RESEARCH ARTICLE | Translational Research in Acute Lung Injury and Pulmonary Fibrosis

Impact of ventilation-induced lung injury on the structure and function of lamellar bodies

Scott Milos,¹,²* Reza Khazaee,¹,²* Lynda A. McCaig,¹ Karen Nygard,⁵ Richard B. Gardiner,⁴,⁵ Yi Y. Zuo,⁶ Cory Yamashita,¹,²,³ and Ruud Veldhuizen¹,²,³

¹Lawson Health Research Institute, Western University, London Ontario, Canada; ²Department of Physiology and Pharmacology, Western University, London Ontario, Canada; ³Department of Medicine, Western University, London Ontario, Canada; ⁴Department of Biology, Western University, London Ontario, Canada; ⁵Biotron Research Centre, Western University, London Ontario, Canada; and ⁶Department of Mechanical Engineering, University of Hawaii at Manoa, Honolulu, Hawaii

Submitted 9 February 2017; accepted in final form 17 May 2017

Milos S, Khazaee R, McCaig LA, Nygard K, Gardiner RB, Zuo YY, Yamashita C, Veldhuizen R. Impact of ventilation-induced lung injury on the structure and function of lamellar bodies. Am J Physiol Lung Cell Mol Physiol 313: L524–L533, 2017. First published May 25, 2017; doi:10.1152/ajplung.00055.2017.—Alterations to the pulmonary surfactant system have been observed consistently in ventilation-induced lung injury (VILI) including composition changes and impairments in the surface tension reducing ability of the isolated extracellular surfactant. However, there is limited information about the effects of VILI on the intracellular form of surfactant, the lamellar body. It is hypothesized that VILI leads to alterations of lamellar body numbers and function. To test this hypothesis, rats were randomized to one of three groups, nonventilated controls, control ventilation, and high tidal volume ventilation (VILI). Following physiological assessment to confirm lung injury, isolated lamellar bodies were tested for surfactant function on a constrained sessile drop surfactometer. A separate cohort of animals was used to fix the lungs followed by examination of lamellar body numbers and morphology using transmission electron microscopy. The results showed an impaired ability of reducing surface tension for the lamellar bodies isolated from the VILI group as compared with the two other groups. The morphological assessment revealed that the number, and the relative area covered by lamellar bodies were significantly decreased in animals with VILI as compared with the other groups. It is concluded that VILI causes significant alterations to lamellar bodies. It is speculated that increased secretion causes a depletion of lamellar bodies that cannot be compensated by de novo synthesis of surfactant in these injured lungs.

as is evident by decreasing levels of blood oxygenation during ventilation (35). These damaging effects of mechanical ventilation are due to repeated opening and collapse at low lung volumes and overdistention of the lung due to the delivered tidal volumes in a heterogeneously injured lung (24). Although ventilation strategies attempting to mitigate these adverse events have improved outcomes in ARDS, avoiding the mechanical ventilation-induced damaged completely is not feasible (5, 37). To further improve outcomes of ARDS patients, it is important to understand the mechanisms by which mechanical ventilation affects the lung.

Animal models, in which the injury is termed ventilation-induced lung injury (VILI), have been used to study the injurious effects of mechanical ventilation (26, 37, 42). These studies have provided a variety of insights into the damaging effects of mechanical ventilation by itself as well as in the context of preexisting injury (37). Among the variety of pathological alterations observed in VILI, alterations to the pulmonary surfactant system appear to represent one of the main mechanisms contributing toward lung dysfunction since they 1) occur early in the disease process, 2) directly affect lung function, and 3) can be prevented by surfactant supplementation (26, 42, 44). Alterations to surfactant also occur in human patients with ARDS (13, 14, 40).

Pulmonary surfactant is a complex lipoprotein mixture lining the air spaces of the lung where it helps maintain normal lung compliance through the reduction of surface tension at the alveolar surface (12). Specifically, by reducing the surface tension to very low values at low lung volumes, surfactant prevents alveolar collapse and allows for inflation with relatively small changes in pressure. Surfactant is synthesized within alveolar type II epithelial cells (ATII) where the phospholipids, neutral lipids, and the surfactant-associated proteins are packaged into lysosomal-like structures, called the lamellar bodies (LBs) (15, 29). Following its packaging into LBs, surfactant is secreted into the air space by exocytosis at the apical membrane of the ATII cell where the LBs quickly unravel and form highly organized phospholipid-protein structures capable of reducing surface tension to very low values.

MECHANICAL VENTILATION is a supportive therapy used in a variety of clinical settings to maintain adequate gas exchange in patients (2). For people with an underlying lung injury, such as patients with acute respiratory distress syndrome (ARDS), mechanical ventilation is essential but can also propagate the lung dysfunction and thereby contribute to disease progression,

* S. Milos and R. Khazaee contributed equally to this work.

