Early Reperfusion Induces Alveolar Injury in Pulmonary Embolism*

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Study objective: To observe (1) whether the reperfusion is one of the causes underlying the development of diffuse alveolar injury following pulmonary embolism, and (2) whether polymorphonuclear leukocyte (PMN) accumulation occurs in the reperfused lobe, and (3) whether the production of superoxide is increased from cells obtained by BAL.

Design: The condition of pulmonary embolism was simulated by occluding the pulmonary artery branch using a balloon catheter in anesthetized closed-chest dogs. The occlusion was maintained for 24 h in the occlusion group, and a 2-h period of occlusion was followed by reperfusion in the reperfusion group. Histologic examination was performed at 24 h after occlusion in both groups (n=8). Using a different group of dogs (n=12), local cellular changes in the occluded and reperfused lobes were evaluated through BAL performed at 1, 2, and 3 h after reperfusion in the reperfusion group and at 3 h after occlusion in the occlusion group. Superoxide generation from BAL cells was measured by the chemiluminescence method.

Results: There was no histologic evidence of alveolar injury in the occluded lobe, but there were numerous leukocytes and erythrocytes along with exudate and damaged alveoli in the reperfused lobe. In the BAL study, the total cell counts recovered by BAL remained unchanged in all groups. However, the number of PMNs increased significantly in the late stages of reperfusion. Enhanced superoxide generation was observed in BAL cells obtained from reperfused lobe.

Conclusion: Reperfusion is one of the causes underlying the development of alveolar injury in pulmonary embolism by triggering immigration of PMNs to alveoli, which results in the increased superoxide generation in BAL cells. (CHEST 1997; 111:198-203)

Key words: bronchoalveolar lavage; pulmonary embolism; reperfusion injury; superoxide

Abbreviations: cps=counts per second; MCLA=2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo[1,2-a] pyrazin-3-one; PA=pulmonary artery; PMN=polymorphonuclear leukocytes; SOD=superoxide dismutase

It is well known that the lungs are relatively resistant to occlusion of the pulmonary artery (PA) because of the double blood supply. However, clinical studies have demonstrated that some patients exhibit pulmonary necrosis subsequent to pulmonary thromboembolism. Therefore, much attention has been focused on the elucidation of the underlying mechanisms that lead to necrosis in pulmonary embolism. A recent study using a pulmonary embolism model in conscious rats showed that the restoration of blood flow after 2-h PA occlusion induces diffuse alveolar injury, although PA occlusion alone causes only minor histologic changes; this finding indicates that natural resolution of the thromboembolus may occur in some patients, which results in reperfusion and induces injury. The study also demonstrated that superoxide is one of the species responsible for the damage, since elimination of superoxide by superoxide dismutase (SOD) attenuated the damage. However, the following two issues...
remain to be clarified in the study. First, does the injury occur in an animal model that is more appropriately designed to be to relevant to clinical pulmonary embolism and second, does superoxide generation actually increase in the reperfused lobe? Thus, the present study was undertaken in a closed-chest canine model and examined the local cellular changes and superoxide generation from BAL cells.

