Physiological effects of oxidized exogenous surfactant in vivo: effects of high tidal volume and surfactant protein A

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ACUTE LUNG INJURY (ALI) can be caused by a number of direct and indirect insults to the lung and has an unacceptably high mortality rate ranging from 30 to 70% (4). It is defined by a number of criteria including arterial hypoxemia and decreased lung compliance (2, 4). Although not included in the definition, ALI is typically accompanied by extensive pulmonary inflammation, including the infiltration of inflammatory cells and the release of mediators such as cytokines, reactive oxygen species, and proteases (2, 4). All patients with ALI require the supportive therapy of mechanical ventilation, which through the use of specific tidal volumes (Vt) and delivery of high oxygen concentrations, improves arterial oxygenation. However, mechanical ventilation has also been implicated in the pathophysiology of ALI by actively contributing to the progression of the disease (7, 10, 28). The physical forces of mechanical ventilation, such as excessive stretch and collapse of the alveolar epithelium, have been shown to disrupt lung mechanics and lung function (10, 28). Alterations of the pulmonary surfactant system have been postulated as one mechanism by which mechanical ventilation can contribute to the progression of ALI (37, 39).

Surfactant is a mixture of phospholipids and surfactant-associated proteins (SP-A, SP-B, SP-C, and SP-D) that reduces surface tension forces at the air-liquid interface of each alveolus, contributing to optimal lung compliance and function (14). Within the air space, surfactant is present in two subfractions: the surface active large aggregates (LA) and the inactive byproducts of respiration, the small aggregates (SA) (29, 40). Typical alterations of the surfactant system during ALI include reduced levels of the surfactant proteins, altered lipid profiles, decreased amounts of LA, and increased amounts of SA subfractions (15, 16, 25, 36). One specific alteration of surfactant that may contribute to surfactant dysfunction is oxidative modifications of the surfactant lipids and proteins due to the increased oxidative stress within the alveolar compartment (1, 31). A variety of studies has demonstrated that in vitro oxidation of a lipid extract of surfactant impaired its biophysical function and that this impairment could be rescued by the addition of 5% SP-A (1, 31). To complement these in vitro studies, we have previously administered an oxidized surfactant to surfactant-deficient rats and observed impaired lung function when compared with animals administered nonoxidized surfactant (3). In these studies, addition of 2.5% bovine SP-A to a low dose of oxidized exogenous surfactant did not rescue the function of this material in vivo.

Because oxidative alterations of surfactant are most likely to occur in situations of mechanical ventilation with high oxygen concentration, our first objective was to investigate whether oxidation of surfactant makes the lung more susceptible to the other deleterious aspect of mechanical ventilation, lung stretch due to high Vt. Furthermore, since addition of 5% SP-A was shown to improve oxidized surfactant function in vitro, the second objective was to examine the effects of this SP-A concentration on oxidized surfactant in vivo.

MATERIALS AND METHODS

Surfactant preparations. Bovine lipid extract surfactant (BLES) was a generous gift from BLES Biochemicals (London, Ontario, Canada). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
To ensure the baseline health status of each animal, initial inclusion criteria were established: arterial partial pressure of oxygen/fractional inspired concentration of oxygen (PaO₂/FIO₂) >400 mmHg, as measured via arterial blood samples (ABL500 Radiometer, Copenhagen, Denmark), and a peak inspiratory pressure (PIP) of <15 cmH₂O. Upon meeting this criteria, the animal was subjected to a series of whole lung lavages, as previously described, until the animal met the following physiological criteria indicative of a surfactant-deficient state: a PaO₂/FIO₂ <100 mmHg that was stable for 10 min and an increase of >3 cmH₂O PIP from the baseline measurement (3, 6).

Experimental groups. Animals were separated into two independent experiments to address the two objectives of this investigation.

Experiment 1. Upon meeting the criteria for the surfactant-deficient state, animals were randomized into one of the following four groups: 1) 20 mg of phospholipid/kg BLES and low Vt ventilation (Vt: 6 ml/kg, PEEP: 5 cmH₂O, rr: 80 breaths/min; BLES-low), 2) 20 mg/kg OX and low Vt ventilation; OX-low, 3) 20 mg/kg BLES and high Vt ventilation (Vt: 12 ml/kg, PEEP: 5 cmH₂O, rr: 44 breaths/min; BLES-high), and 4) 20 mg/kg OX and high Vt ventilation; OX-high. All administered volumes of the surfactant preparations were 1.5 ml.

