 1:1,000 dilution in 1× PBS, prior to being washed for 15 min, then 2 × 5 min in 1× PBS with 0.05% Tween-20, followed by 5-min washing in 5% blocking milk in PBS. Following the washing, the nitrocellulose membranes were incubated for 2 h in donkey anti-rabbit IgG secondary antibody in 1× PBS (1:1,000), prior to being washed again for 15 min, then 3 × 5 min in 1× PBS with 0.05% Tween-20. Finally, supersignal (Biolyxin OptiBlaze WEST picoLUCENT, G-Biosciences, St. Louis, MO) was applied to all nitrocellulose membranes and chemiluminescence was observed. Densitometry was performed and immunoreactivity was expressed as a ratio to the LS1h samples on each respective membrane.

SP-B measurements. A volume equivalent to 10 μg of phospholipid from LA samples were added into a stacking gel and the proteins were separated via 2 h of electrophoresis at 100 V (Bio-Rad Laboratories, Mississauga, ON, Canada). The samples were run alongside 10 μl of human SP-B standard (0.23 μg/μl) and 10-μl molecular weight marker. The proteins were then transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA), and the membranes were blocked overnight at 4°C in NAP-blocker blocking agent (G-Biosciences, St. Louis, MO) at 1:1 dilution in 1× Tris-buffered saline (TBS). The membranes were then incubated for 2 h in SP-B monoclonal antibody (generously supplied by Dr. Suzuki, Kyoto University, Kyoto, Japan) at a 1:1,000 dilution in 1× TBS, prior to being washed for 15 min, then 2 × 5 min in 1× TBS with 0.05%
 Tween-20, followed by 5-min washing in NAP-blocker. Following the washing, the nitrocellulose membranes were incubated for 2 h with goat anti-mouse horseradish peroxidase-IgG secondary antibody in 1× TBS (1:1,000), prior to being washed again for 15 min, then 3× 5 min in 1× TBS with 0.05% Tween-20. Finally, supernignal (Biolynx OptiBlaze WEST picoLUCENT, G-Biosciences) was applied to all nitrocellulose membranes and chemiluminescence was observed. Densitometry was performed and immunoreactivity was expressed as a ratio to the LS1h samples on each respective membrane.

Cytokine analysis. The 1-ml aliquot recovered from the first lavage was centrifuged at 4°C, 200 g, for 10 min. The supernatant was separated into 240-μl aliquots and snap frozen in liquid nitrogen before being stored at −80°C until cytokine analysis. IL-6 and TNF-α were measured by using opti-EIA ELISA kits following the manufacturer’s specifications (Pharmigen, San Diego, CA).

Experiment 2
A separate cohort of 42 animals was utilized in experiment 2 to collect sufficient amounts of LA samples from the four experimental groups described in experiment 1, to examine the effects of the different ventilation strategies on the biophysical properties of surfactant. Animal experimentation and collection of lavage material was identical as described for experiment 1. The entire lavage volume from each animal was centrifuged at 150 g and subsequently at 40,000 g to obtain a LA pellet, which was resuspended in 2 ml of sterile 0.15 M NaCl. Subsequently, LA samples from each group of animals were pooled and further processed into four different preparations: crude LA, purified LA, extracted LA, and extracted + 5% SP-A LA.

Crude LA samples required no additional preparation after centrifugation and resuspension as described above, purified LA samples were prepared via sucrose gradient separation (7). Briefly, samples of crude LA were divided into 2-ml aliquots and then placed onto 15 ml of 0.8 M sucrose, followed by centrifugation at 40,000 g for 15 min. The pellet was removed from the surface of the sucrose, diluted with 0.15% NaCl, and then recentrifuged at 40,000 g for 15 min to remove remaining sucrose. This washing process was performed twice and the final pellet was resuspended in 0.15 M NaCl.

Preparation of extracted LA samples was performed via lipid extraction of separate samples of crude LA samples by the method of Bligh and Dyer (2) using chloroform-methanol (2:1 vol:vol). The chloroform layer, containing the hydrophobic surfactant components, was dried under nitrogen followed by reconstitution of the dried sample in 0.15 M NaCl. Finally, extracted + 5% SP-A LA samples were prepared by supplementation of lipid extracted LA with 5% human SP-A (wt/wt). Human SP-A was obtained from a patient with alveolar proteinosis as described elsewhere (1, 15).

