Mechanical ventilation of isolated rat lungs changes the structure and biophysical properties of surfactant

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Veldhuizen, Ruud A. W., Blayne Welk, Rob Harbottle, Stephen Hearn, Kaushik Nag, Nils Petersen, and Fred Possmayer. Mechanical ventilation of isolated rat lungs changes the structure and biophysical properties of surfactant. J Appl Physiol 92: 1169–1175, 2002. First published November 16, 2001; 10.1152/japplphysiol.00697.2001.—Mechanical ventilation is an essential but potentially harmful therapeutic intervention for patients with acute lung injury. The objective of this study was to investigate the effects of mechanical ventilation on large-aggregate surfactant (LA) structure and function. Isolated rat lungs were randomized to either a nonventilated control group, a relatively noninjurally ventilated group [1 h, 10 ml/kg tidal volume, 3 cmH2O positive end-expiratory pressure (PEEP)], or an injuriously ventilated group [1 h, 20 ml/kg tidal volume, 0 cmH2O PEEP]. Injurious ventilation resulted in significantly decreased lung compliance compared with the other two groups. LA structure, as determined by electron microscopy, revealed that LA from the injuriously ventilated lungs had significantly lower amounts of organized lipid-protein structures compared with LA obtained from the other groups. Analysis of the biophysical properties by using a captive bubble surfactometer demonstrated that adsorption and surface tension reduction were significantly impaired with LA obtained from the injuriously ventilated lungs. We conclude that the injurious mechanical ventilation impairs LA function and that this impairment is associated with significant morphological alterations.

Pulmonary surfactant is a complex mixture of lipids and proteins responsible for stabilizing the lung by reducing the surface tension at the air-liquid interface (5). Two main processes are involved in the reduction of surface tension: 1) the formation of a lipid film at the air-liquid interface via adsorption and 2) a further reduction in surface tension to near-zero values during the repeated compression-expansion cycles occurring during respiration (5). In the lung, this biophysical activity of surfactant is accomplished by one of the two major subtypes of surfactant, the large-aggregate (LA) fraction. This active fraction of surfactant contains organized lipid-protein structures such as tubular myelin, multilamellar structures, and lamellar bodies (31). In contrast, the small-aggregate (SA) subtype of surfactant is not very surface active and consists of small lipid vesicles (31). LA and SA forms have recently been observed in the lung by electron microscopy (17, 20) and can be obtained from the lung by lavaging the lung with saline followed by differential centrifugation (9).

A number of previous studies have examined the surfactant system after MV, both in vivo and in isolated lungs (8, 12, 28–30). However, the analysis of surfactant in those studies did not include a thorough analysis of the major functional component of the active surfactant subfraction, the LA. There are no structural data available on the LA from ventilated lungs, and the limited amount of information available on the surface activity of surfactant from ventilated lungs has been obtained mainly by pulsating bubble surfactometer experiments. Although this is a useful tool in assessing surfactant activity, this technique has its limitations, such as a fixed time (10 s) for adsorption measurements, a fixed degree of area compression (50%) during each pulsation (leading to potential under- and/or overcompression), and the potential of leakage and movement of material from the sample bubble into the chimney of the sample chamber, thereby limiting the accuracy of the measurement. An alternative to the pulsating bubble surfactometer is the captive bubble

MECHANICAL VENTILATION (MV) is a supportive therapy utilized extensively in clinical medicine. However, MV can also damage the lung, especially when large lung volumes are utilized and lung collapse occurs during ventilation (2, 14). In patients with lung injury, these lung-injurious aspects of MV may be difficult to avoid, and it is therefore important to examine some of the mechanisms by which MV affects the lung. One such mechanism is the impairment of the pulmonary surfactant system, as initially suggested by Faridy and colleagues (4). However, the structure-function correlation of the impaired surfactant due to such injury is not clear.

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surfactant (21). Although more labor intensive, the captive bubble surfactometer provides a leak-proof, flexible system to analyze the biophysical properties of surfactant in greater detail than possible with other techniques.

The objective of the present study, therefore, was to specifically investigate the effects of an injurious ventilation strategy on surface activity of the LA by using a captive bubble surfactometer. In addition, we examined the morphology and measured the relative amounts of the molecular species of phosphatidylcholine in the LA subfractions.

