Ozone Stress Initiates Acute Perturbations of Secreted Surfactant Membranes

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Ozone Stress Initiates Acute Perturbations of Secreted Surfactant Membranes


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To identify the early changes of surfactant secretion in response to acute oxidant stress, the authors evaluated morphometrically centriacinar type II cells and lavage fluid surfactant forms obtained immediately after exposure of adult rats to 3 ppm ozone for 1, 2, 4, or 8 hours. In this model, the rat lung develops progressive alveolar edema with significant elevation of lavage fluid proteins at 2 to 8 hours of exposure. Ultrastructural changes in type II cells at 1 and 2 hours included enhanced lamellar body (LB) fusion with significant increase in the compound and vacuolated LB compartments. Parallel changes of lavage fluid surfactant membranes included a sustained, twofold increase in the proportion of loosely coiled multilamellar structures at 1 to 8 hours, with reciprocal decrease in the proportion of tubular myelin from control value of 56% to 34%. The proportion of densely coiled LB-like forms in lavage fluid increased significantly at 4 and 8 hours, whereas the proportions of unilamellar structures remained unchanged. The results indicate that ozone-induced alveolar injury initiates time-dependent defects in the organization of stored and secreted surfactant membranes. The acute ozone stress inhibits unfolding of secreted lamellar body membranes as well as their organization into tubular myelin, thereby perturbing the proportions of extracellular surfactant membranes that are available for adsorption onto the surface film. (Am J Pathol 1991, 138:847–857)

The alveolar type II cell is uniquely specialized to produce and secrete the lipid–protein complex of lung surfactant.1,2 It is well recognized that the surfactant lipids are stored in the form of densely coiled membranous structures within the lamellar body (LB), a multifunctional, compartmented organelle of the type II cell, which incorporates basic components of both secretory pathways and endosomal–lysosomal systems.3–9 At the onset of their secretion, the LB surfactant membranes become loosely coiled,10,11 an event that apparently heralds the unwinding of secreted surfactant membranes. After exocytosis into the hypophase of the alveolar lining layer, the unfolding surfactant membranes are organized into tubular myelin and other morphologically distinct surfactant forms.12–16 There is evidence that the structural organization and spreading of surfactant membranes involves calcium-dependent reactions with active participation of surfactant-associated proteins, and possibly phospholipid transfer proteins and serine proteinases.17–20 Tubular myelin has been found to be associated with optimal adsorption for natural surfactant.21 During the respiratory cycle, membranes from the tubular myelin, and conceivably from other surfactant forms, are believed to spread rapidly onto the alveolar air–liquid interface, where they form a monomolecular surface film composed predominantly of dipalmitoylphosphatidylcholine (DPPC), the primary functional constituent of surfactant. The above scenario serves as a framework to investigate the sequential interrelationships, mechanisms, and significance of changes involving stored and secreted surfactant membranes in response to oxidative and other types of alveolar injury.

Our previous studies have defined an injury-and-repair model induced by acute ozone stress.8,12–22 Using this model, we have obtained evidence that acute oxidative injury results in seemingly defective exocytosis and spreading of surfactant membranes; deficiency of a 14-kd LB protein, recently identified as lysozyme23; depletion of selected LB hydrolases; and persistent elevation in the cholesterol of stored surfactant; followed by progressive increase in both intracellular and extracellular surfactant pools. The present study provides persuasive evidence that acute ozone-induced perturbations of surfactant homeostasis are first manifested by the development

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of structural changes in stored and secreted surfactant membranes, leading to precipitous and sustained deficiency in extracellular tubular myelin.

Materials and Methods

Animal Model

Rats used for these studies were adult, specific pathogen free, male Fischer 344, and weighed 270 ± 10 g. The rats were obtained from Charles River Breeders (Kingston, NY), shipped in filtered crates, housed in cages with filter tops, and acclimated for 1 week before use with free access to food and water. Rats were exposed to ozone for 1, 2, 4, or 8 hours in a specially designed lute exposure chamber. Control rats were exposed to filtered room air. Ozone was generated by passing 100% oxygen through an ozone generator (OREC Model O3V1-0, Ozone Research and Equipment Company, Phoenix, AZ). The ozone was mixed with compressed air to produce 3.0 ± 0.2 ppm ozone concentration. The total gas flow was maintained at 120 l/min to produce approximately 1 air exchange per minute. The ozone concentration within the chamber was continuously monitored with a Mast Model 727-3 ozone monitor (Mast Development Co., Davenport, IA) connected to a strip chart recorder. At the end of the ozone exposure, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and killed by exsanguination by severing the abdominal aorta.