Each LA preparation from the four groups was analyzed for phospholipid phosphorous as described above, to obtain a phospholipid concentration. A total protein assay was also performed on crude and purified LA samples to verify the removal of nonsurfactant proteins.

Biophysical functional analysis. A custom-designed captive bubble surfactometer was used to study the biophysical function of pooled LA samples obtained in experiment 2 (28). All samples were resuspended in a buffer (0.15 mM NaCl, 2 mM Tris-HCl, 1.5 mM CaCl2, pH = 7.4) at a phospholipid concentration of 300 μg/ml and incubated at 37°C for 1 h.

Briefly, a gas-tight chamber was filled with a surfactant suspension and allowed to equilibrate to a temperature of 37 ± 1°C. An air bubble of ~8 mm in diameter was introduced into the suspension and the shape of the bubble was recorded by digital photography as a function of time. The digital images of the bubble were used to measure the area and volume and to calculate the surface tension at the air-liquid interface inside the bubble. Immediately following introduction of the bubble, the change in shape was recorded as a function of time to monitor adsorption of the surfactant material to the air-liquid interface. After an equilibrium surface tension of ~23 mN/m was attained, the chamber was sealed, and one quasi-static compression-expansion cycle was performed to establish the surface area reduction required to obtain minimum surface tension prior to dynamic cycling. Dynamic cycling of the air-liquid interface within the bubble was conducted by first allowing the surfactant to readсорb to equilibrium surface tension, followed by compression and expansion of the bubble at 30 cycles/min between 100 and 110% of the original surface area and the area required to achieve minimum surface tension established during the single quasi-static cycle.

Experiment 3
The third experiment was performed on 20 rats to address the role of the surfactant system in the physiological development and progression of VII. Once inclusion criteria (similar to experiment 1) were met, rats were randomized to receive 1 h of either a LS or a HS mechanical ventilation strategy as described for experiment 1. Following the 1-h period of ventilation rats were further randomized to receive treatment with 20 mg phospholipids/kg body wt of a commercially available exogenous surfactant preparation, BLES (Bovine Lipid Extract Surfactant, BLES Biochemicals, London, ON, Canada), or no treatment as a control group. This dose of surfactant was chosen on the basis of previous studies (1) and preliminary experiments, to increase the surfactant pool size by three- to fourfold. This resulted in four separate experimental groups: 1) 1 h of LS mechanical ventilation with no treatment (LS-NT), 2) 1 h of LS mechanical ventilation with BLES treatment (LS-BLES), 3) 1 h of HS mechanical ventilation with no treatment (HS-NT), and 4) 1 h of HS mechanical ventilation with BLES treatment (HS-BLES).

Surfactant administration involved disconnecting the rat from the ventilator and injecting the BLES solution through the endotracheal tube directly into the lung via a 3-ml syringe. Each instillation was followed by administration of an air bolus and, upon reconnection to the ventilator, a sigh was performed by blocking the expiratory line for two breaths to optimize peripheral distribution. Control rats underwent the same procedure but were administered an air bolus. All rats were subsequently ventilated for an additional 2 h by using only the LS strategy. During ventilation, PIP and mean arterial pressure were monitored and blood gas samples were taken at 15 min intervals. Following this ventilation period animals were euthanized and lavaged as described for experiment 1.

Statistical Analysis
All values are reported as means ± SE. Statistical analysis was performed by a two-way ANOVA to explore any interactive effects followed by a one-way ANOVA with Tukey’s post hoc test for pairwise comparison of groups. Changes over time were compared between groups by repeated-measures ANOVA with a Tukey’s post hoc test. All statistical analysis was performed utilizing SPSS 11.0. Differences between and within groups were considered statistically significant at probability values of <0.05.