Surfactant administration and mechanical ventilation time course. After randomization, the animal was disconnected from the mechanical ventilator and supported in an upright position, and the surfactant preparation was administered intratracheally, followed immediately by a 1.5-ml air bolus. The animal was then placed back in the supine position, and the endotracheal tube was reconnected to the ventilator. After surfactant administration and appropriate amendments to the ventilation strategy, the animal was ventilated for 60 min during which time blood gases, physiological parameters of PIP, and pulmonary shunt were calculated (ABL500 and OSM3 Radiometer) and mean arterial pressure (MAP) was recorded at 15, 30, 45, and 60 min posttreatment.

Immediately after each blood gas measurement, a series of computer software-controlled ventilator piston perturbations was performed to assess lung mechanics (32) (Flexivent version 4.03, Scireq Scientific Instruments). The software paused ventilation for a short period of time to perform a sequence of inspiratory and expiratory movements from which indirect measures of lung pressures and volumes were gauged. These ventilation perturbations comprised a pressure-volume loop from which the software calculated the quasi-static lung compliance (CST) based on the Salazar-Knowles equation with a determination coefficient of >90% to ensure that the data accurately fit within the model. After a 60-min ventilation period, the animal was killed with an overdose of pentobarbital sodium and exsanguination by transection of the dorsal aorta.

Surfactant and total protein measurements. After death, a midline sternectomy was performed to open the chest wall, and the lungs were lavaged with 5 × 10 ml of 0.15 M NaCl. Each lavage was instilled and withdrawn three times, and all the lavages were combined and the total volumes were recorded (3, 37). The lavage material was centrifuged at 150 g for 10 min to remove any cellular debris. The supernatant from this centrifugation was termed the total surfactant fraction (TS), from which 5 ml were saved for phospholipid analysis. The remaining TS was subjected to 15 min of 40,000-g centrifugation, from which 5 ml were saved for phospholipid analysis. The supernatant from this centrifugation was termed the total surfactant fraction (TS), from which 5 ml were saved for phospholipid analysis. The remaining TS was subjected to 15 min of 40,000-g centrifugation, from which 5 ml were saved for phospholipid analysis. 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Bligh and Dyer (5). A Duck-Chong phosphorous assay was performed on extracts to determine the amount of phospholipid within each of the TS, LA, and SA fractions (12). A micro-BCA protein assay was performed on the TS fraction following the manufacturer’s instructions to determine the total amount of protein within the lavage material (Pierce Biotechnology, Rockford, IL).

Statistical analysis. All data are presented as means ± SE. For comparisons between the baseline values and the presurfactant administration values, statistical analysis was performed using Student’s t-test. To test for main effects between the four administration groups at each time point through 60 min, a two-way ANOVA was performed. All comparisons were performed with SPSS version 9.0.0 (SPSS, Chicago, IL) and considered statistically significant at P < 0.05.

A linear regression model was utilized for projecting one PaO2/FIO2 value in the OX-high group that was missing due to technical issues with the blood gas analyzer and for two quasistatic compliance data points missing in the OX-low group. These data points did not meet the >95% coefficient of determination and thus were rejected as inaccurate. All three projected data points were below P < 0.05 with powers >80%. SPSS version 9.0.0 was used to generate the linear regression analysis.

RESULTS

Assessment of surfactant input samples included measurement of a marker of lipid oxidation, malondialdehyde, and the assessment of surface activity on a pulsating bubble surfactometer. After exposure to hypochlorous acid, OX samples had higher concentrations of malondialdehyde associated with the preparation compared with the nonexposed BLES sample (0.74 μM/mg phospholipid vs. 0.0565 μM/mg phospholipids). The OX samples also demonstrated impaired biophysical activity compared with BLES. OX samples had significantly higher surface tension compared with BLES after a 10-s adsorption (33.3 ± 1.9 mN/m vs. 25.5 ± 0.04 mN/m, respectively) and significantly higher minimum surface tension values after 100 pulsations (11.5 ± 1.8 mN/m OX vs. 5.8 ± 0.6 mN/m BLES).

Experiment 1: Physiology. Animals that met entry criteria were randomized into a surfactant administration group. Two animals from the OX-high group did not survive the 1-h ventilation time course. Over the course of the experiment, both of these animals developed very high PIP values (39 and 42 cmH2O) and low MAP values (36 and 22 mmHg). These criteria were indicative of progressive depression of physiological stability in these animals. We postulate that the high airway pressures contributed to depression of venous return and cardiac output. Both of these animals were killed with an overdose of pentobarbital sodium at ~45 min posttreatment. The data from these two animals are included in the last time point before death.