METHODS

Animal model. Eighteen male Sprague-Dawley rats (Charles River Laboratories, St. Constant, PQ, Canada), weighing between 350 and 400 g, were used in this study. All animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (75 mg/kg) and xylazine (5 mg/kg). A tracheotomy was performed by using 0.1 ml of 1% lidocaine hydrochloride (10 mg/ml) for local analgesia and a 14-gauge angiocatheter as an endotracheal tube. A midline sternotomy was then performed to excise the lungs as previously reported (29). The isolated lungs were then randomized to one of three groups: 1) a nonventilated control group (control); 2) a 1-h MV group that underwent “noninjurious” MV using a tidal volume (VT) of 10 ml/kg, a positive end-expiratory pressure (PEEP) of 3 cmH2O, and a respiratory rate of 60 breaths/min (noninjurious); or 3) a 1-h ventilation group undergoing an “injurious” MV strategy using a VT of 20 ml/kg, PEEP of 0 cmH2O, and a respiratory rate of 30 breaths/min (injurious). The two latter groups were ventilated with room air, by using a volume-cycled rodent ventilator (Harvard Instruments, Saint Laurent, PQ, Canada). PEEP was added to the ventilation system by submerging the expiratory tube in water to a depth of 3 cm. During the ventilation period, airway pressures were monitored (model 400 monitor, Sechrist Industries, Anaheim, CA) and recorded on a 400 monitor, Sechrist Industries, Anaheim, CA) and recorded on a

Lavage procedure. The isolated rat lungs were lavaged with saline and processed as previously described (11). Briefly, the lungs were filled with 10–11 ml of 0.15 M NaCl until they appeared fully distended, after which the saline was withdrawn and reinflated two additional times. This procedure was repeated five times with fresh saline for a total of five washes. The total lavage volume was recorded and was spun at 150 g for 10 min. The 150-g pellet, containing mainly cellular debris, was resuspended with saline. The 150-g supernatant was removed, and a 5-ml aliquot of this supernatant (total surfactant) was aliquoted and frozen at −20°C for surfactant analysis. The remaining 150-g supernatant was centrifuged at 40,000 g for 15 min to separate the surfactant LA and SA fractions. The supernatant, containing the SA surfactant, was aliquoted and frozen at −20°C. The LA pellet was resuspended with 2 ml of saline; 0.5 ml of these resuspended LA was fixed for electron microscopic analysis, and the remaining volume was aliquoted and frozen at −20°C.

Measurement of surfactant pool sizes. The amount of surfactant phospholipid was determined in the total lavage, the resuspended 150-g pellet, and total surfactant, SA, and LA fractions in all groups by phospholipid phosphorus measurements. Aliquots of the samples were extracted by the method of Bligh and Dyer (1). Subsequent measurements of phospholipid phosphorus in each of the lipid extracts were determined by the method of Duck-Chong (3). The amounts of surfactant in the total lung lavage were calculated and expressed as milligrams phospholipid per kilogram body weight. In addition, aliquots of the lavage were analyzed for the total amount of protein by using the method described by Lowry and colleagues (10), using bovine serum albumin as a standard.

Electron microscopy. For electron microscopic analysis, resuspended LA were fixed overnight in 4% glutaraldehyde in 150 mM NaCl, 50 mM Tris, and 2.5 mM CaCl2 (pH 7.0). The samples were then centrifuged for 15 min at 10,000 g, and the pellets were processed as previously reported by an electron microscopist who was blinded to the experimental groups (15). Two representative areas of each sample were photographed. The different morphological structures were defined as either tubular myelin, multilamellar structure, lamellar body, or other nonorganized structures, and they were counted by grid counting with the use of a 162-point grid. These counting procedures were also performed blinded with respect to the experimental group of the sample. Data for each different morphological structure were expressed as a percentage of the total counts measure for each sample and an average for each experimental group.