**MATERIALS AND METHODS**

**Animal Preparations**

All animals were managed in accordance with the Guiding Principles for the Care and Use of Animals in the Physiological Sciences proposed by the Physiological Society of Japan and National Institutes of Health guidelines. Dogs were anesthetized with pentobarbital sodium (25 mg/kg IV bolus) followed by continuous infusion; they were intubated and ventilated with room air at 15 breaths/min at a tidal volume of 20 mL/kg. Systemic and pulmonary arterial BP were measured with a fluid-filled catheter placed in the left femoral artery and in the main PA. A PA wedge-type balloon catheter was inserted through the right femoral vein and placed fluoroscopically in the PA branch supplying the left lower lobe. The balloon was used to occlude and reperfuse the PA branch at the indicated time during the monitor of PA or pulmonary capillary wedge pressure. The right lower lobe served as a control lobe. BAL procedures were performed during the course of experiment and histologic evaluation of lungs was done at the end of the experiment. To avoid a possible detrimental effect of the BAL procedure on histologic evaluation, we used a different group of dogs for the histologic study and the BAL study. In the histologic evaluation group, four dogs underwent a 24-h period of PA occlusion (occlusion group), and four dogs underwent a 2-h period of PA occlusion followed by reperfusion (reperfusion group). Dogs were killed by overdose of pentobarbital sodium 24 h after the PA occlusion in both groups, and their lungs were removed from the thorax. In the BAL study group, BAL fluid was obtained from the control lobe and the occluded lobe at the indicated time (3 h after occlusion in the occlusion group [n=3] and 1 h [n=3], 2 h [n=3], and 3 h [n=3] after reperfusion in the reperfusion group, respectively: three separate dogs were used at each different time to avoid repeat BAL procedure). Superoxide generation from BAL cells was measured and the composition of BAL cells was determined.

**Histologic Examination**

The lungs were fixed by injection of 10% formaldehyde solution through the trachea at a pressure of 20 cm H2O and immersed in 10% formaldehyde solution. A 3-mm-thick section was obtained from the center of the each lobe of both lungs. These sections were embedded in paraffin, sectioned serially at 5 μm, and stained with hematoxylin-eosin. The tissue damage was quantitated by evaluation of the presence of abnormal alveoli as described previously. Briefly, all sections were coded randomly, and 50 microscopic fields using a magnification of ×400 were examined in each section. The number of alveoli in each field was totaled. Alveoli containing exudate, more than two erythrocytes or more than two leukocytes, were considered as “damaged alveoli; the percentage of damaged alveoli was calculated over the total number of alveoli.

**Bronchoalveolar Lavage**

BAL was performed as described elsewhere. Briefly, the bronchoscope was introduced through the tracheal tube and wedged into the desired bronchopulmonary segment. Four sequential aliquots (20 mL) of sterile 0.9% NaCl at room temperature were rapidly instilled into and aspirated from the bronchopulmonary segment. The initially recovered fluid was discarded and the three subsequent recovered fluids were pooled for analysis. The cells were collected by centrifugation (2000×g, 10 min) and washed once in Hanks’ balanced salt solution. Superoxide generation from the cells was measured after the total cell number was counted using a hemocytometer. The composition of alveolar macrophages and polymorphonuclear leukocytes (PMNs) in the BAL cells was determined by the smeared sample stained with May-Giemsa.

**Measurement of Superoxide Generation**

Superoxide generation from cells was measured by chemiluminescence method using a specific chemiluminescence probe for superoxide, 2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo[1,2-a] pyrazin-3-one (MCLA) at 37°C as described elsewhere. Briefly, a Petri dish (45-mm diameter) containing 1.0×10⁵ cells and 1 μmol/L MCLA in 3.0 mL Hanks’ balanced salt solution was placed in the photon counter and photon counts were measured continously. Superoxide generation was initiated by phorbol myristate acetate, 300 ng/mL, and terminated by the addition of SOD (50 U/mL). Superoxide generation was quantitated using the peak counts of SOD-inhibitable chemiluminescence expressed as counts per second (cps)/1×10⁵ cells. The actual rate of superoxide generation in this condition was determined by comparing the corresponding value obtained by the cytochrome c reduction method: 7.52×10⁵ cps cells were shown to be comparable to 1 nmol/min.

**Statistical Analysis**

Statistical analysis was performed using the one-way analysis of variance with Newman-Keuls test. All data are expressed as means±SEM. Significance was assigned at p<0.05.

**RESULTS**

**Course and Outcome**

All dogs survived throughout the experimental course. Occlusion and reperfusion of PA did not produce significant changes in the PA pressure, aortic pressure, or blood gas data. Aortic pressure remained above 100 mm Hg and PaO₂ was higher than 90 mm Hg throughout the study (Table 1).

