Pulmonary reexpansion causes xanthine oxidase-induced apoptosis in rat lung

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Saito, Satoshi, Jun-ichi Ogawa, and Yoshihiro Minamiya. Pulmonary reexpansion causes xanthine oxidase-induced apoptosis in rat lung. Am J Physiol Lung Cell Mol Physiol 289: L400–L406, 2005.—The pathogenesis of reexpansion pulmonary edema is not yet fully understood. We therefore studied its mechanism in a rat model in which the left lung was collapsed by bronchial occlusion for 1 h and then reexpanded and ventilated for an additional 3 h. We then evaluated the production of reactive oxygen species in the lungs using fluorescent imaging and cerium deposition electron microscopic techniques and the incidence of apoptosis using the TdT-mediated dUTP-digoxigenin nick end labeling (TUNEL) method. We found that pulmonary reexpansion induced production of reactive oxygen species and then apoptosis, mainly in endothelial and alveolar type II epithelial cells. Endothelial cells and alveolar type I and II epithelial cells in the reexpanded lung were positive for TUNEL and cleaved caspase-3. DNA fragmentation was also observed in the reexpanded lung. In addition, wet-dry ratios obtained with reexpanded lungs were significantly higher than those obtained with control lungs, indicating increased fluid content. All of these effects were attenuated by pretreating rats with a specific xanthine oxidase inhibitor, sodium (5-(o-ethyl-3-((2,6-dimethylidene)decylamino)-1H-phenyl)-1-(2-sulfinylphenyl) pyrazolo[1,5-a]-1,3,5-triazine-4(1H)-one. It thus appears that pulmonary reexpansion activates xanthine oxidase in both endothelial and alveolar type II epithelial cells, producing reactive oxygen species produced by the enzyme induce apoptosis among the endothelial and alveolar type I and II epithelial cells that make up the pulmonary water-air barrier, leading to reexpansion pulmonary edema.

REEXPANSION PULMONARY EDEMA (RPE) is a rare complication of the treatment of lung collapse secondary to pneumothorax, pleural effusion, or atelectasis. Although physicians generally believe that, unlike acute respiratory distress syndrome (ARDS), RPE does not require intensive care, the outcome of RPE is reportedly fatal in 20% of patients (17). Moreover, reexpansion after atelectatic storage for hypothermic preservation of lungs before transplantation may be responsible for postpreservation and postreperfusion lung injury (7).

The mechanism responsible for RPE is not fully understood. It has been suggested that after atelectasis the mechanical stresses related to lung inflation may cause RPE (28). This effect has also been mentioned as a contributor to the pathogenesis of postpreservation lung injury (9). In addition, some evidence suggests changes in alveolar surfactant may contribute to RPE (26, 31), and a number of investigators have reported that neutrophil accumulation in the lung induced by various chemokines (e.g., IL-8 and monocyte chemoattractant protein-1), chemical mediators, and adhesion molecules (24, 30) plays a major role in RPE (33). Consistent with that idea, it is known that the interaction between adherent neutrophils and endothelial cells regulates lung permeability in RPE (10) and that reactive oxygen species (ROS) produced by neutrophils increase pulmonary endothelial permeability. On the other hand, using an animal RPE model Jackson et al. (14) found that exogenous catalase does not prevent RPE, which eliminates extracellular hydrogen peroxide as an important contributor, but they did detect endogenous lung catalase activity and hydrogen peroxide release. This supports the idea that endogenous production of ROS is a key contributor to RPE. In that regard, xanthine oxidase (XOD) is known to be one of the main sources of endogenous ROS, generating superoxide anion and hydrogen peroxide during uric acid production (5), and to be upregulated in the lung by hypoxia-reoxygenation (35).

