Physiological and inflammatory response to instillation of an oxidized surfactant in a rat model of surfactant deficiency

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Physiological and inflammatory response to instillation of an oxidized surfactant in a rat model of surfactant deficiency. J Appl Physiol 96: 1674–1680, 2004. First published December 29, 2003; 10.1152/japplphysiol.01143.2003.—Pulmonary surfactant is a mixture of phospholipids (~90%) and surfactant-associated proteins (SPs) (~10%) that stabilize the lung by reducing the surface tension. One proposed mechanism by which surfactant is altered during acute lung injury is via direct oxidative damage to surfactant. In vitro studies have revealed that the surface activity of oxidized surfactant was impaired and that this effect could be overcome by adding SP-A. On the basis of this information, we hypothesized that animals receiving oxidized surfactant preparations would exhibit an inferior physiological and inflammatory response and that the addition of SP-A to the oxidized preparations would ameliorate this response. To test this hypothesis, mechanically ventilated, surfactant-deficient rats were administered either bovine lipid extract surfactant (BLES) or in vitro oxidized BLES of three doses: 10 mg/kg, 50 mg/kg, or 10 mg/kg + SP-A. When instilled with 10 mg/kg normal surfactant, the rats had a significantly superior arterial PO2 responses compared with the rats receiving oxidized surfactant. Interestingly, increasing the dose five times mitigated this physiological effect, and the addition of SP-A to the surfactant preparation had little impact on improving oxygenation. There were no differences in alveolar surfactant pools and the indexes of pulmonary inflammation between the 10 mg/kg dose groups, nor was there any differences observed between either of the groups supplemented with SP-A. However, there was significantly more surfactant and more inflammatory cytokines in the 50 mg/kg oxidized BLES group compared with the 50 mg/kg BLES group. We conclude that instillation of an in vitro oxidized surfactant causes an inferior physiological response in a surfactant-deficient rat.

One proposed mechanism by which surfactant metabolism and function are altered during ALI is via direct oxidative damage to surfactant. Two lines of evidence support this concept. First, increased production of reactive oxygen species (ROS) has been detected in the lungs of patients with ALI (22, 23, 36, 41). These ROS are produced during the process of inflammation when phagocytic cells are recruited to the lung where they subsequently release a wide variety of highly ROS. This oxidative damage is likely exacerbated via the common supportive intervention for patients with ALI, mechanical ventilation, which is often utilized in combination with high concentrations of oxygen to stabilize gas exchange in these patients. In this setting, mechanical ventilation has been shown to cause further increases in pulmonary inflammation (37), and the increased oxygen provides more substrate for the production of ROS.

The second line of evidence for a potential role of oxidized surfactant in the development of surfactant and lung dysfunction stems from in vitro experiments. Several studies have exposed various isolated surfactant preparations to different oxidizing conditions and have demonstrated the production of phospholipid peroxidation products and impaired surface tension-reducing properties of the oxidized preparations (1, 14, 29, 32). For example, our laboratory has recently exposed a commercial exogenous surfactant that contains a normal phospholipid contingent and only the hydrophobic surfactant-associated proteins (SP-B and SP-C), plus bovine lipid extract surfactant (BLES), to hypochlorous acid and to the Fenton reaction. Analysis of the resulting oxidized surfactant in a captive bubble surfactometer revealed that the surface activity was impaired and that this effect could be overcome by adding SP-A to the oxidized preparation (35). Unfortunately, the in vitro analyses do not take into account all the complex metabolic aspects of surfactant synthesis, secretion, and catabolism that could potentially influence its activity within the lung.

We hypothesized that animals receiving oxidized surfactant preparations would exhibit an inferior physiological and inflammatory response and that the addition of SP-A to the oxidized preparations would ameliorate this response. These studies addressed the following three specific questions: 1) what is the physiological response to oxidized surfactant in a surfactant deficient lung? 2) how is the oxidized surfactant metabolized within the air space? and 3) how does oxidized surfactant affect the inflammatory mediators associated with lung dysfunction?
PHYSIOLOGICAL EFFECT OF IN VITRO OXIDIZED SURFACTANT

MATERIALS AND METHODS

Experimental groups. To examine the physiological impact of an oxidized surfactant system in vivo, rats were made surfactant deficient by repetitive saline lavage and instilled with either BLES or in vitro oxidized BLES (Ox). The dose of 10 mg/kg phospholipid was chosen as one dose because it has been demonstrated to return alveolar levels of phospholipids back to prelavage levels after 2 h of mechanical ventilation (6). Two other doses were also used: 50 mg/kg phospholipid and a 10 mg/kg phospholipid dose that was supplemented with 2.5% bovine SP-A.