RESULTS

Experiment 1

Physiology. Figure 1 shows the PaO2/FIO2 ratio during mechanical ventilation for the four experimental groups. Within the LS groups there was no significant effect of time on PaO2 values, with all values remaining above 420 mmHg. Animals ventilated with the HS strategy had PaO2/FIO2 values that were not significantly different from their respective baseline measurements up to the 90-min time point. At 120 min, the PaO2 values had significantly decreased compared with all other earlier time points. Statistical comparisons between ventilation
Strategy groups revealed no significant differences in PaO₂/FIO₂ values between the LS1h and HS1h groups. However, at 120 min, there was a significantly lower PaO₂/FIO₂ value measured in the HS2h group compared with values recorded from the LS groups.

Values of other physiological outcomes recorded during ventilation are shown in Table 1. PaCO₂ values did not change over time within any group but were significantly lower in both HS groups compared with values recorded from the LS groups. PIP values recorded in both LS ventilation groups also did not change significantly over time. Ventilation with HS resulted in higher PIP values than the LS groups and increased within the HS2h group during the last 30 min of ventilation, such that PIP values after 2 h of HS ventilation were significantly higher compared with all earlier time points. Arterial blood pressure remained constant for all animals at all time points with the exception of the HS2h group, which had significantly lower blood pressure values at 120 min compared with the LS2h group and compared with earlier time points within the HS2h group.

Figure 2 shows the pressure-volume curves measured after euthanasia of animals in the four experimental groups. There were no significant differences in the pressure-volume curves of lungs in animals from the LS1h and LS2h groups. Lung compliance was lower in the HS2h group compared with the HS1h group as determined by the significantly lower volumes at 26 cmH₂O (Vmax). Further comparison between the two ventilation strategies revealed that there were no significant differences between the LS1h and HS1h groups; however, the Vmax was significantly lower in the HS2h group compared with the LS2h group.

**Surfactant analysis.** Values for total surfactant, LA, SA, and %LA are shown in Fig. 3. There was no significant difference in any of these four outcomes when comparing the LS1h and LS2h groups. Comparison of the two HS groups revealed no significant differences in total surfactant and SA pool sizes; however, LA and percent LA were significantly lower in the HS2h group compared with the HS1h group. Comparisons between the two ventilation strategies revealed that, in general, HS ventilation resulted in higher TS pools compared with LS ventilation, which was statistically significant for HS1h compared with LS1h (Fig. 3A). SA pools were also greater and %LA lower in the HS groups vs. LS groups. There were no differences in LA pool sizes between the LS1h and HS1h; however, there was a significantly smaller LA pool obtained from the HS2h group compared with the LS2h group.

Further analysis of the LA fraction was performed to assess the levels of SP-A, SP-B, and cholesterol relative to the amount of phospholipid in this subfraction (Table 2). Comparison of the two time points revealed no significant differences between the two LS groups. Within the HS ventilation groups, the 2-h time point had significantly lower SP-B levels, and significantly higher cholesterol values, compared with the 1-h time point. These SP-B and cholesterol values in the HS2h were also significantly different than the values in the LS2h group.

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**Table 1. PaCO₂, PIP, and blood pressure values at different times during ventilation in the experimental groups described in Fig. 1**

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<tr>
<td>PaCO₂, mmHg</td>
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<tr>
<td>Baseline</td>
<td>42.2±0.7</td>
<td>39.4±0.9</td>
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<tr>
<td>30 min</td>
<td>39.9±1.0</td>
<td>38.9±1.0</td>
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<tr>
<td>60 min</td>
<td>41.3±1.0</td>
<td>39.2±0.8</td>
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<tr>
<td>90 min</td>
<td>39.6±1.1</td>
<td>33.0±1.1‡</td>
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<tr>
<td>120 min</td>
<td>39.4±1.0</td>
<td>33.4±1.5‡</td>
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<tr>
<td>PIP, cmH₂O</td>
<td></td>
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<tr>
<td>Baseline</td>
<td>13.1±0.2</td>
<td>13.3±0.2</td>
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<tr>
<td>30 min</td>
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<tr>
<td>60 min</td>
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<td>13.3±0.2</td>
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<tr>
<td>90 min</td>
<td>13.3±0.2</td>
<td>28.8±1.2‡</td>
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<tr>
<td>120 min</td>
<td>13.5±0.2</td>
<td>36.2±1.2‡</td>
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<td>Blood pressure, mmHg</td>
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<tr>
<td>Baseline</td>
<td>88.9±2.3</td>
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<td>30 min</td>
<td>84.2±3.0</td>
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<tr>
<td>60 min</td>
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<td>90.6±3.8</td>
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<td>120 min</td>
<td>99.0±2.8</td>
<td>65.3±4.9†</td>
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Values are means ± SE. PaCO₂, arterial partial pressure of CO₂; PIP, peak inspiratory pressure. *P < 0.05 for 1-h high-stretch group (HS1h) vs. 1-h low-stretch group (LS1h); †P < 0.05 for 2-h high-stretch group (HS2h) vs. 2-h low-stretch group (LS2h).
There were no significant differences in SP-A levels among the groups.