Within that context, we can easily imagine that XOD contributes to the development of lung injury following pulmonary reexpansion. Moreover, ROS reportedly induce apoptosis, which plays a key role in processes associated with development and cell homeostasis, as well as such ailments as cancer and ARDS (3, 8, 18, 23). The role of ROS-induced apoptosis in RPE has not yet been investigated, however. Our aim, therefore, was to examine the respective roles of ROS, apoptosis, and XOD activity in a rat model of RPE.

MATERIALS AND METHODS

Animal preparation and observation of the intact pulmonary circulation. All protocols for animal experimentation described in this paper were previously approved by the Animal Research Committee of Akita University and adhered to the “Guidelines for Animal Experimentation” of the University.

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). To eliminate artifacts from spontaneous breathing movements, paralysis was induced with 0.15 mg/kg pancuronium bromide and then maintained with smaller doses. The right femoral vein was then cannulated with a polyethylene tube for infusion of agents; the same cannula was also used to continuously infuse saline at a rate of 3 ml·kg⁻¹·h⁻¹. The right femoral artery was similarly cannulated to measure blood pressure and monitor blood gases. A polyethylene cannula (diameter 2.5 mm) was also inserted into the trachea for ventilation with a mechanical ventilator (UGO Basile type 7025, Tokyo, Japan), which delivered a respiratory volume/min of 3 ml at 55 respirations/min, FIO₂ = 0.21. A median incision was made extending to a left lateral thoracotomy along the eighth rib, after which an intralobar site within the left upper lobe was gently bonded to a glass chamber with the bonding agent Aron Alpha (TOA Gosei, Tokyo, Japan). This allowed the intact pulmonary microcirculation to be viewed under intravital fluorescence microscopy (BH2-FRC; Olympus, Tokyo, Japan).

Experimental groups. Male Wistar rats (200–300 g) were divided into three experimental groups: the LBO (left bronchial occlusion) group, the reexpansion group, and the control group. The LBO group was prepared by occluding the left main bronchus. After a 1-h period, the left lung was reexpanded by ventilating with a mechanical ventilator (UGO Basile type 7025, Tokyo, Japan). The reexpansion group was prepared by ventilating the left lung with a mechanical ventilator (UGO Basile type 7025, Tokyo, Japan) and then occluding the left main bronchus for 1 h. The control group was prepared by ventilating the left lung with a mechanical ventilator (UGO Basile type 7025, Tokyo, Japan) and then occluding the left main bronchus for 1 h and then ventilating the left lung with a mechanical ventilator (UGO Basile type 7025, Tokyo, Japan).
group, in which the affected lung was collapsed by ligation of the left bronchus with silk for 1 h and then reexpanded and ventilated with a mechanical ventilator for 3 h; the CT (control) group, which was ventilated with a mechanical ventilator for 4 h; and the BOF (BOF-4272) group, whose rats were intraperitoneally administered 300 μg/kg of sodium (-)-8-(3-methoxy-4-phenylsulfinylphenyl) pyrazolo[1,5-a]-1,3,5-triazine-4(1H)-one (BOF-4272) 1 h before experimentation and then subjected to left bronchial occlusion and reexpansion in the same way as the LBO group.

In vivo visualization and quantification of ROS. Production of ROS was visualized and measured in the intact pulmonary circulation by a digital fluorescence imaging technique developed in our laboratory. On the day of each experiment, rats were injected with 3 mg/kg 2′,7′-dichlorofluorescin-diacetate (DCFH-DA; Molecular Probes, Eugene, OR) suspended in physiological saline. DCFH-DA is membrane permeant and diffuses into cells where it is hydrolyzed into nonfluorescent 2,7-dichlorofluorescein (DCFH), which is rapidly oxidized to highly fluorescent 2,7-dichlorofluorescin (DCF) in the presence of ROS. Fifteen minutes after injection, DCF fluorescence [530 nm emission (DM500 barrier filter) excited by 490 nm illumination (BP490 narrow-band excitation filter)] from the intact pulmonary microcirculation was imaged and recorded as described above. After each experiment, the recorded images were digitized to a resolution of 512 vertical × 512 horizontal pixels and 256 gray levels using an image digitizing card (FRM512; Photoron, Tokyo, Japan) and stored on a hard disk. The fluorescent areas within images were selected by the software. Briefly, the mean gray level and SD for the entire area of interest were calculated for each image. The threshold fluorescence used to assess whether a cell was producing ROS was defined as the mean gray level + 5 SD. Thereafter, for each experimental condition, the number of pixels with values greater than the mean + 5 SD in five images was considered to be an index of ROS production in the pulmonary circulation.

