Ischemia-reperfusion lung injury in rabbits: mechanisms of injury and protection

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Department of Respiratory Medicine, Kanazawa Medical University, Uchinada, Ishikawa 920-0293, Japan; Cardiovascular Research Institute, University of California, San Francisco, California 94134; Department of Veterans Affairs Medical Center, Seattle, Washington 98108; and Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah 84132

Sakuma, Tsutomu, Keiji Takahashi, Nobuo Ohya, Osamu Kajikawa, Thomas R. Martin, Kurt H. Albertine, and Michael A. Matthay. Ischemia-reperfusion lung injury in rabbits: mechanisms of injury and protection. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L137–L145, 1999.—To study the mechanisms responsible for ischemia-reperfusion lung injury, we developed an anesthetized rabbit model in which the effects of lung deflation, lung inflation, alveolar gas composition, hypothermia, and neutrophils on reperfusion pulmonary edema could be studied. Rabbits were anesthetized and ventilated, and the left pulmonary hilum was clamped for either 2 or 4 h. Next, the left lung was reperfused and ventilated with 100% oxygen. As indexes of lung injury, we measured arterial oxygenation, extravascular lung water, and the influx of a vascular protein (125I-labeled albumin) into the extravascular space of the lungs. The principal results were that 1) all rabbits with the deflation of the lung during ischemia for 4 h died of fulminant pulmonary edema within 1 h of reperfusion; 2) inflation of the ischemic lung with either 100% oxygen, air, or 100% nitrogen prevented the reperfusion lung injury; 3) hypothermia at 6–8°C also prevented the reperfusion lung injury; 4) although circulating neutrophils declined during reperfusion lung injury, there was no increase in interleukin-8 levels in the plasma or the pulmonary edema fluid, and, furthermore, neutrophil depletion did not prevent the reperfusion injury; and 5) ultrastructural studies demonstrated injury to both the lung endothelium and the alveolar epithelium after reperfusion in deflated lungs, whereas the inflated lungs had no detectable injury. In summary, ischemia-reperfusion injury to the rabbit lung can be prevented by either hypothermia or lung inflation with either air, oxygen, or nitrogen.

Lung transplantation; pulmonary edema; hypoxia; hypothermia; neutrophil

The mechanisms responsible for ischemia-reperfusion injury to the lung have direct or indirect relevance to clinical lung injury after severe shock, cardiopulmonary bypass, and lung transplantation. Several studies that have explored the mechanisms responsible for ischemia-reperfusion lung injury have improved the methods for preservation of the donor lung for transplantation. However, ischemia-reperfusion lung injury is still a common clinical problem after lung transplantation.

A variety of mechanisms have been identified that may contribute to ischemia-reperfusion lung injury. For example, in some models, ischemia-reperfusion seems to be mediated in part by neutrophils (18, 21, 28, 39). Also, one group reported that elevated levels of interleukin-8 (IL-8), the predominant chemotactic factor for neutrophils, was found in the air spaces of the lungs (39), and another group reported that lung injury after 2 h of ischemia could be prevented by monoclonal antibody treatment against IL-8 (32). In other studies, neutrophils have not been found to be necessary for development of ischemia-reperfusion lung injury (7, 10, 22). In addition, some investigators have related the degree of lung injury to the temperature of the lung (3, 4, 6, 14), the absence of lung inflation (1, 5, 8, 37), or the composition of the alveolar gas (2, 9, 11, 12, 15, 16, 20, 38). However, the relative contribution of each of these factors for prevention of ischemia-reperfusion lung injury has not been examined comprehensively in the same study.

Therefore, the first objective of this study was to establish a time-dependent in vivo rabbit model in which the mechanisms responsible for ischemia-reperfusion lung injury could be examined. Once this was accomplished with a 4-h model of in situ ischemia followed by severe reperfusion permeability pulmonary edema, the second objective was to determine whether inflation of the ischemic lung during the 4 h of ischemia would prevent the reperfusion lung injury. Because lung inflation with 100% oxygen prevented the ischemia-reperfusion lung injury, the third objective was to determine whether the alveolar gas composition contributed to the protective effect of lung inflation. Therefore, the protective effect of inflation with either air or 100% nitrogen was tested. Because cooling of the lung has been reported to attenuate reperfusion lung injury, the fourth objective was to determine the effect of hypothermia on prevention of the reperfusion lung injury in this model. Because the concentration of neutrophils in the blood markedly declined in parallel with the development of reperfusion lung injury, the final objective was to determine whether the neutrophils contributed to the development of ischemia-reperfusion lung injury in this rabbit model. Light and ultrastructural studies of the lungs were included to provide insight into the
magnitude of injury to the endothelial and epithelial barriers of the lung.