Total protein and cytokine levels. Lung lavage material was analyzed for total protein as well as inflammatory cytokines TNF-α and IL-6 (Table 3). There were no differences between the two LS groups in either the total amount of protein recovered from the lavage or the concentrations of inflammatory cytokines. In comparing the two HS groups, there was significantly more protein, TNF-α, and IL-6 in the lavage from animals ventilated for 2 h compared with the 1-h group.

Comparison between the two ventilation strategies revealed no significant difference at the 1-h time point, but significantly more protein, TNF-α, and IL-6 in the HS group after 2 h of ventilation.

Experiment 2

Experiment 2 investigated the biophysical consequences of the alterations to surfactant observed in experiment 1. Functional assessment of the crude (the resuspended 40,000 g pellet) LA samples from the four experimental groups is shown in Fig. 4. The surface tension during adsorption was not significantly different among the four experimental groups.

Table 2. Values of SP-A, SP-B and cholesterol in the LA subfraction for the 4 experimental groups described in Fig. 1

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<tr>
<td>1 h</td>
<td>2 h</td>
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<tr>
<td>SP-A</td>
<td>1.00±0.21</td>
<td>1.11±0.20</td>
</tr>
<tr>
<td>SP-B</td>
<td>1.00±0.04</td>
<td>1.16±0.08</td>
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<tr>
<td>Cholesterol, % wt of PL</td>
<td>8.31±0.47</td>
<td>8.22±0.38</td>
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Values are means ± SE. LA, large aggregates. Surfactant protein-A (SP)-A and SP-B are relative densitometry values from Western blots that had equal amounts of phospholipids (PL) loaded, relative to the LS1h group. *P < 0.05 for HS2h vs. HS1h; †P < 0.05 for HS2h vs. LS2h.

Table 3. Lavage concentrations of 3 markers of lung injury, total protein, IL-6, and TNF-α of the 4 experimental ventilation groups described in Fig. 1

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<td>1 h</td>
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<tr>
<td>Total protein, mg/kg body wt</td>
<td>19±2</td>
<td>27±6</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>70±4</td>
<td>152±23</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>51±25</td>
<td>64±34</td>
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Values are means ± SE. *P < 0.05 for HS2h vs. HS1h; †P < 0.05 for HS2h vs. LS2h.
The results of the surface tension reduction during dynamic compression-expansion cycles are shown in Fig. 4. Comparison of the two LS groups revealed that the surface tension after one compression was significantly higher in the LS2h group compared with the LS1h group, but no other significant differences were noted between these two groups. Comparisons between the two HS groups revealed surface tension values that were not significantly different for cycles 1, 5, and 10 but were significantly higher in the HS2h group during cycle 21 compared with the HS1h group. Comparison between the two ventilation strategies revealed higher surface tensions at the first, fifth, and tenth compression cycles, in the two HS groups compared with their respective LS groups.

Figure 5 shows the biophysical function of LA obtained after a sucrose gradient purification procedure (purified LA). The results of the surface tension reduction during dynamic compression-expansion cycles are shown in Fig. 4B. Comparison of the two LS groups revealed that the surface tension after one compression was significantly higher in the LS2h group compared with the LS1h group, but no other significant differences were noted between these two groups. Comparisons between the two HS groups revealed surface tension values that were not significantly different for cycles 1, 5, and 10 but were significantly higher in the HS2h group during cycle 21 compared with the HS1h group. Comparison between the two ventilation strategies revealed higher surface tensions at general for the two HS groups compared with the LS groups with statistically significant differences noted at cycles 1 and 5 for the 1-h groups and cycle 5, 10, and 21 for the 2-h groups.