![Fig. 1](http://ajplung.physiology.org/)

Fig. 1. 2,7-Dichlorofluorescin (DCF) fluorescence images in the rat lung. To detect production of reactive oxygen species (ROS), rats in the control (CT, A), left bronchial occlusion (LBO, B), and BOF-4272 (BOF, C) groups were injected with 2′,7′-dichlorofluorescin-diacetate (DCFH-DA), after which the DCF fluorescence was imaged. Note that whereas little fluorescence was seen in control rats (A), numerous localized areas of fluorescence (white spots) were detected in LBO rats (B), and that pretreatment with BOF-4272 significantly reduced the numbers of fluorescent spots (C).

![Fig. 2](http://ajplung.physiology.org/)

Fig. 2. Time course of the ROS production in the pulmonary circulation of a rat model of RPE and the effect of BOF-4272. A: time course of ROS production induced by bronchial occlusion. B: effect of BOF-4272 on ROS production induced by bronchial occlusion. Using computerized image analysis, we evaluated ROS production by counting pixels of DCF fluorescence within images of the pulmonary circulation. The specificity of the reaction of DCFH with ROS to form DCF was confirmed by the finding that administration of catalase (5,000 U/kg) completely blocked the response (not shown). ○, CT group; ●, LBO group. The data are expressed as means + SD (n = 5 in each group); *P < 0.05 vs. control rats.
Electron microscopic detection of ROS by the cerium technique. The cerium technique was used to detect ROS in the pulmonary circulation and to identify the cell type(s) producing them (19–21). Briefly, after some experiments a polyethylene cannula was inserted into the pulmonary artery from the infundibulum of the right ventricle, and the blood was washed out of the lung by perfusion with bicarbonate-buffered Krebs-Henseleit solution for 5 min. The lung was then perfused for 10 min with a prewarmed (37°C) cytochemical reaction medium [0.1 M Tris-maleate buffer (pH 7.4) containing 7% sucrose, 1 mM CeCl₃, and 10 mM 3-amino-1H-1,2,4-triazole], perfusion-fixed for 5 min with 2% glutaraldehyde 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% OsO₄, dehydrated, and embedded in epoxy resin. Ultrathin sections were then prepared using an ultramicrotome (LKB 8800 Ultratome III; Bromma, Sweden) and examined under an electron microscope (JEOL 1200 EX; JEOL, Tokyo, Japan).

Apoptosis: TUNEL and caspase-3 staining. Lungs were perfusion fixed for 5 min with a periodate-lysine-paraformaldehyde solution (2% paraformaldehyde, 75 mmol/l disodium hydrogen orthophosphate, 13.5 g/l lysine, and 2.14 g/l sodium periodate), after which tissue blocks were immersion-fixed for 2 h at 4°C with periodate-lysine-paraformaldehyde solution and embedded in soft paraffin. After preparing 3-μm sections, we carried out terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) using an Apop Tag In Situ Apoptosis Detection Kit (Oncor, Gaithersburg, MD) according to the manufacturer’s instructions. The sections were then developed with 3,3’-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin.

Sections were also immunostained with rabbit monoclonal antibody against cleaved caspase-3 (Asp175) (clone 5A1; Cell Signaling Technology, Beverly, MA). In brief, the sections were incubated for 1 h at room temperature with anti-caspase-3 antibody (1:100 dilution), rinsed five times with Tris-buffered saline, and incubated with alkaline phosphatase-labeled polymer anti-mouse/rabbit immunoglobulins (Dako EnVision System; Dako, Carpinteria, CA). After development with fuchsin, the sections were counterstained with hematoxylin.