The CST (ml/cmH2O) and PaO2/FIO2 ratio values for all animals at baseline (BL) and immediately before surfactant administration (PRE) as well as values for the 4 experimental groups over the 60-min ventilation time course. Bovine lipid extract surfactant (BLES)-low (○, n = 7), oxidized (OX)-low (●, n = 5), BLES-high (△, n = 5), and OX-high (▲, n = 6 and n = 4 at 60 min) are shown. + Time death of an individual animal in OX-high group; * P < 0.05 vs. BL measurements; ** P < 0.05 vs. OX groups; b P < 0.05 vs. high tidal volume groups.

The OX-high group had no increase in CST values compared with predadministration. Comparisons between BLES and OX revealed that in both the high Vt and low Vt groups, OX had significantly lower CST values compared with the BLES groups at 15 and 30 min posttreatment. Throughout the entire 60 min, the animals ventilated with the high Vt strategy had significantly lower CST values compared with the two low Vt groups (Fig. 1A).

Figure 1B depicts the mean PaO2/FIO2 ratio values for all groups. All groups had significantly increased PaO2/FIO2 values compared with predadministration values. Comparisons between OX and BLES revealed that for both ventilation strategies, the OX-low groups had lower initial oxygenation response compared with the BLES groups at 15 and 30 min. Similar to the CST data, animals randomized to high Vt had significantly lower PaO2/FIO2 compared with the low Vt animals at the 30- and 60-min time points.

The measurements of PIP, arterial partial pressure of carbon dioxide (PaCO2), shunt, and MAP are summarized in Table 1. When the baseline to presurfactant administration values were compared, there was a significant increase in PIP, PaCO2, and shunt, as well as a significant decrease in MAP, following the repetitive saline lavage procedure. These values returned toward baseline values after surfactant administration with the exception of PIP, which was higher in the high Vt groups due to the Vt utilized. Significantly lower PIP values were observed for the BLES-treated animals compared with their respective OX groups. In addition, at the 15-min time point, shunt and MAP values were significantly different between the OX and
BLES groups. Statistical comparisons between the two ventilation strategies revealed significantly higher PaO2 and MAP values at 15 min, significantly higher MAP values at 60 min, and a significantly lower shunt value at 60 min for low Vt groups vs. high Vt groups.

Lavage analysis. Figure 2A shows the amount of phospholipid per kilogram of body weight in the TS pools, and Fig. 2B represents the percentage of this fraction that was in the LA fraction. There were no significant differences observed between the two groups of animals randomized to the low Vt groups for either TS or the percentage LA. Similarly, there were no differences in TS and the percentage LA in the two groups of animals randomized to the high Vt group. However, comparisons between these two ventilation strategies revealed a significant increase in the amount of TS and a significant decrease in the percentage LA in the high Vt groups compared with the low Vt groups (Fig. 2B). The amount of total protein in the lavage was not significantly different between the BLES-low and OX-low groups (96 ± 33 mg/kg vs. 47 ± 7 mg/kg, respectively). Similarly, protein values were not different between the two high Vt groups (268 ± 35 mg/kg vs. 234 ± 35 mg/kg, BLES-high vs. OX-high). The values of the low Vt groups were significantly lower than the values of the respective high Vt groups.

Experiment 2: Physiology. A total of 22 animals met entry criteria and were randomized to a surfactant administration group; all animals survived the entire ventilation period. The CST (ml/cmH2O) and PaO2/FiO2 ratio mean values for all animals at baseline to preadministration and the subsequent values for each experimental group are shown in Fig. 3. There was a significant decrease in CST from the baseline to preadministration time points following the repetitive saline lavage procedure (Fig. 3A). After administration of the surfactant preparations, there were significant increases in CST values compared with the preadministration values. No significant differences were observed in CST values among groups at any time point. Similarly, compared with baseline, there was a significant decrease in the PaO2/FiO2 values following the repetitive saline lavage procedure, followed by a significant increase after surfactant administration (Fig. 3B). Comparison between the different surfactant preparations demonstrated that the OX group exhibited significantly lower oxygenation values compared with the BLES group throughout the time course of the experiment. The animals administered BLES + SP-A and OX-low (n = 6), BLES-high (n = 5), and OX-high (n = 6) are shown. *P < 0.05 vs. low tidal volume groups. PL, phospholipid.
OX + SP-A had similar oxygenation values to the BLES group throughout the time course of the experiment. At the 45- and 60-min time points, the OX + SP-A group had a significantly superior PaO₂/FIO₂ value compared with the OX group.

The outcomes of PIP, PaCO₂, shunt, and MAP are shown in Table 2. Compared with baseline, there were significant increases in PIP, PaCO₂, and shunt, and a significant decrease in MAP, following the repetitive saline lavage procedure. Similar to experiment 1, all these values returned toward baseline values after surfactant administration. Comparisons among the experimental groups revealed that the OX + SP-A group had significantly decreased PIP and shunt values compared with the OX group at 60 min.