Figure 7 shows the functional properties of extracted LA samples supplemented with 5% SP-A for each of the experimental groups (extracted + SP-A). Adsorption was not significantly different between the two LS groups but was significantly slower in the HS2h group compared with the HS1h group (Fig. 7A). Adsorption of samples from the HS2h group was also significantly slower than the adsorption in the LS2h group (Fig. 4A). The results of the surface tension reduction during dynamic compression-expansion cycles are shown in Fig. 4B. Comparison of the two LS groups revealed that the surface tension after one compression was significantly higher in the LS2h group compared with the LS1h group, but no other significant differences were noted between these two groups. Comparisons between the two HS groups revealed surface tension values that were not significantly different for cycles 1, 5, and 10 but were significantly higher in the HS2h group during cycle 21 compared with the HS1h group. Comparison between the two ventilation strategies revealed higher surface tensions at general for the two HS groups compared with the LS groups with statistically significant differences noted at cycles 1 and 5 for the 1-h groups and cycle 5, 10, and 21 for the 2-h groups.

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group. In general, surface tension reduction of the extracted LA plus SP-A (Fig. 7B) showed a similar pattern as the purified samples shown in Fig. 5B. There were no significant differences between the two LS groups. Samples from the HS2h group had significantly higher surface tensions at each cycle compared with both, the LS2h and HS1h groups.

Experiment 3

Having established alterations and biophysical impairment of surfactant after 1 h of HS ventilation, experiment 3 evaluated the physiological consequences of these alterations by supplementing animals with exogenous surfactant at this time point. Physiological responses over a subsequent 2-h period of LS ventilation were recorded.

Figure 8 shows the PaO$_2$/FiO$_2$ values of the four experimental groups in this experiment. During the first hour of mechanical ventilation all animals had PaO$_2$/FiO$_2$ values that were not significantly different among groups. In the subsequent 2 h of ventilation, animals in the LS-NT and LS-BLES groups maintained PaO$_2$/FiO$_2$ values above 400 mmHg. Animals that received 1 h of HS ventilation and were subsequently switched to 2 h of LS ventilation (HS-NT) had significantly lower PaO$_2$ values compared with the LS-NT group. Treatment with BLES after 1 h of HS mitigated this effect since HS-BLES had significantly higher PaO$_2$/FiO$_2$ values compared with the HS-NT group and these values were not significantly different than the LS-BLES group.

Analysis of the lavage material obtained from these animals confirmed the increase in surfactant LA pools in the BLES-treated animals, with no significant difference between the amounts of LA recovered between the LS-BLES and the HS-BLES groups (data not shown).

DISCUSSION

The majority of studies to date that have investigated alterations to the surfactant system in the setting of VILI have performed measurements when lung injury was severe (7, 31, 36). In this context it is unknown whether the alterations are a consequence of or an active contributor to the development and progression of lung injury. This distinction has important implications for therapeutic interventions such as exogenous surfactant administration. To address this issue we performed three separate experiments. First, we observed alterations of the surfactant system of rats ventilated with a HS strategy at a time before physiological lung dysfunction was apparent and noted that these changes increased in magnitude as lung dysfunction occurred. Second, we determined that there were biophysical consequences of the alterations to surfactant at these two time points and that alterations to the hydrophobic components of surfactant were responsible for this dysfunc-
alveolar surface area associated with the high-VT ventilation, rapidly converted to SA because of the large changes in alveolar metabolism of surfactant would suggest that these LA secretion would increase the LA pool. However, studies on the secretion of surfactant in the short term (9, 22). This increased reports have demonstrated that high-VT ventilation increased the effects of surfactant secretion and conversion. Previous present study. Another potential mechanism would be through directly responsible for the altered pool sizes observed in the important in surfactant metabolism and may therefore be indicated that increased cholesterol levels, as observed in the present study but could come from membrane breakdown of inflammatory cells and/or plasma contamination in the more severely injured HS2h group. Although the increase in cholesterol was relatively modest at the 2 h mark, these changes, in conjunction with the decreased SP-B levels and the increased protein levels at this time point, could have significant consequences. Two of the hydrophobic components may be specifically involved in the observed surfactant impairment in the HS2h samples: cholesterol and SP-B. Several recent studies have indicated that increased cholesterol levels, as observed in the LA from the HS2h group, can impair surfactant function (13, 17, 19). The source of this cholesterol is unknown in the present study but could come from membrane breakdown of inflammatory cells and/or plasma contamination in the more severely injured HS2h group. Although the increase in cholesterol was relatively modest at the 2 h mark, these changes, in conjunction with the decreased SP-B levels and the increased protein levels at this time point, could have significant consequences.