Preparation of Lungs and Lavage Fluid for Morphologic Studies

For studies of type II cells, the lungs from 15 rats, three rats per groups, were processed as described previously. Briefly, the lungs were fixed in situ by intratracheal instillation of prewarmed (37°C) 2.5% buffered glutaraldehyde, volume displacement of total and left lung determined, and the left lung sliced, postfixed with reduced 1% osmium tetroxide, stained en bloc with buffered 1.5% uranyl acetate, and embedded in ‘EM bed 812’ (Electron Microscopy Sciences, Ft. Washington, PA). This technique insures excellent preservation of lamellar bodies and extracellular surfactant forms. For preparation of lavage fluid, an incision was made in the necks of 15 rats (three rats per group) under general anesthesia, the trachea exposed, a cannula inserted into the trachea, and the chest and the abdomen were opened to facilitate exsanguination. Approximately 10 ml of Hank’s balanced salt solution (HBSS) at 37°C was instilled intratracheally and immediately withdrawn from the lung. The returned lavage was combined with an equal amount of 5% glutaraldehyde, also at 37°C, to make a final concentration of 2.5% HBSS-buffered glutaraldehyde. The fixed lavage was kept at 37°C for 4 hours, stored at 4°C overnight, then aliquoted into 1.5-ml microfuge tubes and centrifuged at 7200g for 10 minutes. The supernatants were discarded and the pellets washed in buffer. Care was taken not to disrupt the pellets after the initial centrifugation. After postfixation with buffered 1% osmium tetroxide reduced with 1.5% potassium ferrocyanide, the pellets were en bloc stained with 1.5% uranyl acetate, pH 6.3, washed in saline, dehydrated with a graded series of acetone, and embedded in ‘EM bed 812.’

Lung Tissue and Surfactant Membrane Morphometric Measurements

Ultrastructural morphometry of alveolar type II epithelial cells was performed as previously described. Briefly, two 3-mm² square blocks were selected randomly from the centriacinar region of the left lung for semithin toluidine-blue–stained sections and thin sections for EM. Random profiles of type II cells were photographed at 5700× magnification, printed, and enlarged to 16,500×, and the areas of type II cells and their organelles were measured by the point counting method using a multipurpose test grid system. Volume density of lamellar bodies was determined by the formula $V_v = \frac{P_i}{P_T}$, where $P_i$ = points of a randomly placed grid that hit the lamellar bodies and $P_T$ = total points of the grid that hit the cell.

For morphometric studies of lavage fluid surfactant membranes, 9 to 12 blocks of pelleted material were obtained from each rat. Five blocks per rat, for each time period (control, 1, 2, 4, and 8 hours), were randomly selected and ultrathin sections cut for evaluation in the electron microscope. Three random electron micrographs were taken per block of cell-free regions at 4500× and printed at 15,000×. A total of 15 pictures for each rat and 45 pictures for each period were used for the morphometric evaluation of surfactant membranes. A simple square lattice test grid was employed to quantitate the surfactant membranes, which were classified into the following subtypes: 1) LB-like form—circular, densely coiled multilamellar body, similar to lamellar body but devoid of limiting membrane; 2) multilamellar structure—consists of multiple, loosely coiled lamellae that are arranged in various configurations; 3) tubular myelin—characteristic organization with a lattice pattern; and 4) unilamellar structure—lamellae arranged singly in various configurations. Point counts were summed for each surfactant membrane subtype over all the photos taken for each rat, and the percentage of each subtype analyzed was calculated.
Lavage Protein

Total protein concentrations in lavage fluids were measured by a modification of the Lowry method that ensures solubilization of surfactant lipids and proteins.\textsuperscript{25}