Address for reprint requests and other correspondence: R. Veldhuizen, Lawson Health Research Institute, Rm. F4-110, Western Univ., 268 Grosvenor St., N6A 4V2, London, ON, Canada (e-mail: rveldhui@uwo.ca).

L524 1040-0605/17 Copyright © 2017 the American Physiological Society http://www.ajplung.org

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Previous studies on the alterations to surfactant during VILI have mainly focused on alveolar surfactant (26, 28, 42). In these studies, surfactant is obtained via lung lavage after a period of high tidal volume ventilation and analyzed for pool sizes, composition, and surface tension-reducing ability. These studies have demonstrated that surfactant obtained from animals with VILI consists of a smaller fraction of functional surfactant that is altered in its composition. Most notably, surfactant from animals with VILI contained more cholesterol as compared with surfactant from control animals (26, 31, 42). Furthermore, these changes, as well as the effect of serum proteins that have leaked into the lung during injury, result in an impaired surface tension reducing activity of the recovered surfactant (34).

Whereas these previous studies have provided insight into the alterations of the alveolar surfactant, not much is known about the pathological changes to surfactant that occur intracellularly. During the injurious ventilation the ATII cell will be exposed to shear stress as well as stretch, both of which may impact intracellular pathways associated with LB synthesis and secretion. It was therefore hypothesized that following VILI there would be alterations to LB number, composition, biophysical function, and morphology. This hypothesis was tested using an established rat model of VILI in which LBs were isolated from lung tissue for biochemical and functional analysis.

**MATERIALS AND METHODS**

Animals experimentation. A total of 32 male Sprague-Dawley rats aged 6–10 wk weighing 300–550 g (Charles River, St Constant, PQ, Canada) were used for this study. All procedures were approved by the Animal Care Committee at the University of Western Ontario and are in agreement with the guidelines of Canadian Council of Animal Care. Before being used for the experiments, rats were acclimatized for a minimum of 3 days in the housing facility, during which time they had access to standard food and water ad libitum. Following acclimatization, rats were randomized into one of three experimental groups: 1) naive nonventilated controls (NV), 2) control ventilation (CV), and 3) ventilation-induced lung injury (VILI). Rats grouped to CV and VILI were prepared for mechanical ventilation, while NV animals were immediately euthanized. For animals in the CV and VILI groups, animal surgery, intubation, and ventilation procedures were performed as previously reported (26, 42). The weight of each rat was recorded, and animals were anesthetized with intraperitoneal injections of ketamine (70–100 mg/kg) and dexmedetomidine (1 mg/kg) in sterile 0.15 M NaCl. The left and right jugular veins were exposed and catheterized to continuously deliver anesthetic (0.5–2.0 mg·kg⁻¹ · h⁻¹ propofol), neuromuscular blocker (cisatracurium), and sterile fluids (sterile saline with 100 IU heparin/l) at a rate of 0.5–1.0 ml·100 g⁻¹ · h⁻¹. The carotid artery was exposed and catheterized to obtain blood samples for blood gas measurements (ABL 500; Radiometer, Copenhagen, Denmark), to monitor heart rate and blood pressure, and to allow continuous delivery of heparinized saline using an infusion pump (Model 22; Harvard Apparatus, St. Laurent, PQ, Canada). Following exposure of the trachea, a 14-gauge endotracheal tube was inserted and tightly secured. Rats were given a dose of neuromuscular blocker (100 μg/kg cisatracurium) and were immediately connected to a volume cycled rodent ventilator (Model 683; Harvard Apparatus) set at a tidal volume (Vₜ) = 8 ml/kg, a respiratory rate (RR) = 54–56 breaths/minute, a positive end expiratory pressure (PEEP) = 5 cmH₂O, and a fraction of inspired oxygen (F₀₂) = 1.0. All animals were given two breath holds (or sighs) to promote alveolar recruitment and were ventilated at these settings for 15 min before sampling of baseline arterial blood gas measurements with only animals meeting the inclusion criteria of PaO₂ > 400 mmHg being used for further study. Animals that met the inclusion criteria were ventilated with either a CV or an injurious VILI strategy. Rats randomized to CV were ventilated without alteration of the baseline ventilator settings (Vₜ = 8 ml/kg, RR = 54–58 beats/min, PEEP = 5 cmH₂O, and F₀₂ = 1.0) for 180 min. Rats randomized to the VILI group were switched to a second rodent ventilator (Model 683; Harvard Apparatus) set to deliver ventilation settings of Vₜ = 25–30 ml/kg, RR = 15–18 beats/min, PEEP = 0 cmH₂O, and F₀₂ = 1.0, for 180 min. Peak inspiratory pressure was measured throughout ventilation with a pressure monitor (Model Criterion 40; Caradyn, Galway, Ireland) connected with the rodent ventilator. Blood gas measurements were recorded every 15 to 30 min during the ventilation. Following completion of 180 min of mechanical ventilation, ventilated rats were euthanized with an intravenous overdose of pentobarbital sodium (110 mg/kg). Following euthanization, the lungs from the rats were either used for LB isolation and biophysical activity (experiment 1, n = 6/8 per group) or for electron microscopic analysis of LBs (experiment 2, n = 3 per group).