Surface activity analysis. A captive bubble surfactometer was used to evaluate surface activity of the resuspended LA samples from the different lungs (16, 21). Briefly, the captive bubble surfactometer contains a glass chamber with an agar plug, and it uses a pressure-driven plunger to compress the fluid in the chamber. An air bubble of ~3–4 mm in diameter was pulled into the diluted sample suspension in the chamber, which was held at 37°C. The bubble rested against the agar plug, which was sufficiently hydrophilic to prevent loss of material from the interface. The change in bubble geometry was recorded with an infrared light source and a camera for 10–15 min after the introduction of the bubble to the sample to monitor the adsorption of the surfactant to the bubble’s air-liquid interface. After adsorption, the bubble chamber was sealed, quasi-static compression and expansion of the bubble was performed in discrete steps (at a rate of ~5% of bubble volume every 10 s) for five cycles, and the change in bubble shape was recorded. In a subsequent experiment, dynamic compression and expansion of the bubble area was performed by cycling the bubble between 10 and 110% of original area at a rate of 30 cycles/min. Images documenting changes in bubble area were recorded during each experiment, and their shapes were analyzed by using custom-designed software. The surface tension of the bubble was calculated on the basis of shape of the air bubble. The relative area of the bubble was plotted as a function of surface tension to generate the surface tension-area isotherms of the films formed at the air-liquid interface. Individual LA samples were analyzed at a phospholipid concentration of 250 μg/ml in 150 mM NaCl, 2 mM Tris·HCl, and 1.5 mM CaCl2 (pH 7.0) on the basis of phospholipid phosphorus measurement.

Phosphatidylcholine compositional analysis. Electrospray ionization-mass spectrometry was utilized to determine the different molecular species of phosphatidylcholine in the surfactant samples (7, 18). This analysis was carried out on a triple-quadrupole instrument of the electrospray ionization-mass spectrometer (model API 365, Sciei, Concord, ON, Canada) by using the positive-ion mode. Approximately 100
µg of the surfactant LA were extracted by the method of Bligh and Dyer (1). The chloroform extract was dried, and the lipids were resuspended in 900 µl chloroform-methanol (1:8 vol/vol). Immediately before analysis, 100 µl of 0.01 M NaOH were added to the sample, and the sample was injected into the mass spectrometer at a liquid flow rate of 10 µl/min. The peak intensities on the mass spectrum were used to calculate the relative amounts of the major phosphatidylcholine species.

Statistics. All values are reported as means ± SE. For comparisons between three groups of samples, statistical significance was determined by using a one-way ANOVA followed by a Tukey’s post hoc test. Comparisons between two groups were made by using Student’s t-test. The statistical analysis was performed by using SPSS 9.0 statistical software. A P value < 0.05 was considered significant.

RESULTS

Eighteen rats were randomized to one of the three experimental groups. As shown in Table 1, there were no significant differences between the experimental groups. In the two ventilation groups, peak inspiratory pressure (PIP) was measured at the start and end of the 1-h ventilation. PIP was significantly higher in the lungs ventilated with the injurious strategy compared with the noninjurious strategy at both time points (Table 1). Furthermore, within the injurious group, PIP increased significantly over the 1-h time period. The volume of air used to inflate the lungs to 25 cmH2O during generation of pressure-volume curves is shown in Table 1. The injuriously ventilated lungs had significantly lower volume than both other groups. Noninjuriously ventilated lungs had lower volumes than control lungs.

There was no significant difference between the total lavage volume recovered from each of the groups (Table 1). The amounts of the two surfactant subfractions, the LA and SA, recovered from the lung lavage material are also shown in Table 1. All three groups had similar amounts of LA recovered from the lavage. The injuriously ventilated lungs had significantly more SA than the other two groups. Because the LA component of surfactant is responsible for the surface tension-reducing ability, this subfraction was investigated in more detail.

| Table 1. Characteristics of experimental groups and amounts of surfactant subfractions |
|-----------------------------------------------|-------------------|-------------------|
| Control | Noninjurious | Injurious |
| n | Weight, g | 374 ± 2 | 365 ± 7 | 379 ± 12 |
| PIP at 0 min, cmH2O | 20.0 ± 2.2 | 26.2 ± 1.1† |
| PIP at 60 min, cmH2O | 21.2 ± 1.6 | 32.3 ± 1.4‡ |
| Volume at 25 cmH2O, ml/kg | 39.4 ± 2.0 | 32.3 ± 2.3‡ | 25.0 ± 2.5§ |
| Lavage volume, ml | 46.3 ± 1.3 | 44.7 ± 0.3 | 44.3 ± 0.3 |
| LA, mg phospholipid/kg | 1.7 ± 0.2 | 2.1 ± 0.3 | 2.4 ± 0.4 |
| SA, mg phospholipid/kg | 2.8 ± 0.4 | 2.5 ± 0.2 | 4.2 ± 0.5§ |

Values are means ± SD; n, no. of rats; PIP, peak inspiratory pressure; LA, large-aggregate surfactant; SA, small-aggregate surfactant. †P < 0.05 vs. noninjurious. ‡P < 0.05 vs. PIP at 0 min. §P < 0.05 vs. control. §P < 0.05 vs. control and noninjurious.