Statistical Analysis

The data were all analyzed using a one-way analysis of variance (ANOVA). Duncan’s multiple range test (MRT) was used to look for differences among groups when an F value, determined by ANOVA, was significant ($P < 0.05$). To satisfy the assumption of homogeneity of variances when using ANOVA, the Burr-Foster Q-test was used. When the variances were found to be heterogeneous, the log transformation was applied to the data, and the ANOVA and Duncan’s MRT rerun on the transformed data. Statistical analysis was accomplished using a microcomputer and *SPSS/PC +* V-3.1 software (SPSS, Inc., Chicago, IL).

Results

Lavage Fluid Protein

Increasing duration of ozone exposure resulted in progressive accumulation of plasma proteins in lavage fluid (Table 1). A significant elevation in protein values was first noted at 2 hours of exposure. The concentration of proteins in lavage fluid increased 6 times and 20 times at 4 and 8 hours of exposure, respectively.

General Morphologic Observations

Fixation of lavage fluid before centrifugation resulted in superior preservation of suspended surfactant forms. With this method, surfactant membranes in lavage fluid retained the configuration of alveolar surfactant forms, which were observed in lungs fixed by the airways (Figure 1; compare with Figure 2). Furthermore this method enabled identification and quantitation of all formed elements present in lavage fluid, thereby avoiding biases that are inherent to various fractionation procedures.

Tubular myelin from control lungs was the predominant extracellular form in both lavage fluid and alveolar spaces. Additional surfactant forms included unilamellar structures, loosely coiled multilamellar structures, and LB-like forms (Figures 1a, 2a). In ozone-exposed rats, both lavage fluid and the alveolar spaces showed prominent accumulation of multilamellar structures at the expense of tubular myelin. These changes appeared as early as after 1 hour of exposure to 3 ppm ozone (Figures 1b, 2b). In addition, there was a striking increase in the proportion of LB-like forms at 4 and 8 hours (Figures 1c, d, and 2c, d), often in association with fibrin deposits (Figure 2d). Similar alterations of surfactant membranes were observed at the air–liquid interfaces of trapped air bubbles that were often found within edematous alveoli (Figure 3).

Ultrastructural changes of type II cells included the presence of partially vacuolated areas within the lamellar bodies, which were frequently fused and often contained eddylike cores. These changes were observed most frequently during the first 2 hours of ozone exposure (Figure 4), and were accompanied by accumulations of secreted LB-like forms in seemingly distended alveolar pouches (Figure 5). It is of interest that the secreted LB-forms at 4 hours often contained eddylike cores, apparently as a reflection of ozone-induced exocytosis. The alveolar pouches were located in alveolar corners beneath overhanging type I epithelial cells, and they were inconspicuous in control lungs.

### Table 1. Concentration of Protein in Lavage Fluid from Control Rats and Rats Exposed to 3 ppm Ozone

<table>
<thead>
<tr>
<th>Duration of ozone exposure (hours)</th>
<th>Control</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 22</td>
<td>n = 5</td>
<td>n = 5</td>
<td>n = 5</td>
<td>n = 8</td>
</tr>
<tr>
<td>Protein (μg/ml)</td>
<td>71.7 (61–85)</td>
<td>97.7 (85–113)</td>
<td>124.6 (92–168)</td>
<td>452.0† (360–567)</td>
<td>1612.1‡ (1315–1997)</td>
</tr>
</tbody>
</table>

Values expressed as means with 95% confidence intervals shown in parenthesis. Duncan’s Multiple Range test was used to test for significant differences at $P < 0.05$. n = number of rats.