**Experiment 1: LB isolation and analysis.** Isolation of LBs from whole lung tissue was performed as previously described (6, 11, 32). All solutions used during LB isolation were 4°C, saline free, and contained 0.01 M HEPES (pH = 7.4). Briefly, the pulmonary circulation was perfused by injection of 10 ml of 0.32 M sucrose solution from the inferior vena cava out a hole cut in the left ventricle of the heart. Following clearance of the pulmonary circulation, lungs were excised out of the chest cavity and briefly rinsed in 1.0 M sucrose. Visible large airway tissue was removed from lungs before weighing and tissue homogenization. Subsequently, lungs were cut into smaller pieces and chopped length and width wise using a tissue chopper (McIlwain Tissue Chopper; Mickle Laboratory Engineering). The tissue was then added to a 1.0 M sucrose solution producing a 10–20% wt/vol lung homogenate suspension. Homogenization was carried out using five passes of a serrated, 0.15- to 0.23-mm clearance pestle in a Potter-Elvehjem homogenizer to promote the release of intracellular contents from lung tissue. The collected homogenate was filtered through 4-ply gauze to remove large cellular debris. Smaller debris was pelleted and removed from the homogenate solution via centrifugation at 500 g for 15 min at 2°C.

LBs were isolated and purified from homogenate through the use of discontinuous sucrose gradients (32). Briefly, 1.0 M sucrose tissue homogenate was layered below volumes of 0.32 M sucrose and 0.68 M sucrose before centrifugation in a SW28 rotor at 64,000 g for 2 h at 4°C with no brake in a Beckman Coulter Optima L-80 XP ultra-centrifuge. The LBs containing interfacial band between 0.32 and 0.68 M sucrose was collected. With the aid of a refractometer (ABBE Refractometer, model A300A; Fisher Scientific, Waltham, MA), concentrated 1.0 M sucrose was added to the collected interfacial materials to produce a sucrose solution of 0.58 M. The LB-containing 0.58 M sucrose solution was then layered beneath 0.32 M sucrose and 0.45 M sucrose before being centrifuged again at identical settings. Purified LBs were collected at the 0.45–0.58 M sucrose interfacial band. Subsequently, the isolated LB solution was diluted to 0.2 M sucrose and LBs were pelleted by centrifuging the collected materials at 40,000 g for 15 min at 4°C. The resulting LB pellet was resuspended in 300 μl of saline and frozen at −20°C for subsequent analysis.

Three separate animals were used to confirm the LB isolation and purity by TEM. Briefly, the isolated LBs were collected in 0.2 M sucrose containing 0.1 M sodium cacodylate buffer (pH 7.3) and 1.2% glutaraldehyde and 1.2% formaldehyde as primary fixatives (33). LB-containing samples were centrifuged at 40,000 g for 15 min at 4°C to produce a pellet. Further processing was carried out as previously described (9) including postfixation for 1 h with 1% osmium tetroxide.
in 0.1 M Cacodylate buffer and en bloc staining overnight (12–18 h) at 4–8°C with half-saturated aqueous uranyl acetate. Specimens were dehydrated in an ascending series of acetone solutions and embedded in Epon-Araldite resin at 60°C for 3–5 days. Ultra-thin (70 nm) sections were cut (Ultramicrotome Reichert-Jung Ultracut E; Leica Microsystems, Wetzlar, Germany) and poststained with 2% uranyl acetate and lead citrate. TEM imaging of LB samples was performed at low and high magnifications (Fig. 1). Low-magnification micrographs showed isolated LB organelles without the presence of other identifiable cellular ultrastructures, such as mitochondria, or the presence of other surfactant components, such as tubular myelin. High magnifications of isolated LBs displayed characteristic features such as dense osmiophilic folds of phospholipids surrounded by an intact limiting membrane.