Surfactant preparations. BLES was obtained from BLES Biochemicals (London, ON, Canada), and the oxidation process with hypochlorous acid was performed according to previously established conditions (35). To avoid variable levels of oxidation, this oxidation process was performed on the entire amount of surfactant required for our studies. The nonoxidized control surfactant utilized was from the same production batch as the oxidized material. The SP-A was extracted from natural bovine alveolar surfactant and purified by chromatography with a mannose column as described by Cockshutt et al. (9).

Animal experimentation. Male Sprague-Dawley rats, weighing between 350 and 500 g, were used for these experiments (Charles River, St. Constant, PQ, Canada). All procedures were approved by the animal use subcommittee at the University of Western Ontario, following the guidelines of the Canadian Council of Animal Care. Animals were acclimatized for 3 days, during which time they were group housed and allowed free access to water and standard chow. The mass was recorded, and the animals were anesthetized (75 mg/kg ketamine and 5 mg/kg xylazine in sterile 0.15 M saline) with an intraperitoneal injection. Once anesthetized, the animal received an analgesic (0.05–0.1 mg/kg buprenorphine) intramuscularly. The right jugular vein and the right carotid artery were exposed and cannulated with PE-50 tubing. The venous line was used for further administration of drugs. The carotid line was used to take measures of heart rate and blood pressures, to obtain samples for arterial blood-gas measures, and for the instillation of fluids (sterile 0.15 M saline and 1,000 IU heparin/l) via an infusion pump at a rate of 1 ml/100 g body weight/1 h. The trachea was exposed, and an endotracheal tube (14 gauge) was secured in place with 2-0 surgical silk. Immediately after the endotracheal tube was secured, the animal was administered a neuromuscularly anesthetized (2 mg/kg pancuronium bromide) to inhibit spontaneous respiratory movements. The endotracheal tube was connected to a volume cycle rodent mechanical ventilator (Harvard Instruments, St. Laurent, PQ, Canada) set at a tidal volume of 7 ml/kg, °C saline (0.15 M NaCl and 1.5 mM CaCl2). The samples were incubated for 1 h at 37°C until analysis. The proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were both measured by using opti-EIA ELISA kits and following the manufacturer’s instructions (Pharmigen, San Diego, CA).

Surface tension-reducing activity. On the basis of phosphorous analysis, LA samples were resuspended at 2.5 mg phospholipids/ml in 0.15 M NaCl and 1.5 mM CaCl2. The samples were incubated for 1 h at 37°C, and surface tension reduction properties were assessed on a pulsating bubble surfactometer (12).

Statistical analysis. All data are presented as means ± SE. Between two experimental groups statistical analysis was performed by using a Student’s t-test, and between three experimental groups a one-way ANOVA with a Tukey’s post hoc test was performed with SPSS software package for Windows, version 9.0.0 (SPSS, Chicago, IL). All comparisons were considered statistically significant at probability values <0.05.

RESULTS

There were significant differences in surface tension-reducing ability between the inputs of normal BLES and the oxidized BLES as analyzed on the pulsating bubble surfactometer (Fig. 1). After the brief adsorption time the BLES samples had a significantly lower surface tension, which remained significantly lower throughout the subsequent 60 pulsations. Al-
through the trend persisted beyond 60 pulsations, no further significant differences were observed between the two input samples.

The mean mass of all the animals utilized in these studies was 436 ± 6 g, and it was not different among the experimental groups. The total number of preadministration lavages and the volume of total lavaged material recovered were not different between all groups (data not shown). All animals that met the inclusion criteria had similar physiological indexes reflective of a surfactant-deficient state: PaO₂ of 73.0 ± 1.3 Torr, arterial partial pressure of carbon dioxide (PaCO₂) of 53.8 ± 0.8 Torr, and PIP values of 22.1 ± 0.3 cmH₂O.