**Histologic Examination**

Microscopic examination in the control lobe (the right lower lobe) revealed that the alveolar structure was normal, and alveoli did not contain leukocytes or erythrocytes. In the occluded lobe (the left lower lobe of the occlusion group), alveolar structure also remained unchanged and alveoli contained no leukocytes, erythrocytes, or exudate (Fig 1). In the reperfused lobe (the left lower lobe of the reperfus-
sion group), there were numerous leukocytes and erythrocytes along with damaged alveolar framework. The accumulated leukocytes were identified as PMNs by the lobulated nucleus. The presence of exudate was also demonstrated by the hyalin in the intra-alveolar space.

For quantification of the severity of the damage, each alveolus was examined. The total number of alveoli examined in 50 microscopic fields of the left lower lobe and control lobe was 268±12 and 265±5 in the reperfusion group and 273±22 and 278±25 in the occlusion group, respectively. These findings indicated that the quality of tissue fixation and the examination method were identical in all lobes of both groups. The percentages of alveoli with exudate, erythrocytes, and leukocytes in the perfused, control, and occluded lobes are shown in Figure 2. The percentage of alveoli containing exudate was significantly higher in the reperfused lobe than in the occluded lobe (control lobe: 0.2±0.2% [2/278]; occluded lobe: 0.2±0.2% [1/273]; and reperfused lobe: 16.6±12.4% [46/268]) (Fig 2). The percentage of alveoli containing erythrocytes was also significantly higher in the reperfused lobe (control lobe: 0.8±1.0% [2/278]; occluded lobe: 1.4±0.9% [2/273]; and reperfused lobe: 23.0±18.1% [63/268]). The percentage of alveoli containing leukocytes was also higher in the reperfused lobe (control lobe: 12.0±3.6% [32/265]; occluded lobe: 12.1±4.2% [33/273]; and reperfused lobe: 57.0±26.4% [155/268]).

Cell Composition of BAL and Superoxide Generation

The total cell counts recovered by BAL were 1.53±0.4, 1.1±0.64, 1.07±0.31, 1.77±0.9, and 1.4±0.35×10⁷ in the control, occlusion, 1-h, 2-h, and 3-h reperfused lobes, respectively. There was no significant difference in BAL cell numbers among groups. The macrophage numbers showed a tendency to decrease in the occluded and reperfused lobes (1.4±0.4, 1.0±0.66, 0.93±0.22, 1.13±0.66, and 0.87±0.23×10⁷ in the control, occlusion, 1-h, 2-h, and 3-h reperfused lobes, respectively [Fig 3]). The number of PMNs increased in the reperfused lobe. The increase in PMN number was more significant in the late stages of reperfusion ([2 h and 3 h]; 0.13±0.23, 0.2±0.2, 1.5±1.0, 6.5±2.7, and 5.4±1.8×10⁷ in the control, occluded, 1-h, 2-h, and 3-h reperfused lobes, respectively). Superoxide generation from BAL cells was significantly increased in the reperfused lobes, which was more marked in the later stage of reperfusion (0.41±0.03, 0.34±0.08, 1.23±0.55, 3.82±1.41, and 3.41±0.60×10⁶ cps/10⁵ cells, in the control lobe, occluded lobe, 1-h, 2-h, and 3-h reperfused lobes, respectively; Fig 4).

**Discussion**

The present study in canine closed-chest model demonstrated the following: (1) histologic changes compatible with pulmonary diffuse alveolar injury occurred in the lobe which underwent 2-h PA occlusion followed by reperfusion but not in the lobe without reperfusion; (2) the PMN fraction increased in BAL cells which was recovered from the lobe with reperfusion, but it remained unchanged in BAL cells recovered from the control lobe or the lobe which was only occluded; the number of macrophages in BAL cells did not increase in either the lobes with occlusion or reperfusion; and (3) the superoxide generation markedly increased in BAL cells obtained from the reperfused lobe but not from the occluded lobe.