For LB isolations used for analysis, phospholipid pools were measured after lipid extraction using a modified Duck-Chong phospholipid-phosphorus assay (4, 8). The amount of surfactant recovered in the isolated LB fractions was expressed as milligrams phospholipid per unit body weight. Total cholesterol levels in isolated LBs were quantified using an enzymatic colorimetric Amplex Red cholesterol assay kit, as per manufacturer’s instructions (Thermo Fisher Scientific, Waltham, MA).

The biophysical function of the isolated LBs was assessed using a constrained sessile drop surfactometer (CSD) as previously described (28, 38). Briefly, aliquots of LBs samples were centrifuged at 21,000 g for 15 min at 2°C to concentrate samples at 2 mg/ml phospholipid in buffer (140 mM NaCl, 2.5 mM HEPES, and 1.5 mM CaCl2, pH 7.4). Glass beads were added to samples to promote mixing, and samples were heated at 37°C for at least 1 h before biophysical assessment on the CSD. A 9-μl drop of sample was placed on the CSD pedestal within an environmental controlled chamber at 37°C and atmospheric humidity. Samples were allowed to equilibrate over 3 minutes without manipulation of the sample drop. Samples were then exposed to 20 repeated compression-expansion cycles on the CSD at a rate of 30 cycles/min. Drop images were recorded during compression/expansion cycles and were analyzed by Axisymmetric Drop Shape Analysis software to obtain an accurate measurement of surface tension (19). LB samples from each rat had three to four technical replicates exposed to dynamic compression-expansion cycles.

**Fig. 1.** Representative transmission electron microscopy (TEM) images of isolated lamellar bodies (LBs) from nonventilated (NV) rat lungs; image shows intact LBs at magnifications of ×4,600 (A) and ×92,000 (B) without other identifiable structures present. Scale bar in B = 100 nm.

**Experiment 2: TEM analysis.** In the second experiment, nine rats were used to assess the impact of various ventilation strategies on LB number and area within the ATII cell as well as LB morphology in situ within the lung tissue. Three rats from each ventilation strategy had lungs processed as previously described (10). Briefly, at the end of ventilation, lungs were fixed in situ at a pressure of 20 cm water column by intratracheal instillation using the primary fixative solution. The fixative solution contained 1.5% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C (pH 7.3) (33). Following filling the airways by instillation of the primary fixative solution via the trachea into the lungs, the trachea was clamped, and the intact lungs were submerged in the same fixation solution for 24 h (at 4°C). Further processing and sampling of lungs was carried out as described previously (10). Following the separation into the right and left single lung, each lung was cut from the apex to the base into parallel slices with a thickness of 3 mm using a tissue slicer. Random tissue sampling was done using a transparent Plexiglas grid with 5-mm holes placed over the collected slices. Parts of the tissue underlying the holes were excised using a 5-mm colonic biopsy punch and cut into small 1- to 2-mm blocks.

Further tissue processing was carried out as described previously (9). Blocks were postfixed for 2 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.3) and then stained in en bloc with 2% uranyl acetate and 2% lead citrate. Samples were dehydrated in an ascending acetone series and embedded in Epon-Araldite resin at 60°C for 3–5 days (22). Ultra-thin (70 nm) sections were cut (Ultramicrotome Reichert-Jung Ultracut E; Leica Microsystems) and poststained with 2% uranyl acetate and 2% lead citrate.

Imaging of isolated LBs and ATII epithelial cells was carried out using a Transmission Electron Microscope CM10 (Philips Electron Optics, Eindhoven, The Netherlands). Morphological and quantitative image analysis in all experimental groups was carried out via Image-Pro Premier software (Media Cybernetics, Rockville, MD) to obtain three types of measurements: 1) relative area and number of LBs within the ATII cells; 2) morphological features of LBs including aspect ratio, roundness, mean diameter, and J) intensity of intra-LB-stained materials as a representation of their phospholipid content. In total, 200 images/experimental group for morphological analysis and 90 images per experimental group for quantitative analysis were analyzed. For distribution histograms, LB area and staining intensity values were separated into seven intervals and the percentage of images in each interval was expressed for each experimental group.

**Statistical analysis.** Data are expressed as means ± SE. All statistics were performed using statistical analysis software GraphPad Prism (GraphPad Software, La Jolla, CA). Comparisons between ventilation strategies were made using a one-way ANOVA with a Tukey’s post hoc test. P < 0.05 was considered statistically significant.