10 mg/kg Dose. Figure 2A shows the arterial oxygenation (PaO₂) during the 120-min mechanical ventilation time course after intratracheal instillation of either 10 mg/kg BLES or 10 mg/kg oxidized BLES. Fig. 2B shows the surfactant aggregate phospholipid levels, and Fig. 2C shows the surface tension reducing activity of the LA subfraction. The 10 BLES group had PaO₂ levels that were significantly higher than the animals instilled with 10 Ox (Fig. 2A). This difference was consistent throughout the entire 120 min of mechanical ventilation. There was no difference in the PaCO₂ levels between the 10 BLES and the 10 Ox groups at 5 min (51.1 ± 3.5 vs. 56.0 ± 4.8 Torr) or 120 min (42.4 ± 3.4 vs. 44.5 ± 4.1 Torr) after instillation. Similarly, no significant differences were observed in the measurements of PIP between the 10 BLES and 10 Ox groups at 5 min postinstillation (17.0 ± 0.5 vs. 21.0 ± 1.5 cmH₂O) and 120 min (16.8 ± 0.8 vs. 18.1 ± 1.3 cmH₂O).

There were no significant differences in the phospholipid levels measured in the TS, LA, or SA between the 10 BLES and the 10 Ox groups (Fig. 2B).

When the LA were assessed for surface tension-reducing ability, the 10 BLES group achieved lower surface tensions with fewer pulsations compared with the LA recovered from the 10 Ox group; however, this did not reach statistical significance (Fig. 2C).

Table 1 shows the total protein values, TNF-α, and IL-6 concentrations measured in the lavage of the no-Rx, 10 BLES, and 10 Ox groups, of which there were no differences among all three groups.

50 mg/kg Dose. Figure 3A shows the PaO₂ response, Fig. 3B shows the surfactant aggregate phospholipid levels, and Fig. 3C shows the surface tension-reducing activity of the LA subfraction after instillation with 50 mg/kg BLES or 50 mg/kg oxidized BLES and 120 min of mechanical ventilation. PaO₂ levels were the same between animals instilled with 50 BLES and 50 Ox throughout the entire 120 min of mechanical ventilation (Fig. 3A). There was significantly more phospholipid within the TS and LA fractions in the 50 Ox group compared with the 50 BLES group and no differences in the levels of SA (Fig. 3B).

The LA recovered from the 50 BLES group achieved lower surface tensions than the LA recovered from the 50 Ox group. This difference was statistically significant at pulsations 50–80 (Fig. 3C).

Table 1. Total amount of protein, TNF-α, and IL-6 in the lavaged material from animals instilled with 10 mg/kg dose

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Protein, mg/kg</th>
<th>TNF-α, pg/ml</th>
<th>IL-6, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>no-Rx</td>
<td>32 ± 15 (4)</td>
<td>608 ± 252 (5)</td>
<td>3,162 ± 678 (5)</td>
</tr>
<tr>
<td>10 BLES</td>
<td>37 ± 10 (3)</td>
<td>676 ± 223 (4)</td>
<td>4,453 ± 1,147 (5)</td>
</tr>
<tr>
<td>10 Ox</td>
<td>45 ± 8 (4)</td>
<td>1,218 ± 615 (4)</td>
<td>9,562 ± 2,916 (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses are no. of animals. TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; no-Rx, 1-ml air bolus; 10 BLES, 10 mg/kg bovine lipid extract surfactant (BLES); 10 Ox, 10 mg/kg oxidized BLES.
There was no significant difference in the total protein and IL-6 concentrations in the lavaged material between the 50 mg/kg group and 50 mg/kg oxidized BLES group (Table 2). There was a significantly higher concentration of TNF-α in the lavage material of the 50 mg/kg group compared with the 50 BLES group.

10 mg/kg Dose + SP-A. Similar to the 10 mg/kg groups, 5 min after instillation with BLES + SP-A, PaO₂ levels were significantly higher than the animals administered Ox + SP-A (Fig. 4A). Superior oxygenation was consistent throughout the ventilation time course, although it was not statistically different at the 60- and 120-min time points.

There were no significant differences between the phospholipid levels within the TS, LA, and SA between either of the 50 mg/kg BLES group and 50 mg/kg Ox group (Table 2). There was a significantly higher concentration of TNF-α in the lavage material of the 50 OX group compared with the 50 BLES group (Table 2).

The LA recovered from the BLES + SP-A group had significantly lower initial surface tensions and significantly lower surface tensions throughout the pulsation time course compared with the LA recovered from the Ox + SP-A group (Fig. 4C).

There were no differences in the concentrations of TNF-α and IL-6 in the lavage material recovered from both the BLES + SP-A and Ox + SP-A groups (Table 3).