The reduced amounts of SP-B, as noted in the HS2h group, combined with the results from the measurements of lipid reduction within the lung leading to decreased lung function. This would then progress over time with further alterations of the surfactant system and the upregulation of other indexes of lung injury, such as increases in inflammatory mediators.

To further assess the effects of HS on surfactant function, the second experiment examined the biophysical properties of the LA. Most previous studies have utilized the crude LA fraction to determine surfactant activity (14, 31). We examined this fraction as well as a sucrose gradient-purified LA, a chloroform-extracted LA, and the extracted LA with SP-A. One of the main mechanisms by which surfactant impairment occurs is thought to be via protein inhibition (7, 16, 29). Many in vitro and some in vivo studies have shown that addition of serum protein to surfactant results in poor biophysical function and can impair lung function (7, 16, 18, 23, 29, 35). In the present study we examined this phenomenon by purifying the LA over a sucrose gradient, thereby removing serum protein from the samples. Purification of the crude HS1h samples was sufficient to rescue the surface tension-reducing ability to functional levels similar to LA from both LS samples that were purified. These findings, in conjunction with the data shown in Figs. 6 and 7 showing abnormal surface tension properties in the extracted HS1h samples and rescue of these alterations in the extracted + SP-A HS1h samples, suggest that the impaired activity of the LA from the HS1h group is likely due to protein inhibition in conjunction with a functional alteration to the hydrophobic components of surfactant. Interestingly, removal of serum protein from the HS2h samples did not rescue function.

The complete analyses of the various LA samples isolated from these lungs at both time points revealed that alterations to the hydrophobic components of surfactant significantly contributed to surfactant impairments noted at both time points of HS mechanical ventilation. As noted, lipid extraction was performed to isolate the hydrophobic components of surfactant. This process removed all hydrophilic components, including serum protein as well as the surfactant-associated protein SP-A, from the crude LA samples of each group (39). In general the lipid-extracted samples had slower adsorption times and were less surface active over consecutive compression expansion cycles compared with the crude and purified LA samples. The hydrophobic components of surfactant include the surfactant-associated proteins SP-B and SP-C and all lipid components of surfactant, including the neutral lipid cholesterol. The observed impairments following lipid extraction in the HS samples implicates these proteins and lipids as contributing to the observed biophysical dysfunction.

The specific alterations to surfactant observed after 1 h of HS ventilation included an increase in SA in the lavage and a decreased percentage of LA. Furthermore, the biophysical analysis showed that these LA had an impaired surface tension-reducing ability. These changes continued over time, such that after 2 h of HS ventilation, when lung dysfunction was evident, all relative changes had increased in magnitude including a severe functional impairment of the LA fraction. At this 2-h time point, the amounts of SP-B and cholesterol present in the LA were also significantly altered. Furthermore, markers of lung injury, such as total lavage protein, IL-6, and TNF-α, were significantly increased after 2 h of HS ventilation, but not after 1 h. Thus it appears that high Vt initially changes the surfactant system by changing the pool sizes of the surfactant subfractions. Several mechanisms may account for such changes. For example, it has been shown that HS ventilation can rapidly affect the activity or numbers of alveolar macrophages in the alveolar space (10, 26). These cells are very important in surfactant metabolism and may therefore be indirectly responsible for the altered pool sizes observed in the present study. Another potential mechanism would be through the effects of surfactant secretion and conversion. Previous reports have demonstrated that high-Vt ventilation increased secretion of surfactant in the short term (9, 22). This increased secretion would increase the LA pool. However, studies on the alveolar metabolism of surfactant would suggest that these LA rapidly converted to SA because of the large changes in alveolar surface area associated with the high-Vt ventilation, leading to the observed increase in SA (12, 32, 33). Over time, this decrease of LA, combined with the impaired function of these LA, would contribute to an impaired surface tension