* Significantly different from control.
† Significantly different from control, 1 hour and 2 hours.
‡ Significantly different from control, 1, 2, and 4 hours.
Figure 1. Representative electron micrographs of lavage fluid surfactant membranes from control rats (A) and rats exposed to 3 ppm ozone for 1 hour (B), 4 hours (C), and 8 hours (D). TM, tubular myelin; MS, multilamellar structures; US, unilamellar structures; LBF, LB-like forms. Quantitative changes of surfactant membranes are given in Table 2 (× 11,500).
Figure 2. Alveolar spaces from control rats (A) and rats exposed to ozone for 1 hour (B), 4 hours (C), and 8 hours (D). The proportions and ultrastructural features of extracellular surfactant forms are similar to those observed in lavage fluid at corresponding time points. A, alveolar space; C, capillary lumen; LB, lamellar body; LBf, LB-like form; MS, multilamellar structure; TM, tubular myelin; US, unilamellar structure; F, fibrin (x 7000).
Figure 3. Edematous alveolus from a rat exposed to ozone for 4 hours. The air-liquid interface of a trapped air bubble (A) shows a partially disrupted surface film (SF). The alveolar hypophase (H) contains granular precipitate of edema fluid, and focal accumulation of surfactant membranes, primarily LB-like forms (LBf) and multilamellar structures (MS), with no tubular myelin present. C, capillary lumen (× 25,000).

Figure 4. Type II cell from rat exposed to ozone for 1 hour. Some lamellar bodies (LB) are fused (at arrows), contain eddylike core structures, and are partially vacuolated. N, nucleus of type II cell; A, alveolar space; C, capillary lumen (× 10,000).
expressed in square microns) at 2, 4, and 8 hours, respectively. A persistent decrease was also noted in the electron-dense multivesicular bodies (MVB), whereas the electron-lucent MVB did not change in response to ozone stress. By contrast, both vacuolated and compound LB compartments increased more than twice the normal values at 1 to 2 hours, with return to control range after longer periods of exposure.

In ozone-exposed rats, the lavage fluid showed more than a twofold increase in the proportion of multilamellar structures, from control value of 15% to more than 30% at 1 to 8 hours of exposure (Table 3). Conversely, the proportions of tubular myelin showed a sustained decrease from control value of 56% to 34%, 38%, 34%, and 31% at 1, 2, 4, and 8 hours’ ozone exposure, respectively. Significant increases (17% and 19%; control value 10%) in LB-like forms were found at 4 and 8 hours, but the proportions of unilamellar structures did not change with increasing duration of ozone exposure.

Discussion

The results of this study indicate that acute, oxidative stress to the alveoli rapidly initiates striking alterations in both stored and secreted surfactant membranes. In response to 3 ppm ozone exposure for 1 to 2 hours, the lamellar bodies demonstrate eddylike transformations of their membranes, as well as significant expansion of the vacuolated and compound LB compartments. These findings confirm earlier suggestion that initial effects of ozone stress involve enhanced fusion of lamellar bodies with accelerate mobilization of surfactant membranes through the intercommunicating LB compartments. These events are apparently linked to augmented emptying of lamellar bodies into the alveoli, as evidenced by the early accumulation of LB-like forms in alveolar pouches, followed by a measurable reduction in the area of the total LB compartments at 2 to 8 hours of exposure.

In spite of the above effects on the storage and secretion of surfactant membranes, the ozone stress does not acutely alter the content and composition of surfactant phosphatidylcholine in either lavage fluid or isolated lamellar bodies. This is not surprising, in view of the remarkable ability of type II cells to maintain surfactant homeostasis through multiple mechanisms including endocytosis and reuse of surfactant constituents.

Significant perturbations of the lung surfactant have been reported to occur in experimental models involving prolonged exposure of animals to oxidative stress. As shown by the data presented, however, the structural organization, rather than biochemical composition, of the extracellular surfactant membranes is highly sensitive to acute oxidant injury. These data enabled us to identify two major, time-dependent defects in the organization of extracellular surfactant. The earliest defect is characterized by intra-alveolar accumulation of loosely coiled multilamellar structures at the expense of tubular myelin, with unilamellar structures unchanged. This lesion is maintained throughout the period of 1 to 8 hours’ ozone exposure. The second defect consists of progressive accumulation of LB-like forms at 4 to 8 hours after exposure. As reported previously, the progressive accumulation of LB-like forms in alveolar spaces persists during the reparative stage of ozone-induced diffuse alveolar damage (DAD), in association with abnormal endocytosis of densely coiled, rather than loosely coiled, lamellar bodies.