Table 2. Total amount of protein, TNF-α, and IL-6 in the lavaged material from animals instilled with 50 mg/kg dose

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Protein, mg/kg</th>
<th>TNF-α, pg/ml</th>
<th>IL-6, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 BLES</td>
<td>32±11 (4)</td>
<td>344±112 (4)</td>
<td>3,135±728 (5)</td>
</tr>
<tr>
<td>50 Ox</td>
<td>65±15 (5)</td>
<td>746±113* (5)</td>
<td>5,024±556 (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses are no. of animals. 50 BLES, 50 mg/kg BLES; 50 Ox, 50 mg/kg oxidized BLES. *P < 0.05 vs. 50 BLES.

Table 3. Total amount of protein, TNF-α, and IL-6 in the lavaged material from animals instilled with 10 mg/kg dose + SP-A

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Protein, mg/kg</th>
<th>TNF-α, pg/ml</th>
<th>IL-6, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 BLES + SP-A</td>
<td>42±15 (5)</td>
<td>336±180 (4)</td>
<td>6,753±3,649 (4)</td>
</tr>
<tr>
<td>10 Ox + SP-A</td>
<td>55±7 (6)</td>
<td>631±74 (5)</td>
<td>4,640±1,819 (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses are no. of animals. BLES + SP-A, 10 mg/kg BLES + surfactant-associated protein A (SP-A) (2.5% of phospholipids by weight); Ox + SP-A, 10 mg/kg oxidized BLES + SP-A.
DISCUSSION

Because of its locale within the air space, the presence of unsaturated phospholipids and associated proteins, pulmonary surfactant has been identified as a potential target of ROS (1, 8, 35). Furthermore, on the basis of in vitro studies, the oxidation of surfactant has been proposed as a mechanism by which surfactant metabolism and biophysical function are altered and can contribute to the pathophysiology of ALI (1, 14, 29, 30, 35). In this study, we examined the physiological effects of an in vitro oxidized exogenous surfactant preparation supplemented with and without SP-A. We observed that when surfactant-deficient rats are instilled with 10 mg/kg normal surfactant-deficient with and without SP-A. We observed that when surfactant-deficient rats are instilled with 10 mg/kg normal surfactant, the PaO$_2$ response is significantly superior compared with the rats receiving oxidized surfactant. Interestingly, increasing the dose fivefold mitigated this physiological effect, whereas the addition of SP-A to the surfactant preparation had little impact on improving oxygenation. This study provides in vivo evidence that oxidation of surfactant may contribute to lung dysfunction in ALI.

Numerous investigators have reported increased oxidative stress as a factor that contributes to the pathophysiology of ALI (8, 13, 17, 22). Accordingly, in vivo models have been utilized to mimic the oxidative stress that is accrued during the progression of ALI, such as exposure to prolonged hyperoxia and ozone (2, 3, 10, 31). A limitation of these types of in vivo studies is that it is difficult to ascertain the specific contribution of an altered surfactant system to the lung injury vs. the other contributing factors that are affected by hyperoxia exposure such as oxidative damage to the pulmonary epithelium or endothelium and the resulting inflammatory response. One approach that has been utilized to circumvent this issue is to examine the biochemical and biophysical consequences of an in vitro oxidized surfactant (1, 16, 32). Our experiment has extended those previous studies by examining the physiological response to oxidized surfactant by instilling normal and oxidized surfactant preparations into the surfactant-deficient lungs of saline lavaged rats (5, 7, 18). A limitation of this approach is that the lung lavage protocol may induce some edema formation in addition to the surfactant deficiency. Although the physiological responses to the surfactant may have been affected by this additional damage, the observation that the total protein concentrations in the lung lavages were not different among the groups would indicate that edema formation was similar in all groups.

Another important aspect of our experiment was the specific oxidizing condition utilized. We used the previously established conditions of hypochlorous acid exposure (35), which represented a high pathophysiological exposure level as suggested by Merritt and colleagues (1, 32). It should be considered, however, that oxidation was limited to one oxidizing agent, hypochlorous acid, and one surfactant, BLES. Previous studies have demonstrated that the effect of reactive oxygen and nitrogen species exposure on surfactant activity may depend on the surfactant composition as well as the specific oxidizing agent utilized (1, 8, 16, 35). These differences are due to the fact that different oxidizing agents, such as the Fenton reaction, peroxynitrite, or hypochlorous acid, may have different targets within a specific surfactant preparation. For example, inactivation of surfactant by peroxynitrite was more severe in a surfactant preparation containing only SP-B and -C compared with a natural surfactant preparation containing SP-A, -B, and -C (8). It has also been shown that the impairment of surfactant by peroxynitrite was in part attributed to damage to the small hydrophobic surfactant proteins (16). Our laboratory has previously demonstrated that exposure of BLES to hypochlorous acid led to the formation of oxidized phosphatidylcholine species (35). Thus, although the present study provides a first indication of the in vivo consequences of an oxidized surfactant, further studies investigating the surfactant proteins as a potential target for the oxidizing agent as well as the in vivo responses of other surfactant preparations and/or other oxidizing conditions are warranted.