**Lavage analysis.** Figure 4 depicts the amount of phospholipid per kilogram of body weight in the total surfactant pools and the percentage LA. There were no significant differences between the BLES group and the OX group. Animals administered surfactant supplemented with SP-A had significantly lower amounts of total surfactant compared with the BLES and OX groups (Fig. 4A). There were no significant differences in the percentage LA among all groups (Fig. 4B). The total amount of protein in the bronchoalveolar lavage was not significantly different among the experimental groups (37 ± 7, 74 ± 43, 65 ± 20, and 42 ± 9 mg/kg for BLES, OX, BLES + SP-A, and OX + SP-A, respectively).

**DISCUSSION**

Based on the evidence that both excessive lung stretch and oxidative damage to surfactant can independently impact lung function, we assessed whether the adverse effects associated with a higher degree of lung stretch due to a high Vt would be accentuated in animals that were administered in vitro oxidized surfactant. This first objective was addressed by evaluating the response of surfactant-deficient rats that were administered normal or oxidized surfactant and then subjected to low Vt (6 ml/kg) or high Vt (12 ml/kg) mechanical ventilation. The most pronounced effect on lung function was observed in animals subjected to the high Vt ventilation compared with the low Vt groups. This effect was evident from severely impaired blood oxygenation, significantly increased total amounts of surfactant phospholipids accompanied with lower percentage LA, and higher total protein within the air space. We conclude that in this experimental model, 12 ml/kg of Vt causes severe physiological dysfunction regardless of the oxidative status of the surfactant system.

Our results from the nonoxidized groups were surprising since preliminary results had demonstrated that a 12 ml/kg Vt or even a 20 ml/kg is well tolerated by normal, nonlavaged rats over this time period (results not shown). The impaired physiology in the high Vt groups is therefore likely related to the

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**Table 2. Physiological outcomes of PIP, PaCO₂, shunt, and MAP for baseline measurements, and 15 and 60 min postventilation strategy change from experiment 2**

<table>
<thead>
<tr>
<th>Measure</th>
<th>Measure</th>
<th>Group</th>
<th>15 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIP, cmH₂O</td>
<td>11.8±0.1</td>
<td>BLES (n = 6)</td>
<td>15.5±0.6</td>
<td>15.6±0.4*</td>
</tr>
<tr>
<td>PaCO₂, mmHg</td>
<td>41.7±0.8</td>
<td>OX (n = 5)</td>
<td>16.2±0.8</td>
<td>15.7±0.6</td>
</tr>
<tr>
<td>% Shunt</td>
<td>10.7±0.3</td>
<td>BLES+SP-A (n = 6)</td>
<td>14.6±0.4</td>
<td>14.4±0.3*</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>84.6±1.5</td>
<td>OX+SP-A (n = 5)</td>
<td>16.1±0.7</td>
<td>13.8±0.6*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BLES</td>
<td>51±3.1</td>
<td>47.8±1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OX</td>
<td>50.5±1.6</td>
<td>46.4±3.0</td>
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<tr>
<td></td>
<td></td>
<td>BLES+SP-A</td>
<td>52.4±1.6</td>
<td>46.6±1.6</td>
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<tr>
<td></td>
<td></td>
<td>OX+SP-A</td>
<td>49.8±5.2</td>
<td>48.7±2.0</td>
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<tr>
<td></td>
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<td>BLES</td>
<td>18.0±0.8</td>
<td>12.1±3.8*</td>
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<tr>
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<td></td>
<td>OX</td>
<td>20.1±1.6</td>
<td>19.3±0.8</td>
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<td>15.0±1.0</td>
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<tr>
<td></td>
<td></td>
<td>OX+SP-A</td>
<td>16.0±1.0</td>
<td>13.2±0.8*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BLES</td>
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<td>81.2±4.9</td>
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<tr>
<td></td>
<td></td>
<td>OX</td>
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<td>72.0±5.3</td>
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<td></td>
<td>BLES+SP-A</td>
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<td>82.7±2.1</td>
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<td></td>
<td>OX+SP-A</td>
<td>75.8±6.1</td>
<td>78.0±1.5</td>
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Values are means ± SE. *P < 0.05 vs. baseline measurements; *significant vs. OX groups; *significant combined effect of surfactant and supplementation with surfactant protein A (SP-A). The baseline and PRE measures consist of all the animals from the administered groups.
combinatorial effects that high Vt had on the exogenous material and on the lung tissue after repetitive saline lavage. Compared with the lower Vt groups, high Vt resulted in markedly higher peak airway pressures that may have contributed to the injury. Furthermore, higher Vt was associated with lower percentages of the biophysically active LA. In the lavage model, endogenous surfactant pools are depleted such that this decrease in LA cannot be rescued by endogenous surfactant secretion. In addition, our protein values indicate that high Vt increases the permeability of the pulmonary endothelium-epithelial barrier and thus causes influx of serum proteins that can potentially inhibit surfactant function (11, 18). These two aspects together significantly impaired the biophysical function of the exogenous surfactant system regardless of the oxidative status of the surfactant. This surfactant dysfunction is the probable cause of our observed increases in pulmonary shunt, decreases in lung compliance, and increases in airway pressures, all of which likely contributed to the inferior oxygenation observed in both the high Vt groups.