Table 1. Cerium deposition

<table>
<thead>
<tr>
<th>Cerium Positive, %</th>
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<tbody>
<tr>
<td>Type I cells</td>
</tr>
<tr>
<td>Type II cells</td>
</tr>
<tr>
<td>Endothelial cells</td>
</tr>
<tr>
<td>Neutrophils</td>
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</table>

Data are expressed as means ± SD. Type I cells, alveolar type I epithelial cells; type II cell, alveolar type II epithelial cells. Shown are the numbers of cells examined in 5 randomly selected areas in 4 LBO rats under electron microscopy (magnification, ×1,000) and the percentage of cells showing cerium deposition.
balance, dried for 24 h in an oven at 95°C, and weighed again. The wet-dry ratios were then calculated from the two weights.

Statistics. All data were expressed as means ± SD. Groups were compared by two-way analysis of variance in combination with least-square methods (JMP IN version 5.1.1; SAS Institute, Cary, NC). The significance of individual differences was evaluated by contrast tests. Values of $P < 0.05$ were considered significant.

RESULTS

Production of ROS in the pulmonary circulation. We first examined the time course of ROS production in the pulmonary circulation of reexpanded rat lungs. Little DCF fluorescence was detected in the lungs of rats in the CT group, indicating the presence of little or no ongoing ROS production under control conditions (Fig. 1A). Indicative of a substantial increase in the production of ROS, however, we found marked increases in DCF fluorescence in the LBO group (Figs. 1B and 2). Moreover, this effect was almost completely blocked by pretreatment with the XOD inhibitor BOF-4272 (Figs. 1C and 2B).

Bronchial occlusion alone did not induce production of ROS, and DCF fluorescence in the LBO group was almost completely eliminated by pretreatment with catalase, verifying the specificity of the reaction of DCFH with ROS (not shown).

We next used electron microscopy and the cerium technique to determine the source of the ROS. We found that cerium was deposited almost exclusively around alveolar type I and II epithelial cells and endothelial cells of rats in the LBO group (Fig. 3B). Little or no cerium deposition was observed in the CT and BOF groups (Fig. 3, A and C, respectively). Further quantitative analysis of the cerium deposition in the lungs of LBO rats showed that the main sources of ROS were alveolar type II epithelial cells and endothelial cells (Table 1).

Apoptosis in the pulmonary circulation. We initially evaluated the incidence of apoptosis among cells in the lung by the TUNEL method. Whereas few apoptotic cells were detected in CT or BOF lungs (Fig. 4, A and C), numerous apoptotic cells were identified in LBO lungs (Fig. 4, B and D). This effect was
confirmed by DNA electrophoreses, which showed the characteristic ladder pattern of apoptosis in LBO lungs (Fig. 4E, lane 2), but not in CT or BOF lungs (Fig. 4E, lanes 1 and 3). Quantitative analysis of the incidence of apoptotic cells identified in the lungs of LBO rats by the TUNEL method or immunohistochemical staining of cleaved cytoplasmic caspase-3 suggested alveolar type I and type II epithelial cells, neutrophils, and endothelial cells all were affected (Fig. 4, F and G; Table 2). Having said that, however, we found it difficult to be certain precisely which cell types were affected using the TUNEL method at the light microscopic level; in particular, it is virtually impossible to distinguish endothelial and alveolar type I epithelial cells. Therefore, to determine more specifically which cell types were affected by apoptosis, we carried out electron microscopic TUNEL assays. With this approach, we continued to detect no apoptotic cells in CT lungs (Fig. 5A). By contrast, gold particles indicating apoptosis were readily detected among alveolar type I and type II epithelial cells, endothelial cells, and neutrophils of LBO lungs (Fig. 5B).