**RESULTS**

**Physiology.** For physiological data, animals used for the biophysical measurements and electron microscopy analysis were combined for each of the three experimental groups. Body weight was not significantly different among the groups (data not shown). The arterial oxygenation values over the 180-min ventilation period of the two mechanically ventilated groups are shown in Fig. 2. There were no significant differences in arterial oxygenation between CV and VILI at any time from baseline to 135 min. At 150 min through to the end of the duration of mechanical ventilation, animals exposed to VILI had significantly reduced arterial oxygenation compared with CV rats. Rats exposed to CV did not experience any significant decrease in oxygenation at any time point as compared with baseline, while rats exposed to VILI had a significant decline in
oxygenation as compared with baseline at times 150 throughout to the end of ventilation.

Table 1 displays additional physiological parameters assessed during ventilation. At the baseline, there was no difference in any measured physiological parameter between the rats exposed to either ventilation strategy. Following 180 min of ventilation, Peak inspiratory pressure was significantly increased in VILI-exposed animals compared with CV. Furthermore, following 180 min of ventilation, blood pressure was reduced, and peak inspiratory pressure was increased as compared with baseline in all ventilated rats, while heart rate was only increased in CV rats as compared with baseline.

Experiment 1: LB isolation and analysis. Following isolation, the isolated LB samples were analyzed for the quantity of their phospholipid contents and the relative cholesterol content (% phospholipid). As shown in Fig. 3A, CV rats ventilated for 180 min had significantly more total phospholipid levels recovered in the LB pellet following the isolation process as compared with both NV rats and VILI rats. However, there was no difference in recovered LB phospholipid content between spontaneously breathing NV animals and those exposed to VILI. The percentage of cholesterol, relative to phospholipid, was not different among the three groups (Fig. 3B).

Subsequently, the isolated LB samples had their biophysical properties assessed on the CSD. Representative surface tensions vs. surface area isotherms of isolated LBs are shown in Fig. 4. LBs isolated from NV and CV rats displayed low surface tensions upon the initial compression (<5 mN/m), while rats exposed to VILI had an impaired ability to reduce surface tension, reaching a surface tension of 15 mN/m at cycle 1 (Fig. 4). Repeated dynamic surface area cycling lowered the minimum surface tension achieved by LB samples isolated from all ventilation groups compared with the first respective dynamic compression cycle (cycle 1 vs. cycle 10). In addition, isotherms constructed at different compression cycles demonstrated that LBs isolated from rats grouped to NV generally had a substantial reduction in hysteresis area following repeated cycling, a phenomenon not observed in LBs isolated from mechanically ventilated rats. Whereas these representative isotherms demonstrate the overall surfactant surface tension changes achieved during cyclical compression and expansion of the representative samples, the quantification and statistical comparisons of minimum surface tension and other biophysical parameters from all LB samples are shown in Fig. 5 and Table 2, respectively.

Minimum surface tensions of LB samples obtained from rats from the three experimental groups are shown in Fig. 5. LBs

Table 1. Lung physiology of rats at baseline and after exposure to 3 h of mechanical ventilation

<table>
<thead>
<tr>
<th>Time point, min</th>
<th>PIP, cmH2O</th>
<th>PCO2, mmHg</th>
<th>Blood pressure, cmH2O</th>
<th>Heart rate, beats/min</th>
<th>Blood pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>11.11 ± 0.45</td>
<td>34.8 ± 1.6</td>
<td>165.8 ± 8.6</td>
<td>259.7 ± 4.6</td>
<td>7.40 ± 0.01</td>
</tr>
<tr>
<td>180 min</td>
<td>15.33 ± 0.2 †</td>
<td>37.3 ± 1.3</td>
<td>80.2 ± 3.8 †</td>
<td>270.8 ± 6.6 †</td>
<td>7.33 ± 0.02</td>
</tr>
<tr>
<td>Control Ventilation</td>
<td>10.89 ± 0.46</td>
<td>32.2 ± 2.1</td>
<td>159.2 ± 6.7</td>
<td>247.9 ± 12.0</td>
<td>7.41 ± 0.02</td>
</tr>
<tr>
<td>VILI</td>
<td>31.61 ± 1.18 * †</td>
<td>44.4 ± 3.5</td>
<td>48.0 ± 5.1 * †</td>
<td>243.1 ± 15.5</td>
<td>7.28 ± 0.02</td>
</tr>
<tr>
<td>Baseline</td>
<td>11.11 ± 0.45</td>
<td>34.8 ± 1.6</td>
<td>165.8 ± 8.6</td>
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<td>243.1 ± 15.5</td>
<td>7.28 ± 0.02</td>
</tr>
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</table>

Values are means ± SE; n = 9/group. VILI, ventilation-induced lung injury; PIP, peak inspiratory pressure. *P < 0.01 vs. control ventilation (CV). †P < 0.05 vs. baseline time.
from rats exposed to VILI had significantly higher minimum surface tensions achieved compared with associated controls. The impairment associated with VILI LB samples was statistically different at cycles 1–10 and cycles 1–14 and 18 compared with the minimum surface tension of surfactant obtained from CV and NV rats, respectively (Fig. 5). There were no significant differences in minimum surface tension achieved upon compression of isolated LBs between control NV and CV samples. Comparing minimum surface tensions achieved during repeated compression expansions, it was demonstrated that there were significant reductions in minimum surface tension achieved over the course of dynamic cycling as compared with cycle 1 for both NV (cycle 2 and onward)- and VILI (cycle 3 and onwards)-exposed animals.