Representative electron micrographs of the LA fractions from the three experimental groups are shown in Fig. 1. Quantification of the different morphological structures is depicted in Fig. 2. LA from control lungs contained ~10% tubular myelin, 5% lamellar bodies, and 10% multilamellar structures; the remaining structures were nonorganized lipid-protein structures. These percentages obtained in the control group were not significantly different from those obtained from the noninjurious group. In contrast to the other two groups, the LA from the injuriously ventilated lungs contained very low amounts of organized lipid structures, specifically tubular myelin. The percentages of these structures in the injurious group were statistically significant compared with the control group for tubular myelin and multilamellar structures and compared with the noninjurious group for lamellar body structures.

Figure 3 shows the surface tension during adsorption of isolated LA from each of the groups, as analyzed...
on a captive bubble surfactometer. LA samples from the control and noninjurious groups reduced surface tension to equilibrium (21–23 mN/m) in ~4 min. The adsorption to equilibrium was significantly slower for samples from the injurious group compared with the other two groups. The samples from the injurious group reached equilibrium surface tension after ~10 min of adsorption.

The ability of LA samples to reduce surface tension to low values during quasi-static and dynamic compression-expansion cycles was also assessed on the captive bubble surfactometer. The results from quasi-static cycling (Fig. 4A) showed that LA from the control and noninjurious groups reached a minimum surface tension that was lower than the samples from the injurious group, and this was statistically significant after the third and fifth cycle. During the dynamic cycling experiments, LA from control and noninjurious also reached significantly lower minimum surface tension values than the samples from the injuriously ventilated lungs (Fig. 4B). The compression needed to reach minimum surface tension during the first quasi-static cycle was not significantly different among the three groups (Table 2). Comparison of the values for cycles 3 and 5 revealed that the area compressions in the control and noninjurious groups were significantly lower than values for the injurious group. Overall, similar differences in percent area compressions were obtained for the samples undergoing the dynamic cycling (results not shown).

Figure 5 shows the results of the measurement of the molecular species of phosphatidylcholine by mass spec-

![Fig. 2. Morphometric analysis of the electron micrographs. Different structural forms of pulmonary surfactant were counted by grid counting. Lam bod, lamellar body; Multi lam, multilamellar structure. $P < 0.05$ vs. control. $#P < 0.05$ vs. noninjurious.](image)

![Fig. 3. Surface tension during adsorption. Resuspended large-aggregate surfactant from the 3 experimental groups was utilized to measure adsorption to equilibrium surface tension on the captive bubble surfactometer. $P < 0.05$ vs. control and noninjurious.](image)

![Fig. 4. Minimum surface tensions of resuspended large-aggregate surfactant. Samples from the 3 experimental groups was resuspended at 250 μg/ml and analyzed on the captive bubble surfactometer. Quasi-static cycling (A) and dynamic cycling (B) were performed as described in METHODS $P < 0.05$ vs. control and noninjurious.](image)

![Table 2. Compression needed to reach minimum surface tension](image)
The relative amounts of phosphatidylcholine molecular species were not significantly different among the three groups. In each of the groups, the dipalmitoyl species represented ~40% of the total phosphatidylcholine species.

DISCUSSION

The major finding of this study was that injurious MV did not result in a significant decrease in the amount of LA obtained from the lung but that it did result in significant changes in the morphological structure and biophysical properties of these LA. These observations provide further evidence that MV can significantly affect pulmonary surfactant function and thereby impact lung function.

In the present experiments, we utilized isolated rat lungs to examine the effects of ventilation on pulmonary surfactant similar to a variety of other studies investigating different aspects of MV (14, 24, 29). On the basis of previous studies, we selected an injurious ventilation strategy and compared the surfactant system from these ventilated lungs with a nonventilated control group. A noninjurious ventilation strategy was also investigated to control for surgical procedures and ischemic time. It should be noted that the noninjurious strategy still resulted in a significant decrease in compliance, indicating that these procedures were not completely innocuous. The main objective was then to perform a detailed analysis on the functional subfraction of surfactant, the LA.