It should be noted that the earliest defect, ie, reduction of tubular myelin with reciprocal elevation of multilamellar structures, is already established before manifested pulmonary edema. Therefore, the mechanism of this surfactant alteration is not directly connected to transudated plasma proteins, which have been found to effect various types of biophysical surfactant inactivation. It is conceivable, however, that transudated proteins play a role in the progressive accumulation of the densely coiled LB-like forms in edematous alveoli, during the exudative/reparative stages of ozone-mediated DAD.

It is relevant that abundant intra-alveolar accumulation
of LB-like forms, in association with prominent lamellar bodies in type II cells, was first described in newborn lungs with hyaline membrane disease (HMD) at the reparative stage. Subsequent studies have documented the virtual absence of tubular myelin (with abundant lamellar bodies) in HMD, suggestive of an abnormality in the conversion of lamellar bodies to tubular myelin. There is also evidence that in HMD type II cells are deficient in SP-A and SP-B, which has been proposed that the lack of tubular myelin in HMD is due to deficiency of surfactant-associated proteins. This concept is consistent with in vitro studies demonstrating that structures similar to tubular myelin can be reconstituted from synthetic lipids and surfactant-associated proteins.

It is of interest that the proportions of surfactant subtypes change in response to physiologic events, such as hyperpnea, which also promote significant increase in alveolar surfactant. In these conditions, surfactant homeostasis is apparently achieved by maintaining constant or increased levels of the tubular myelin-rich fraction. It should be emphasized, however, that at present there is little information about the in vivo regulation of extracellular surfactant forms.

It is known that in vitro formation of tubular myelin is dependent on calcium ions, surfactant-associated proteins, and possibly phospholipid transfer proteins. There is also evidence that serine proteinase activity is required for the in vitro conversion of tubular myelin to a 'vesicular' surfactant form, which may represent 'spent' surfactant. These vesicular forms are composed of unilamellar membranes and they may represent components of unilamellar structures that we found in both lavage fluid and alveoli. Because the proportion of these structures did not change in response to ozone expos-

![Figure 6. Morphometric estimates of lamellar body volume densities. Ozone exposure does not significantly influence the volume density of total lamellar body compartment. Standard error of the mean is shown in parenthesis above bar.](image-url)
sure, it is unlikely that alterations in serine proteinase activity affected the ozone-mediated perturbations of surfactant membranes. In this respect, our results are consistent with the concept that acute oxidative injury selectively depletes important, functional components of lung surfactant, which promote spreading and organization of surfactant membranes.

A large body of data indicate that oxidant lung injury is primarily the outcome of an imbalance between the generation of free radicals and the antioxidant defenses. There is also evidence that free-radical mechanisms are responsible, at least in part, for the ozone-induced oxidation of proteins and polyunsaturated fatty acids. It is plausible, therefore, that the ozone stress initiates through various mechanisms the accumulation of radicals within the alveolar hypophase, thereby inactivating proteins involved in the extracellular organization of surfactant. It is also conceivable that endocytosis by type II cells of extracellular surfactant, containing toxic oxidation products, results in amplification of injury within stored surfactant by initiating depletion of oxidant-sensitive lamellar body proteins, such as lysozyme and selected hydrolases.

By identifying ozone-induced sequential defects in stored and secreted surfactant membranes, this study has provided new information concerning the morphogenesis of important processes, supporting the following scenario. In normal type II cells, stored surfactant membranes begin to unwind during exocytosis, and the secreted membranes are preferentially organized into tubular myelin, which is believed to be the primary source for membranes spread onto the surface film. The acute ozone stress apparently inhibits the unfolding of the rapidly secreted lamellar body membranes, as well as their extracellular organization into tubular myelin. Under these circumstances, the alveolar air–liquid interface is preferentially receiving membranes from multilamellar structures and LB-like forms, which are not expected to spread on the surface film as efficiently as tubular myelin.

The above concept clearly underscores the need for novel in vivo approaches to study the regulation of extracellular surfactant forms during the development and progression of alveolar injury.

### References

on the mode of release of lamellar bodies. Tissue Cell 1975, 7:587–599