The additional outcomes of the biophysical assessment of the isolated LBs are shown in Table 2. First, there were no significant differences between VILI and controls with respect to the surface tension measured following 120- to 180-s LB

![Fig. 4. Representative surface tension (mN/m) vs. surface area (fraction of the initial area) isotherms at dynamic compression-expansion cycles 1 and 10 of lamellar bodies isolated from NV rats (top) or rats exposed to 3 h of CV (middle) or VILI (bottom). Within each isotherm, open symbols represent compression of the surface film and closed symbols represent the expansion of the film.](image)

![Fig. 5. Minimum surface tension (mN/m) of isolated lamellar bodies during 20 dynamic compression-expansion cycles from NV rats, or rats exposed to CV or VILI. #: P < 0.05: VILI vs. NV; *P < 0.05: VILI vs. CV; n = 6-8/treatment group.](image)
sample adsorption on the CSD pedestal. Second, all LB samples regardless of origins were compressed to similar extents during the functional assessment, as indicated by maximum compression at cycle 1. Lastly, there was no difference in the maximum surface tensions achieved between LB samples isolated from rats exposed to various ventilation strategies at either cycle. Furthermore, there was no difference in maximum surface tension achieved comparing cycle 1 and 10 within each ventilation strategy.

Experiment 2: in situ imaging of LBs. TEM imaging of ATIIIs and LBs was performed on lung tissue samples from all three experimental groups. Representative images for all groups are shown in Fig. 6. Relatively low magnifications micrographs provided images of LBs within the ATII structures. Higher magnifications were used to visualize the various morphological details of LBs (Fig. 6B).

Quantitation of the LB number, size, and area occupied within the ATII cells is shown in Fig. 7. Statistical comparisons revealed that the number of LBs in ATII cells significantly increased in CV compared with NV group. The number of LBs in VILI animals decreased compared with both NV and CV groups by approximately threefold (Fig. 7A). The relative area of ATII occupied by the LBs (Fig. 7B) was significantly lower in animals from the VILI group as compared with both CV and NV groups. In addition, the area occupied in the CV showed a significant increase compared with the NV group.

Further morphological comparisons of LBs of rats exposed to various ventilation strategies are shown in Table 3. Comparing the size and shape aspects of LBs visualized from all three ventilation strategies, it was found there were no significant differences among groups regarding roundness and aspect ratio, while significant differences were observed in mean LB area and diameter between CV and NV groups. Staining intensity reflects the mean values of intensity levels per pixel on the images of LBs in each group. Black color, which represents a fully stained area, has a value of zero and white (absence of any staining) represents 255 in this measurement. The significant increase in the gray scale in VILI group indicates a decrease of staining compared with the LBs from CV group (Table 3). To further explore the significant changes in morphology, distribution histograms of LB area and staining intensity are shown in Fig. 8. Increases in LB area overall in the CV group are shown to be due to a decrease in the relatively small LBs and an increase in larger (1.0 μm and larger) LBs (Fig. 8A). Differences in staining intensity are due to an overall increase in staining values (i.e., lighter staining) in the LBs from the NV group as compared with the two other groups (Fig. 8B).

DISCUSSION

Mechanical ventilation is a common supportive therapy with, under some conditions, the potential of damaging the lung (2). This injury, termed VILI in animals, is in part mediated through alterations to the pulmonary surfactant system (26, 28, 41, 42). A variety of studies have examined the impact of VILI on surfactant’s pool sizes and biophysical function by examining the extracellular surfactant obtained via

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![Fig. 6. Representative electron micrographs from each of the experimental groups at two different magnifications. Top row: alveolar type II epithelial cells (ATII) with LBs inside, ×5,800; scale bar = 2 μm. Bottom row: LB, ×64,000; scale bar = 100 nm.](http://ajplung.physiology.org/)

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LAMELLAR BODIES IN VENTILATION-INDUCED LUNG INJURY

AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00055.2017 • www.ajplung.org
lung lavage. The current study has added to these previous findings by examining the effect of VILI on the intracellular source of surfactant, the LB. It was hypothesized that there would be alterations to LB number, basic composition, biophysical function, and morphology following exposure to VILI. The results demonstrated that minimum surface tension reduction of unraveled LBs isolated from rats exposed to VILI was significantly impaired during early dynamic compression-expansion cycles compared with noninjured ventilator controls. Furthermore, LB number and area occupied within the ATII decrease after VILI compare with CV and NV groups. Taken together, it is concluded that VILI causes significant alterations to LBs and we speculate that these observed changes contribute to impairments in surface tension reduction within the alveolar hypophase.