A variety of studies have investigated how pulmonary surfactant is affected by MV. For example, Verbrugge and colleagues (30) reported that MV of rats with high pressures resulted in lung dysfunction and changes in the relative amounts of surfactant subfractions (30). In addition, using the same experimental setup as the present study, our laboratory has previously demonstrated that the amounts of total surfactant and the subfractions can be affected by the ventilation strategy and that this is influenced by the VT used as well as the level of PEEP (29). Consistent with these former studies, we observed that injurious ventilation increased the amount of surfactant phospholipid. This is likely related to stretch-related increases in surfactant secretion occurring in the lungs ventilated with the relatively high VT (13). In the present study, there was no change in the amount of LA at the end of the ventilation period, which is likely related to conversion of secreted LA into SA during the 1 h of ventilation with the specific injurious ventilation strategy utilized. Consistent with this conclusion was the significant increase in the SA fraction of surfactant in the injuriously ventilated lungs. It is not known whether the increased amount of SA contributes to the lung dysfunction.

Although the recovered amount of LA did not change among the three groups, the structure and function of the recovered LA were significantly affected by injurious MV. It is important to realize that for these measurements LA were isolated as a pellet of a centrifugation at 40,000 g for 15 min. This technique has been used in a variety of studies, and it has been demonstrated that it effectively and consistently isolates the functional subfraction of surfactant (27, 29, 31). Some studies have utilized the more time-consuming sucrose gradient to obtain the different subfractions; however, in general, the results obtained with this technique appear to be similar to those of differential centrifugation (6, 26).

Regardless of the separation technique, the large aggregates obtained from normal lung have been demonstrated to contain organized lipid-protein structures, such as tubular myelin. Interestingly, after injurious MV, the percentage of the highly organized lipid-protein structures was significantly decreased compared with noninjurally ventilated and control samples. Thus, although equal amounts of LA were obtained from the three groups, the morphology of the LA differed significantly. Because it has not been possible to purify and study the specific morphological structures of surfactant LA, such as tubular myelin, is very difficult to conclusively determine the relevance of our observed morphological changes. However, it seems reasonable to suggest that the reduction in organized structures in the injuriously ventilated lung was, in part, responsible for the reduced activity of these LA. The altered structure of the surfactant may be related to changes in surfactant protein composition occurring because of the injury and/or because of other factors produced by the injured lung that interfere with the organized lipid-protein structures.

The activity of the LA was measured by using a captive bubble surfactometer (21). The captive bubble surfactometer has been utilized to determine the mechanisms by which surfactant performs its biophysical function (19, 22, 23, 25); however, it has not been extensively utilized to investigate material from injured lungs. Previous studies have demonstrated impaired surface activity of surfactant obtained from injuriously ventilated lungs by using a pulsating bubble surfactometer (29, 30). The present results provide further insight into the specific impairments that occur to the activity of LA obtained from injuriously ventilated lungs. First, the adsorption of the surfactant was
slower in the injured group than the other two groups. It should be noted, however, that even the LA from the injured group eventually reached equilibrium surface tension of 22–24 mN/m. This is important because this meant that the subsequent quasi-static cycling of these samples was all performed at a similar starting point in terms of the surface tension of the film. The quasi-static cycling showed that, in addition to the impaired adsorption, LA from the injured group could not reach the same low surface tension values as the other two groups. Interestingly, during the first quasi-static cycle, the samples behaved similarly. However, during subsequent cycles, the samples from noninjuriousy ventilated and control lungs reached lower surface tension, whereas the LA from the injured group behaved similarly to the first cycle. The general view on the mechanism by which surfactant reduces surface tension is that it forms a surface film that, during the compression-expansion cycles, becomes enriched in the main surface tension-reducing agent of surfactant, dipalmitoylphosphatidylcholine. Thus the observation that the injured sample behaved similarly to the other samples during the first cycle but did not improve during the five quasi-static cycles would indicate that the initial adsorbed film was similar in composition among the three groups; however, the injurious material was not capable of further dipalmitoylphosphatidylcholine enrichment of the film during the subsequent cycles. Analysis of the relative amount of phosphatidylcholine species by mass spectrometry revealed no difference among the groups, indicating that it was not a lack of dipalmitoylphosphatidylcholine that was responsible for the difference in surface tension reducing activity.

Overall, this study demonstrated that 1 h of ex vivo MV can significantly impact the morphology, increase the adsorption time to reach equilibrium, and impair the surface tension-reducing activity of LA surfactant. Further studies are required to investigate which specific components are responsible for these alterations of surfactant. Furthermore, although this study specifically focused on the LA substraction, additional investigations into the effects of SA on the activity of surfactant are also warranted.

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