**METHODS**

**Surgical Preparation**

All experiments were approved by the University of California, San Francisco Animal Care Committee. New Zealand White rabbits (2.5–3.5 kg) were anesthetized with 1% halothane. An endotracheal tube was inserted in the trachea through a tracheotomy. The rabbits were ventilated with 100% oxygen at 15 ml/kg of tidal volume, with a peak airway pressure of <25 cmH2O and a positive end-expiratory pressure of 2 cmH2O. Carotid arterial and central venous lines were inserted to measure systemic and venous blood pressure and to collect arterial blood. A sternotomy was done followed by opening of both pleural spaces. The pulmonary ligament was dissected between the left lung and the mediastinum, and umbilical tape was placed around the entire left pulmonary hilum (including the left main bronchus, the left pulmonary artery, and the left pulmonary vein). In additional experiments, Japanese White rabbits (2.5–3.0 kg, n = 4 rabbits) were anesthetized with 20 mg/kg of pentobarbital sodium intravenously. Ventilation and surgical preparation were the same as above except that the left main bronchus was isolated from the pulmonary artery and vein so that perfusion could be continued to a deflated lung in some studies (see Specific Protocols). The rabbits were covered by a thermostatically controlled pad to maintain the lung temperature at 37–38°C.

**General Protocol**

In all experiments, a 30-min baseline of stable blood pressure and heart rate was required before clamping of the left pulmonary hilum. Sodium heparin (500 U/kg) was administered 10 min before clamping. Next, the left pulmonary hilum was clamped with the umbilical tape for either 2 or 4 h. The lungs were completely deflated for either 2 or 4 h after ischemia for either 2 or 4 h. At the end of reperfusion and reventilation, the rabbits were exsanguinated, and the lungs were excised separately. The water-to-dry weight ratio was measured by drying separated lungs to a constant weight at 70°C for 72 h. In rabbits with severe lung injury (4-h studies), samples of alveolar edema fluid were collected with a 5-Fr feeding tube.

**Specific Protocols**

**Group 1:** Effect of the ischemic period on reperfusion lung injury. To determine the effect of duration of the ischemic period on the reperfusion injury, the left lung was exposed to ischemia for either 2 (n = 4 rabbits) or 4 h (n = 4 rabbits). The left pulmonary hilum was clamped at the end of expiration. The lungs were completely deflated for either 2 or 4 h after clamping. The right lung was ventilated to maintain arterial PCO2 between 35 and 45 mmHg. Next, the left lung was undamped and exposed to reperfusion and reventilation with 100% oxygen for 4 h. To confirm that there was no blood flow to the clamped left lung, the left lungs were excised at the end of ischemia for 4 h and homogenized (n = 3 rabbits). The quantity of the vascular tracer (131I-albumin) that had been injected after clamping of the left pulmonary hilum was measured in the homogenized lung samples.

To be certain that the injury depended on both ischemia and deflation, four rabbits were studied for 4 h after deflation of the lung without interruption of blood flow to the lung. A 30-min baseline of stable blood pressure and heart rate was required before clamping of the left main bronchus. Sodium heparin (500 U/kg) was administered 10 min before ligation of the left main bronchus. Next, the left main bronchus was ligated for 4 h, and the left lung was collapsed. Perfusion to the left lung was continued during ligation of the left main bronchus. Ventilation of the left lung was resumed for 4 h after collapse. At the end of reventilation for 4 h, the rabbits were exsanguinated, and the lungs were excised separately. The water-to-dry weight ratio was measured by drying.

**Group 2:** Effect of inflation on ischemia-reperfusion lung injury. To determine whether inflation of the ischemic lungs with 100% oxygen affected reperfusion lung injury, we clamped the left hilum when the lungs were inflated at an airway pressure of 30 cmH2O. Ischemia (no blood flow) was maintained for 4 h (n = 4 rabbits); the lungs were then exposed to reperfusion and reventilation with 100% oxygen for 4 h. The lungs were inflated 4 h after clamping.