To investigate the impact of VILI on intracellular surfactant within ATII, we used an established model of VILI (26, 42). Lung injury in this model was verified by indicators of pulmonary function, such as reduced PaO2:FIO2 and other physiological parameters similar to those previous studies on VILI. Subsequently, two aspects of the LBs were investigated; first, we examined the biophysical function of isolated LBs using the constrained sessile drop surfactometer, and second, we performed a morphological analysis of LBs through TEM of fixed lung tissue. Together, these complementary approaches allowed for a thorough examination of the LB within the different experimental groups and support our overall conclusion that some of the alterations of surfactant due to mechanical ventilation are initiated intracellularly.

Table 3. Morphological analysis of LBs visualized within ATII from the three ventilation groups

<table>
<thead>
<tr>
<th>Lamellar body morphological parameter</th>
<th>NV</th>
<th>CV</th>
<th>VILI</th>
</tr>
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<tbody>
<tr>
<td>Average intensity per pixel†</td>
<td>110.3 ± 6.7</td>
<td>97.7 ± 13.8</td>
<td>125.3 ± 5.4*</td>
</tr>
<tr>
<td>Area, μm²</td>
<td>0.37 ± 0.01</td>
<td>0.81 ± 0.14#</td>
<td>0.54 ± 0.12</td>
</tr>
<tr>
<td>Diameter, μm</td>
<td>0.64 ± 0.01</td>
<td>0.92 ± 0.08#</td>
<td>0.75 ± 0.1</td>
</tr>
<tr>
<td>Circularity‡</td>
<td>1.12 ± 0.01</td>
<td>1.12 ± 0.02</td>
<td>1.40 ± 0.2</td>
</tr>
<tr>
<td>Aspect ratio§</td>
<td>1.40 ± 0.03</td>
<td>1.37 ± 0.04</td>
<td>1.39 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3/group. ATII, alveolar type II epithelial cells. *P < 0.05 vs. CV, #P < 0.05 vs. NV. †Measure of brightness of each pixel on LB image. ‡Circularity = (4 × pi × area). §Aspect ratio = ratio or major axis to minor axis of an ellipse.
group, have elegantly shown the adsorptive capacity and unraveling at the air-liquid interface of LBs (17, 18); our study builds on these findings by subjecting LBs isolated in vivo to dynamic compression-expansion cycles. It demonstrates that isolated LBs, under conditions which have promoted its unraveling, can reduce surface tension values to near zero values similar to isolated surfactant from the alveolar space. Furthermore, our experiment examined this biophysical function following exposure to VILI and demonstrated impairment in this surface tension activity. Several studies have demonstrated compositional and structural differences in the LBs following induction of lung injury (21, 22); however, our study is the first to do so from a biophysical perspective.

In our experiments, impairments in surface tension reduction were observed in VILI samples during the first few compression-expansion cycles; however, continued cycling improved these minimum surface tensions achieved. Thus LBs isolated from VILI-exposed rats could overcome their initial impairment in surface tension reduction by repeated compressions and expansions, as would occur during continuous respiration. The phenomenon of improved surfactant surface tension reduction during repeated lateral compression is likely explained by interfacial film remodeling, involving the removal (or “squeeze out”) of components interfering with reaching low surface tension (20). The process, includes the removal of fluidizing, unsaturated phospholipid, cholesterol, and potentially other components from the surface film resulting in the formation of a dipalmitoylphosphatidylcholine-enriched film capable of reaching low surface tensions (20). We previously reported that elevated cholesterol was a contributing factor to the inhibition of alveolar surfactant obtained by lavage by affecting the fluidity of the surfactant film (31, 42). In the current study, analysis of the relative cholesterol levels in the isolated LB fraction demonstrated no differences among groups. Our data lead us to suggest that upon film formation at the air-liquid interface, LB surfactant isolated from NV or CV rats could immediately form a dipalmitoylphosphatidylcholine-enriched film upon the first compression, while LBs from VILI-exposed rats required several compressions to result in a more purified surface film. However, this functional impairment is not due to elevated levels of cholesterol.