Although the lungs are known to be resistant to PA occlusion because of the double blood supply, increasing evidence indicates that reperfusion following ischemia can cause damage in the lungs. However, most studies that demonstrated the reperfusion injury in the lungs utilized animal models in which both ventilation and perfusion are interrupted, or the ischemic time is as long as 24 or 48 h since these are relevant for lung transplantation models. To examine the effect of reperfusion in a pulmonary embolism model, it is important to use a model in which the PA is occluded for a shorter duration without interfering with ventilation. Our previous study of rat pulmonary embolism model fulfilled the above-mentioned criteria and suggested that one of the underlying mechanisms in the development of pulmonary injury is the superoxide-related reaction. In that model, however, the PA was obstructed by tying the PA branch from outside...
Figure 1. Light microscopic view of the lung in occluded and reperfused lobes. Top: occluded lobe (magnification ×60); center and bottom: reperfused lobes (magnification ×60 and ×600, respectively). Structure of alveoli is normal in the occluded lobe. The reperfused lobe shows hemorrhagic alveolar injury with PMN. Bar=500 μm in top and center. Bar=50 μm in bottom.

Figure 2. Quantitative assessment of lung injury. Percentages of alveoli with (A) exudate, (B) erythrocytes, and (C) PMNs are shown. There were no significant differences in the percentage of alveoli with exudate, erythrocytes, and PMNs between the control and occluded lobes. Reperfused lobes disclosed a significant increase in the percentage of alveoli with exudate, erythrocytes, and PMNs. Asterisk=significant difference of reperfused lobes vs control and occluded lobes (p<0.05).

of the vessel, and thus it is not an ideal model of pulmonary embolism.14 In addition, superoxide generation was not evaluated. Therefore, in the present study, we used closed-chest dogs with the balloon occlusion method to obstruct from the intraluminal side and measured superoxide generation from BAL cells. It may be argued that for an analogous model of pulmonary embolism, the autologous clot
Asterisk=significant
dagger=each lobe vs occluded lobe; double dagger=2-h vs 1-h reperfused lobes (p<0.05).

Figure 3. Effects of occlusion and reperfusion on BAL cells. The number of macrophages did not differ among the lobes, but the number of PMNs increased in the 2-h and 3-h reperfused lobes. Asterisk=significant difference of each lobe vs control lobe (p<0.05).

Method\textsuperscript{15} should be applied in place of the balloon occlusion method. However, the clot method does not allow distinction of a specific site of obstruction or control of the timing of reperfusion. Concerning the length of ischemia, we chose 2 h with the intent to confirm in dogs our previous finding in rats\textsuperscript{5} and also because a currently performed therapeutic maneuver\textsuperscript{16} employs an early application of thrombolysis.

Although we focused on the measurement of superoxide and did not examine the actual role of superoxide in reperfusion injury in this study, we assume that the increase in superoxide generation is one of the causative factors in lung injury since we have already demonstrated the effect of SOD against reperfusion injury in a previous study.\textsuperscript{5} The present findings support our hypothesis; one of the underlying mechanisms in the development of pulmonary diffuse alveolar injury following pulmonary embolism is the natural resolution of thrombus, although there is no direct relation between this study and the actual data on thrombus resolution in patients with necrosis.\textsuperscript{2-4} This hypothesis remained to be proved by detailed histopathologic examination in patients with pulmonary necrosis or pulmonary edema after pulmonary artery thromboendarterectomy.\textsuperscript{17}

Figure 4. Superoxide generation from BAL cells. The peak superoxide generation from 1×10\textsuperscript{6} cells increased markedly in BAL cells obtained from the reperfused lobes. Asterisk=significant difference of each lobe vs control lobe; dagger=each lobe vs occluded lobe; double dagger=2-h vs 1-h reperfused lobes (p<0.05).

References