**Group 3:** Effect of alveolar gas composition on ischemia-reperfusion lung injury. Because inflation with 100% oxygen (group 2) prevented most of the lung injury, we determined the effect of alveolar gas composition in the ischemic lung on the reperfusion lung injury. The ischemic lungs were inflated with either air (n = 3 rabbits) or 100% nitrogen (n = 4 rabbits). To inflate the left lung with air before clamping of the left pulmonary hilum, the rabbits were ventilated with air, and the left pulmonary hilum was clamped when the lungs were inflated at an airway pressure of 30 cmH2O. To inflate the ischemic lung with nitrogen, we inserted an 8-Fr feeding tube in the left main bronchus. Oxygen in the left lung was replaced with nitrogen over 5 min, and then the left hilum was clamped. The left lung was inflated with 100% nitrogen at an airway pressure of 30 cmH2O.

**Group 4:** Effect of hypothermia on ischemia-reperfusion lung injury. To cool the ischemic deflated lung (n = 4 rabbits), the left lung was immersed in a cooled isosmolar solution (66% of Ringer lactate plus 33% of physiological saline solution) that was circulated (Master Flex Pump Controller; Cole-Parmer Instruments, Chicago, IL) around the lung in the bag for 4 h immediately after clamping of the left pulmonary hilum. The temperature of the solution around the lungs was maintained at 6–8°C. After 4 h of ischemia, the lungs were exposed to reperfusion at normal temperature and reventilation with 100% oxygen for 4 h.

**Group 5:** Effect of depletion of neutrophils on ischemia-reperfusion lung injury. Because the number of neutrophils decreased after reperfusion in the rabbits with deflated lungs during ischemia, we determined whether depletion of neutrophils affected the ischemia-reperfusion lung injury. To deplete neutrophils, vinblastine (0.75 mg/kg) was administered through the ear vein 4 days before the experiments, as we have done previously (13). The lungs were inflated (n = 3 rabbits) or deflated (n = 3 rabbits) during ischemia over 4 h and then were exposed to reperfusion and reventilation with 100% oxygen over 4 h.

**Measurements**

**Extravascular lung water.** Right and left lungs were homogenized separately. The homogenate from each lung was then centrifuged at 30,000 g at 4°C for 1 h to obtain clear supernatant. The water content of lung homogenate, supernatant, and whole blood was measured by drying duplicated samples to a constant weight at 70°C for 72 h. The hemoglobin concentration in whole blood and the lung supernatant was measured using the cyanomethemoglobin method. The ratios of the supernatant to whole blood by 10.220.33.2 on August 27, 2017 http://ajplung.physiology.org/ Downloaded from
of extravascular water content to blood-free dry weight of the lung were calculated as reported previously (30, 33).

Plasma equivalents. To estimate influx of plasma in the interstitium and air spaces, we measured the total extravascular \(^{131}\)I-albumin accumulation in the experimental lung (left) and the control lung (right). This was done with the following equation

\[
^{131}\text{I-albumin}_{\text{extravascular}} = \frac{^{131}\text{I-albumin}_{\text{lung}}}{^{131}\text{I-albumin}_{\text{plasma}}} - ^{131}\text{I-albumin}_{\text{intravascular}}
\]

To obtain the \(^{131}\)I-albumin\(_{\text{extravascular}}\), \(^{131}\)I-albumin counts in the last plasma sample were multiplied by whole blood volume. Plasma equivalents were calculated by the equation

\[
\text{Plasma equivalent} = \frac{^{131}\text{I-albumin}_{\text{extravascular}}}{^{131}\text{I-albumin}_{\text{plasma}}}
\]

Plasma \(^{131}\)I-albumin counts were averaged over the time course of the experiment. This ratio provides a good index of equilibration of the vascular protein tracer in the extravascular compartment, as we have reported previously (13, 35).

Hemodynamics, airway pressure, and arterial blood gases. Heart rate, systemic and venous blood pressure, and airway pressure were continuously monitored. Arterial blood gas analyses were carried out in blood samples during baseline, at the end of ischemia, and hourly after reperfusion for 4 h (Blood Gas Analyzer; Ciba Corning Diagnostics, Medfield, MA).

Neutrophils. Blood samples were collected during baseline, at the end of ischemia, and every hour for 4 h after reperfusion. The total number of neutrophils in the circulating blood was counted with a hemocytometer with a differential on smears of samples to determine the percent neutrophils. The total number of neutrophils was also counted in the pulmonary edema fluid from the reperfused rabbit lungs that had been deflated (alveolar edema fluid was not obtainable from the other experimental groups).