Although it was not our original objective, the above findings may shed some light on the variable results of exogenous surfactant trials for patients with ALI. Many of the clinical trials have reported transient increases in oxygenation but no overall reduction in mortality (25, 33, 34). Based on our short-term results and interpretation, we can speculate that when the exogenous material is converted to SA forms, this material may not be adequately recycled back into functional LA forms, and the condition of the surfactant system as well as lung function reverts back to the impaired status present before treatment.

Whereas the above discussion focused on the effects observed in the high Vt groups, the protective mode of ventilation utilized with the low Vt conditions provided us with the opportunity to examine the potential impact of oxidized surfactant on lung physiology. Both experiments demonstrated that in vitro oxidized surfactant had inferior activity in vivo compared with nonoxidized material. These results support our previous study in which different doses of this material were tested (3) and are likely a consequence of oxidative modification of SP-B and SP-C, as suggested by the in vitro studies by Rodriguez-Capote et al. (30). Together with evidence that oxidative stress is a major component of the inflammatory processes in ALI (23, 26), these studies suggest that further investigations into reducing oxidative stress within the lung and specifically on the surfactant system are warranted.

More importantly, based on the in vitro evidence that 5% SP-A rescued the biophysical function of oxidized surfactant (31), we tested whether supplementation with SP-A would ameliorate the impaired physiological response to the administration of oxidized surfactant. The primary finding from this experiment was that of a significantly superior physiological response of SP-A containing oxidized surfactant compared with oxidized surfactant alone. This result confirms the previous in vitro findings, and, combined with older studies demonstrating SP-A’s protective effects against protein inhibition, provides strong support for an important contribution of SP-A to the biophysical function of surfactant, particularly in the setting of lung injury (8, 35). The biophysical mechanism by which SP-A improves surfactant activity is not exactly known, but it has been demonstrated that the presence of SP-B is required for SP-A to improve surface tension-reducing properties (27). Interestingly, since the published description of normal lung physiology of the SP-A-deficient mice and their susceptibility to pulmonary bacterial infections, the role of SP-A has recently been mostly defined as immunomodulatory (20, 21). Certainly, there is very strong evidence that host defense is an important function of this protein (9, 24); however, the biophysical impact of SP-A on surfactant function has been observed both in vitro and in vivo and likely represents a second major function of this protein (8, 35).

For both experiments, we analyzed the amount of surfactant aggregates that could be recovered from the lavage after the 60-min ventilation. We did not find any differences between oxidized and nonoxidized surfactant in any of the experimental conditions. Thus oxidized surfactant appeared to be metabolized similarly to nonoxidized surfactant. This implies that metabolism of surfactant is not selective for oxidized or less functional material. However, within each of the two experiments, we observed differences in these outcomes between experimental groups. For example, the high-stretch ventilation caused a significant decrease in percentage LA, and this finding is consistent with our previous studies indicating that higher Vt causes increased conversion of LA into SA (38). In the second experiment, we observed that significantly lower amounts of surfactant were recovered in the groups administered surfactant supplemented with SP-A compared with the surfactant without SP-A. This decreased recovery of surfactant could be due to the effect of SP-A on receptor-mediated secretion and uptake of phospholipids by the alveolar type II cells, although nonspecific SP-A-mediated aggregation to the alveolar epithelium cannot be excluded as a possibility (19, 22, 41). Regardless of

Fig. 4. Total amount of surfactant (A) and %LA (B) recovered from the lung lavage obtained after ventilation. The BLES-administered groups are represented by the filled bars, and the OX-administered groups are represented by the open bars (n = 5 or 6 for all experimental groups). *P < 0.05 vs. non-SP-A groups.
the specific mechanism, supplementation of surfactant with SP-A improved the physiological responses even though it also resulted in lower amounts of surfactant present in the air space.

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GRANTS

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