Fig. 8. PaO2/FiO2 values of animals ventilated with either a LS (VT = 8 ml/kg, PEEP = 5 cmH2O, RR = 54–56 bpm) or a HS (VT = 30 ml/kg, PEEP = 0 cmH2O, RR = 14–16 bpm) strategy for 1 h. Subsequently, animals were randomized to either no treatment (NT) control or a surfactant administration (BLES) procedure, followed by an additional 2 h of LS ventilation. *P < 0.05 for HS-NT vs. LS-BLES; †P < 0.05 for HS-NT vs. LSNT.
extracts supplemented with SP-A, suggests that SP-B is also an important factor at 2 h. Past studies have shown that the hydrophobic protein SP-B is necessary for SP-A to enhance the biophysical function of surfactant (6, 25). In the present study, addition of SP-A to extracted LA samples resulted in an improvement in function of the LS and HS1h extracted samples compared with extracted samples lacking SP-A, indicating that these extracted samples contained functional SP-B that facilitated the enhancement of biophysical properties when SP-A was added. Conversely, the poor function observed in the HS2h samples following addition of SP-A provides evidence supporting alterations to SP-B as contributing to surfactant dysfunction in the latter HS time point. The mechanisms responsible for the decreased SP-B in the present study are unknown but are currently being investigated.

To further address the cause or effect issue of the surfactant alteration in VILI, the final series of experiments were performed in which animals were switched from HS ventilation to LS ventilation, with or without surfactant treatment after the first hour of mechanical ventilation. The subsequent 2 h of LS ventilation, as performed in experiment 3, led to an immediate decrease in PaO₂ values in animals not receiving exogenous surfactant. This result demonstrated that the 1 h of ventilation with the HS strategy resulted in a subsequent deterioration in oxygenation despite utilization of a more protective ventilation strategy over the latter time period. Furthermore, since the administration of exogenous surfactant mitigated these subsequent effects of 1 h of HS, it is concluded that the alterations of the surfactant system at the 1-h time point represent an important mechanism by which HS ventilation affected the lung over time.

The experimental model we utilized to examine the role of surfactant during ventilation was a rat model of VILI. The specific ventilation strategy implemented was based on preliminary studies designed to develop a consistent decrease in oxygenation within a 2-h time period and involved a Vt of 30 ml/kg without PEEP. This HS ventilation strategy was compared with a LS strategy. One apparent limitation of the present study is that the high Vt values utilized are much greater than those used in clinical settings (4). Nevertheless, the application of even relatively low Vt values to injured lungs with a heterogeneous distribution of injury may result in regional overstretching of the more compliant areas of the lung, similar to the overstretching observed in the entire lung in our model. In addition, the high concentrations of oxygen in conjunction with the ventilation strategies used could also have contributed to the changes noted in this study, although animals in all groups received the same concentrations of oxygen, and the durations of exposure were relatively short.

In conclusion, previous studies have shown that surfactant obtained from severely injured lungs of both patients with acute respiratory distress syndrome and animals with VILI exhibited impaired biophysical function compared with surfactant obtained from healthy lungs. The present study has expanded on this knowledge by demonstrating that alterations to the pulmonary surfactant system occur prior to evidence of lung dysfunction, suggesting that these changes are a consequence of the ventilation itself. We have also demonstrated that these relatively early alterations of surfactant directly contribute to lung dysfunction. Finally, we have demonstrated that although the initial impairment of surfactant function is primarily caused by inhibition by serum protein, as suggested in the literature, alterations to the hydrophobic components of surfactant contribute to the dysfunction and are in fact the main mechanisms for surfactant impairment at the stage of severe lung dysfunction in our model of VILI.

ACKNOWLEDGMENTS

The authors thank Dr. Nils Petersen for helpful discussions. We also acknowledge Lynda McCaig and Dr. Li-Juan Yao for technical assistance and Nevena Markovic for proofreading the manuscript.

GRANTS

These studies were supported by a grant from the Canadian Institute of Health Research.

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