IL-8 levels in plasma and pulmonary edema fluid. The concentrations of rabbit IL-8 (rIL-8) in plasma and pulmonary edema fluid were measured with ELISA. Briefly, goat anti-rIL-8 fusion antibody was diluted to 200 µg/ml with 0.1 M bicarbonate buffer, pH 9.6. Polystyrene 96-well plastic plates (Corning) were coated with the antibody solutions and incubated at 4°C. The plates were rinsed two times with Dulbecco's PBS after antibodies were removed and then were incubated for 1 h at 37°C with diluent (5% nonfat milk in Dulbecco's PBS containing 0.05% Tween 20) for blocking. The samples were diluted in assay diluent and added in 100 µl/well aliquots, and then the plates were incubated at 37°C for 2 h. The plates were washed four times with washing solution (Dulbecco's PBS containing 0.05% Tween 20). Next, biotinylated goat anti-rIL-8 fusion protein IgG was added to each plate and then incubated at 37°C for 2 h. The plates were incubated with streptavidin-biotin-peroxide complex reagent (Zymed, S. San Francisco, CA) at 37°C for 1 h. Finally, peroxidase substrate (3,3',5,5'-tetramethylbenzidine; KPL, Gaithersburg, MD) was added at 100 µl/well and incubated at 37°C for 1 h. The reaction was stopped with 100 µl/well of 1 M phosphoric acid, and the optical density (450 nm) in each well was read in a microtiter plate reader (Dynatech, Chantilly, VA).

Morphological examination. For morphological examination, lungs were obtained from rabbits with the left lungs deflated during ischemia for 4 h followed by reperfusion for 10 min (n = 2 rabbits) or from rabbits with the left lungs inflated with 100% oxygen during ischemia for 4 h followed by reperfusion for 10 min (n = 2 rabbits). The right lungs were used as controls. For light microscopy, clamped and inflated lobes were immersed in Carnoy's fixative and were placed at 4°C. For transmission electron microscopy, small amounts of fixative (2.5% glutaraldehyde + 1% paraformaldehyde) were injected into the lobes with a TB syringe and 27-gauge needle as we have done previously (30). The clamped lung lobes were immersed in the same fixative and were stored at 4°C. The lung samples (0.5 × 1 × 1 mm) were postfixed in 1% osmium tetroxide, en bloc stained with 5% uranyl acetate, dehydrated through a graded series of acetone, and embedded in Polybed 812. Random thin sections (80 nm) were cut from all five embedded blocks with a diamond knife and were counterstained with uranyl acetate and lead citrate. Slices of the lung were examined by a transmission electron microscope (JEM-1200FXII; Japan Electron Optics Laboratory, Tokyo, Japan).

Statistics

Data are expressed as means ± SD. Analysis of variance was used to compare the data between the groups. A Student's paired t-test was used to compare the data in the same group. Significance was P < 0.05.

RESULTS

Group 1: Effect of the Ischemic Period on Reperfusion Lung Injury

Severe ischemia-reperfusion lung injury occurred in the lungs that were deflated during ischemia for 4 h. All rabbits died within 1 h after reperfusion, with a large quantity of alveolar edema fluid that had a protein concentration similar to plasma (Fig. 1). In the experiments with ischemia for 4 h, extravascular lung water increased to 16.1 ± 3.1 g H₂O/g dry lung in the left lung compared with 7.9 ± 0.7 g H₂O/g dry lung in the right lung (Fig. 2A); plasma equivalents increased to 28.4 ± 10.0 ml in the left lung compared with 10.7 ± 4.3 ml in the right lung (Fig. 2B).

Fig. 1. Edema fluid-to-plasma protein concentration ratio in the rabbits that developed severe pulmonary edema (see Fig. 2) and large quantities of alveolar edema fluid after 4 h of ischemia and lung deflation in rabbits with normal numbers of neutrophils (group 1) or those with neutrophil depletion (group 5).
In the experiments with ischemia for 2 h, extravascular lung water increased to 5.7 ± 1.1 g H₂O/g dry lung in the left lung compared with 3.9 ± 0.3 g H₂O/g dry lung in the right lung (Fig. 2A); the plasma equivalents increased to 3.0 ± 1.1 ml in the left lung compared with 0.5 ± 0.3 ml in the right lung (Fig. 2B). In contrast to the 4-h experiments, no alveolar edema fluid could be suctioned from the air spaces of the lung.