The main physiological result from the present study was that the administration of 10 mg/kg BLES resulted in significantly higher PaO$_2$ levels than the instillation of 10 mg/kg oxidized BLES. This observation supports the concept that an oxidized surfactant in the air space is less capable of maintaining lung function than a nonoxidized surfactant. The analysis of the lavage material indicated that, at least for this dose, oxidized and normal BLES were metabolized similarly because surfactant pool sizes were similar. Despite the difference in blood-gas values, the LA’s biophysical function after the 2 h of mechanical ventilation was not significantly different between the two groups. It is likely that this apparent difference between the activity in vitro and in vivo results was related to differences in the experimental conditions. In general, the activity of surfactant in vivo can be affected by distribution of the surfactant within the lung and the presence of other material in the alveolar space, such as serum proteins. In contrast, surface activity of LA in vitro is performed with isolated LA at a specific concentration. Thus it is possible, for example, that oxidized surfactant is more susceptible to protein inhibition than nonoxidized surfactant. This would affect the in vivo function, but it may not be easily detected when analyzing the activity of isolated LA. The correlation between physiological responses to instillation of a specific surfactant preparation and in vitro activity requires further study.

In addition to the 10 mg/kg dose, we tested a fivefold higher dose because studies in vitro have demonstrated a concentration dependence of surfactant activity when tested by itself as well as in the presence of inhibitory proteins (15, 19). In support of these in vitro observations, our results showed that the 50 mg/kg dose had similar oxygenation values between oxidized BLES and BLES and that these values were comparable to the 10 BLES. Interestingly, more surfactant was recovered from rats instilled with 50 mg/kg oxidized BLES compared with BLES. This difference was largely due to an increased recovery of LA. Although further metabolic studies are required, this result would suggest that oxidized surfactant is removed more slowly from the air space. The fact that no difference in pool sizes were observed in the 10 mg/kg groups may be due to the possibility that these groups had already reached a metabolic equilibrium after 2 h of ventilation.

Our third experiment involved the addition of SP-A to BLES and oxidized BLES based on the observation that in vitro SP-A could mitigate the biophysical impairment of oxidized surfactant (8, 16, 35). In view of these in vitro results, it was surprising that surfactant-deficient rats administered SP-A containing oxidized surfactant did not have an improved physiological response. This result was similar to the results observed with the preparations without supplemental SP-A (compare
Physiological Effect of In Vitro Oxidized Surfactant

These results suggest that SP-A may not improve the function of oxidized surfactant in vivo; however, some caution is warranted with regard to this conclusion because our experiments utilized bovine SP-A in rat lungs. Although a variety of studies have demonstrated cross-species activity of SP-A (4, 28, 33, 38), it is possible that in our experimental setup bovine SP-A behaves differently from rat SP-A. It should also be noted that the in vitro experiments utilized 5% (by weight) SP-A in a calcium-containing buffer (35), whereas we utilized 2.5% SP-A in saline because of the quantities required.

For each of the three experiments, we also analyzed TNF-α and IL-6 as indexes of pulmonary inflammation and lung injury. Although statistical significance was only reached for the TNF-α concentrations in the 50 mg/kg groups, in general the animals receiving oxidized surfactant had higher concentration of the two inflammatory cytokines. It is known that pulmonary surfactant has immunomodulatory properties (40), and it is possible that, in addition to the biophysical impairment, oxidation also affects this secondary function of surfactant.

In summary, our study was based on evidence of oxidative stress in patients and animals with ALI and on the reported in vitro impairment of oxidized surfactant. Our study contributes to these observations by demonstrating that oxidized surfactant has an impaired function in vivo. We also provided preliminary indications that oxidized surfactant had an altered metabolism and affected the concentrations of inflammatory cytokines in the lung. Overall, these observations provide further support for a role of oxidative damage to surfactant as a contributing mechanism to lung dysfunction in acute lung injury. Future studies are required to address the occurrence and relative importance of oxidative damage to surfactant in injured lungs.

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