Wet-dry ratio. Finally, we measured the wet-dry ratios of lungs harvested from rats in each group to assess the effect of the observed apoptosis on their fluid content (Table 3). We found that pulmonary reexpansion significantly increased wet-dry ratios, indicating development of pulmonary edema and that the effect was significantly inhibited by BOF-4272.

DISCUSSION

In the present study, we found that pulmonary reexpansion leads to increases in ROS production, increases in the incidence of apoptosis among alveolar epithelial and endothelial cells, and increases in the fluid content of the lung and that all of these effects are inhibited by pretreating subjects with an XOD inhibitor (BOF-4272). Together, these findings strongly suggest that XOD-mediated ROS production induces apoptosis among alveolar epithelial and endothelial cells, which in turn leads to dysfunction of the blood-air barrier and the development of pulmonary edema.

Table 2. Kind of apoptotic cell

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total Count Cell Number</th>
<th>Percentage of Apoptotic Cell, %</th>
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<tbody>
<tr>
<td>Alveolus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>4</td>
<td>47.3±2.1</td>
<td>—</td>
</tr>
<tr>
<td>LBO</td>
<td>4</td>
<td>46.3±2.6</td>
<td>—</td>
</tr>
<tr>
<td>BOF</td>
<td>4</td>
<td>48.0±1.8</td>
<td>—</td>
</tr>
<tr>
<td>Type I cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>4</td>
<td>119.0±1.4</td>
<td>0.4±0.5</td>
</tr>
<tr>
<td>LBO</td>
<td>4</td>
<td>118.5±2.4</td>
<td>16.1±3.1*</td>
</tr>
<tr>
<td>BOF</td>
<td>4</td>
<td>119.0±0.8</td>
<td>2.1±0.5</td>
</tr>
<tr>
<td>Type II cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>4</td>
<td>162.5±2.4</td>
<td>0.5±0.3</td>
</tr>
<tr>
<td>LBO</td>
<td>4</td>
<td>163.3±4.3</td>
<td>62.5±1.5*</td>
</tr>
<tr>
<td>BOF</td>
<td>4</td>
<td>164.3±1.9</td>
<td>3.8±0.6</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>4</td>
<td>103.3±5.6</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>LBO</td>
<td>4</td>
<td>104.5±1.7</td>
<td>46.0±2.0*</td>
</tr>
<tr>
<td>BOF</td>
<td>4</td>
<td>101.8±2.1</td>
<td>3.0±0.8</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>4</td>
<td>5.5±1.7</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>LBO</td>
<td>4</td>
<td>7.0±2.7</td>
<td>14.8±14.1</td>
</tr>
<tr>
<td>BOF</td>
<td>4</td>
<td>5.3±2.1</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD. Shown are the numbers of cells examined in 5 randomly selected areas in 4 LBO rats under light microscopy (×400) and the percentage of apoptotic cells. * P < 0.05 vs. control (CT) determined by ANOVA followed by Tukey’s honestly significant-difference adjustment for multiple comparisons.
Macrophages and neutrophils, mechanical stress, and production of ROS by the alveolar type II epithelial cells plays a major role in the pathogenesis of RPE. We plan to test that hypothesis in future experiments.

To better understand the mechanism of RPE, we examined ROS production within individual cells in the lung using an intravital DCF digital fluoro-imaging technique developed in our laboratory (19–21). This method entails measuring ROS production as a function of the oxidation of DCFH to DCF (2). In an earlier study, Bass et al. (2) demonstrated the utility of this reaction for detecting hydrogen peroxide generated from isolated neutrophils. More recent findings indicate that DCFH is not oxidized solely by hydrogen peroxide, but also by the hydrogen peroxide-derived reactive intermediates (6, 19, 29). Nonetheless, DCFH remains a useful probe with which to assess overall oxidative stress.