The number of circulating neutrophils decreased by ~70% in the experiments with ischemia for 4 h (Fig. 3). In the experiments with ischemia for 2 h, the number of neutrophils did not change significantly after reperfusion compared with that during ischemia.

Arterial oxygenation decreased to <100 mmHg after reperfusion and reventilation in the experiments with lungs that were deflated during ischemia for 4 h. In the experiments with lungs deflated during ischemia for 2 h, arterial oxygenation decreased significantly and did not recover during 4 h after reperfusion and reventilation.

In the experiments that were done to confirm no blood flow to the ischemic lung, there was no vascular tracer (¹³¹I-albumin) in the homogenized ischemic lungs. However, 0.59 ± 0.06 ml of vascular tracer was measured in the right lung.

All rabbits survived for 4 h after the 4-h deflation of the left lung in the presence of pulmonary perfusion. There was no difference between the water-to-dry weight ratio in the collapsed left lungs and the contralateral right lungs. Water-to-dry weight ratios in the left lungs were 5.52 ± 0.41 g/g in the collapsed left lungs and 5.06 ± 0.20 g/g in the contralateral right lungs. The final arterial blood gas after reventilation was 352 ± 87 mmHg. Thus the severe lung injury following reperfusion of the collapsed, ischemic lung (Figs. 1 and 2) could be prevented by maintaining blood flow to the collapsed lung.

**Group 2: Effect of Inflation on Ischemia-Reperfusion Lung Injury**

Inflation of the ischemic lungs with 100% oxygen for 4 h prevented ischemia-reperfusion lung injury. Extravascular lung water was reduced to 5.5 ± 0.2 g H₂O/g dry lung compared with 16.1 ± 3.1 g H₂O/g dry lung in the experiments with deflated ischemic lungs (Fig. 4A). Plasma equivalents were reduced to 1.9 ± 0.9 ml compared with 28.4 ± 10.0 ml in the experiments with deflated ischemic lungs (Fig. 4B). Extravascular lung water and plasma equivalents were normal in the right control lungs in this group. Also, inflation of the ischemic lungs with 100% oxygen prevented the decrease of circulating neutrophils after reperfusion and reventilation (Fig. 5). Inflation of the ischemic lung with 100% oxygen also prevented the decrease in oxygenation after reperfusion and reventilation (Fig. 6).
Group 3: Effect of Alveolar Oxygen Concentration on Ischemia-Reperfusion Lung Injury

The group 2 experiments established a remarkable protective effect of inflation with 100% oxygen. Therefore, these group 3 experiments were designed to determine the contribution of alveolar gas composition to the protective effect. Inflation of the lungs with air or 100% nitrogen at an airway pressure of 30 cmH₂O reduced the increased extravascular lung water and plasma equivalents as much as inflation of the lung with 100% oxygen (Fig. 7).

Although the number of neutrophils decreased 1 h after reperfusion from the number of neutrophils at the end of the ischemic period, the decrease was less in the experiments with lungs inflated with oxygen, air, or nitrogen compared with the decrease in the experiments with deflated lungs (data not shown). In summary, inflation of the ischemic lung at an airway pressure of 30 cmH₂O with any gas (oxygen, air, or nitrogen) prevented ischemia-reperfusion lung injury.

Group 4: Effect of Cooling of the Ischemic Lung on Ischemia-Reperfusion Lung Injury

Hypothermia of the ischemic lung abolished ischemia-reperfusion lung injury. Although the left lung was completely deflated, extravascular lung water and plasma equivalents were normal in the hypothermia experiments (Fig. 8). The number of neutrophils and the level of oxygenation after reperfusion were not significantly different from those during baseline in the hypothermia experiments (data not shown).

Fig. 4. Effect of inflation of the ischemic lung on reperfusion lung injury. A: extravascular lung water; B: plasma equivalent. *P < 0.05 vs. corresponding values in the deflated left lungs in the 4-h ischemia experiments.

Fig. 5. Effect of inflation of ischemic lung on blood neutrophil counts. *P < 0.05 vs. neutrophil counts 1 h after reperfusion in the 4-h ischemia experiments with inflated left lungs during ischemia.

Fig. 6. Effect of inflation with 100% oxygen during ischemia on arterial Po₂ (PaO₂). *P < 0.05 vs. the corresponding values in deflated left lungs in the 4-h ischemia experiments.