A second novel aspect of our investigation was assessing the intra-alveolar LB surfactant content through TEM image analysis in VILI. The results obtained included reduced numbers and relative area occupied by LBs in VILI, as well as structural assessments such as shape and staining intensity. Distribution histograms of the LBs showed that the VILI group had relatively more smaller LBs and that these LBs were lighter in color after staining, suggesting lower phospholipid content. The previous investigations into LBs in the context of lung injuries have mostly focused on lung transplantation and ischemia reperfusion (7, 10, 23). These studies, using extensive stereological analyses, showed an association of decreased levels of LBs within the type II cell with lung injury. In both scenarios, it is feasible that the lower number of LBs is the result extensive exocytosis of surfactant phospholipids and proteins. Support for this notion stems from the numerous studies investigating surfactant secretion, in which alveolar stretch appears to be one of the most effective signals for exocytosis (1, 27). Furthermore, in a previous study from our group, we showed an increase in alveolar pool size of surfactant following VILI, which was followed by increased alveolar metabolism and inactivation of the surfactant (26). Together with the current observations of a decreased staining of LBs in VILI and impaired function of the isolated LBs, the results support the conclusion that the secretion of surfactant due to mechanical ventilation cannot be compensated for by synthesis/secretion of de novo LBs in these injurious conditions.

The focus of our analysis of the LBs was on the biophysical function and morphology, with only a limited compositional analysis of our samples. Nevertheless, both the functional and morphological alterations observed in the LBs from the VILI group are, by definition, due to compositional alterations and modifications. The literature on compositional analysis of LBs, or on proteins and genes involved in surfactant and LB biosynthesis, in the context of VILI is relatively scarce. For example, gene array studies have identified many genes that are altered in VILI; however, the genes identified are representative of all a mixture of all lung cells, in which changes to LB metabolism may not provide a strong enough signal to be detected (45). Indeed, the majority of genes identified with these techniques involve generic pathways of inflammation, wound healing, stress response, among others (45). A more targeted approach has yielded a bit more insight and should be used in future studies. For example, we have previously reported decreases of mRNA levels of surfactant proteins B and C in an isolated rat model of VILI (39), which could have an impact on the biophysical function. In addition, stretching of a type II cell-like cell line has yielded information on phospholipase A2 activation (25), which plays a crucial role in surfactant metabolism, as well as the induction of synthesis of the main phospholipid of surfactant, phosphatidylcholine (30). In terms of LB formation and morphological alterations, the role of surfactant lipid transporter ABCA3 should be investigated further. A conditional null mutation of this transporter resulted in altered LB formation, morphological alterations, and altered PL composition (3). Although these changes did not completely replicate our VILI observations, they do support a role for ABCA3 in lung injury. Furthermore, it has been shown that heterozygotes for the null mutation of this protein were more susceptible to VILI (16). Overall, the exact genes, proteins, and pathways involved in mediating the functional and structural alteration of LBs in VILI are unknown. It is speculated that alterations in the production of phospholipids and surfactant proteins, as well as changes in ABCA3 lipid transporter, likely contribute to the LB morphological and biophysical alterations observed between VILI-exposed samples and controls.

It should be noted that there are inherent limitations associated with our studies. For example, our model of VILI is based on a short time period of high tidal volume ventilation to reflect a clinical condition that progresses with lower tidal volumes over prolong periods of time in human patients who have an underlying lung disease. Nevertheless, several previous observations in animal models of VILI, such as ventilation-induced lung inflammation (biotrauma) and elevated cholesterol within surfactant, have subsequently been confirmed in clinical situations (31, 36, 46). Our analysis was limited to a time point at which lung injury was severe; it would be interesting to analyze LBs at a stage before significant lung injury has been reached to assess if the changes in LBs precede those physiological changes. Such studies are considered a future direction. In addition, our visualization of LBs and ATII were obtained...
from two-dimensional image analysis; future studies should consider complementary volumetric and topographic stereology of LBs and ATII.

In conclusion, whereas previous studies have primarily focused on the role of alveolar surfactant in VILI, this study has expanded this knowledge by investigating the intracellular impairments of surfactant. Our studies showed a decrease in LB content within the lungs of animals with VILI and that these remaining LBs had impaired biophysical properties when analyzed on a constrained sessile drop surfactometer. These alterations suggest that overstretching and collapse due to injurious ventilation promote secretion of LBs but do not result in increased synthesis of LBs, and as a consequence, subsequent impairment of the secreted surfactant during ventilation cannot be compensated or overcome by further secretion of de novo surfactant.

ACKNOWLEDGMENTS

We thank Drs. Fred Possmayer and Jim Lewis for helpful discussions.

GRANTS

Funds for these studies were provided by an operating grant from the Canadian Institutes of Health Research and Grants-in-Aid from the Ontario Thoracic Society.

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