Previously proposed mechanisms for the pathogenesis of RPE include changes in surfactant, accumulation of alveolar macrophages and neutrophils, mechanical stress, and production of ROS (10, 24, 26, 30, 31, 33). That we found BOF-4272 to inhibit ROS production in LBO rats (Fig. 2B) means that the observed effects of reexpansion are almost entirely XOD dependent and not the result of neutrophil respiratory bursts. In fact, we found that cerium was deposited almost exclusively around endothelial and alveolar type II cells rather than neutrophils (Fig. 3, Table 1), which is in stark contrast to what is seen in the endotoxin-infused lung (19–21). We therefore believe that ROS produced by XOD in endothelial and alveolar type II cells plays a major role in the pathogenesis of RPE.

Our quantitative analysis enabled us to observe that cerium was not deposited around alveolar type I epithelial cells (Table 1), which means that these cells did not produce ROS in the reexpanded lung. Nevertheless, alveolar type I epithelial cells did become apoptotic (Table 2), and the apoptosis was inhibited by BOF-4272. We therefore suggest that apoptosis among alveolar type I epithelial cells was induced by membrane-permeant hydrogen peroxide produced by other cell types (e.g., alveolar type II epithelial cells and/or endothelial cells) in the vicinity of the type I epithelial cells. We plan to test that hypothesis in future experiments.

Allopurinol is another XOD inhibitor that often has been used to study the enzyme’s function and has been shown to effectively attenuate lung reoxygenation injury (1, 34, 35). However, it has also been shown that oxypurinol, a major metabolite of allopurinol, is a scavenger of the highly reactive hydroxyl radical and of hypochlorous acid. Consequently, effects obtained with allopurinol in in vivo models need not be entirely due to XOD inhibition, making interpretation of the findings difficult (22). In the present study, therefore, we used BOF-4272, which directly binds to the xanthine binding site of XOD and acts as a competitive antagonist, leaving the NADH binding site unaffected (25). In addition, we previously showed that BOF-4272 does not affect superoxide production by neutrophils, nor does it scavenge superoxide (23). We therefore believe that our findings with BOF-4272 enable us to conclude that pulmonary reexpansion leads to XOD-mediated ROS production and apoptosis.

Apoptosis is a highly regulated physiological process that leads to cell death via two major pathways: the intrinsic pathway, which is mediated by the mitochondria, and the extrinsic pathway, which is mediated by death-signaling ligands (e.g., TNF-α or Fas ligand) and subsequent caspase-8 activation (3, 8, 12). We did not determine which pathway was activated in our model. Both pathways share downstream activation of caspase-3, however, which prompted us to look for the presence of cleaved cytoplasmic caspase-3 as a way of confirming induction of apoptosis. On the other hand, oxidant-induced damage to DNA is known to activate the intrinsic pathway, resulting in permeabilization of the outer mitochondrial membrane and release of molecules normally confined to the mitochondrial intermembrane space, such as cytochrome c; procaspases-2, -3, and -9; and apoptosis-inducing factor (3, 8, 12). Furthermore, XOD reportedly plays a key role in the ROS production that precedes the apoptotic cascade and cell death associated with reductions in mitochondrial cytochrome c content and caspase-3 activity (11, 32), and production of ROS followed by the release of cytochrome c and activation of caspase-9 reportedly causes mitochondria-regulated cell death in alveolar epithelial cells (27). We therefore suggest that it is most likely the intrinsic pathway that mediates apoptosis in our model.

In summary, we have shown that XOD-induced apoptosis among the endothelial and alveolar epithelial cells that make up the blood-air barrier in the lung is a major contributor to RPE.

GRANTS

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REFERENCES


Table 3. Wet-dry ratio of the lung

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Wet-dry ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>4</td>
<td>3.5±0.3</td>
</tr>
<tr>
<td>LBO</td>
<td>4</td>
<td>6.5±0.4*</td>
</tr>
<tr>
<td>BOF</td>
<td>4</td>
<td>4.8±0.2</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD; *P < 0.05 vs. CT group.