Fig. 7. Effect of alveolar gas composition on indexes of reperfusion lung injury, specifically extravascular lung water (A) and plasma equivalents (B), the index of accumulation of plasma protein in the extravascular space of the lung. * and †P < 0.05 vs. the corresponding values in the ischemic lungs deflated during ischemia.
Group 5: Effect of Depletion of Neutrophils on Ischemia-Reperfusion Lung Injury

Depletion of neutrophils by vinblastine did not affect ischemia-reperfusion lung injury in either deflated or inflated lungs. Severe pulmonary edema occurred in the lungs deflated during ischemia even though these rabbits were severely neutropenic. Also, inflation of the ischemic lungs in these neutropenic rabbits still prevented the reperfusion injury (Fig. 9). There was no significant difference between the number of circulating neutrophils before reperfusion and those after reperfusion in the experiments with deflated lungs (Fig. 10).

IL-8 Concentrations in Pulmonary Edema Fluid

In the rabbits with 4 h of ischemia with lung deflation, the concentration of IL-8 did not increase significantly in the pulmonary edema fluid compared with the concentration in the plasma. The level of IL-8 was $0.69 \pm 0.46$ ng/ml in plasma during ischemia, $0.44 \pm 0.37$ ng/ml in plasma after reperfusion, and $0.78 \pm 0.48$ ng/ml in the pulmonary edema fluid. In these rabbits, the number of neutrophils in the edema fluid was $591 \pm 287/\mu l$, approximately fourfold less than in the circulating blood at the same time (Fig. 3).

Morphological Examination

Light-microscopic evaluation demonstrated extensive alveolar edema in the reperfused lungs that had been deflated for 4 h. In contrast, the reperfused lungs that had been inflated for 4 h showed no evidence of alveolar edema.

The ultrastructural examination of the 4-h deflated lungs followed by reperfusion for 10 min demonstrated both capillary endothelial and alveolar epithelial injury (Fig. 11, A and B). There was vacuolization and fragmentation of the capillary endothelium, and there was also blebbing, fragmentation, and vacuolization of the alveolar epithelium. Furthermore, there was sloughing and denuding of significant portions of the alveolar epithelium in the 4-h deflated lungs followed by 10 min of reperfusion. In contrast, the reperfused lungs from inflated lungs that had been ischemic for 4 h demonstrated no overt injury to the capillary endothelium or the alveolar epithelium. Alveolar epithelial type I cells were well preserved, and the alveolar epithelial type II cells had a completely normal appearance (Fig. 11, C and D).

DISCUSSION

In the present study, we developed an in vivo rabbit model and determined the effect of the ischemic period,
lungs inflation, the alveolar gas composition, temperature, and neutrophils on the development of severe reperfusion pulmonary edema. One advantage of this experimental preparation was that samples of alveolar edema fluid could be obtained from the distal air spaces of the ischemia-reperfused lungs. The analysis of alveolar edema fluid provides important information. For example, it is possible to differentiate increased permeability from hydrostatic edema (24). In this study, the edema fluid-to-plasma protein concentration was high (0.95), indicating that the mechanism of pulmonary edema was from an increase in permeability to protein.

Although the 2-h duration of ischemia induced some lung injury (Fig. 2), the injury was mild and not lethal. However, 4 h of ischemia resulted in severe lung injury with 100% mortality. Previously, a duration of ischemia for 2 h has been used in many in vivo experimental models (26, 32). However, severe lung injury with large quantities of alveolar edema fluid has not been reported in prior ischemia-reperfusion models. Therefore, we used the ischemic period of 4 h for the remainder of the experiments.

The most striking initial discovery was that static inflation of the lung with 100% oxygen almost completely prevented the lung injury. The decline in arterial oxygenation was prevented with inflation (Fig. 6), and extravascular lung water was nearly normal compared with the deflated lung (Fig. 4A); also, lung vascular permeability to protein was nearly normal in the inflated lung (Fig. 4B). Interestingly, the protective effect of inflation did not depend on the composition of the alveolar gas mixture since either air or 100% nitrogen also prevented the injury (Fig. 7).

There have been different reports on the effect of the gas concentration during ischemia on reperfusion lung injury. In one study, edema was greater in the lung inflated with 100% nitrogen than in the lung inflated with oxygen (38). Also, the levels of ATP in the lung were preserved in the lung inflated with oxygen or air compared with ATP levels in deflated lungs (1, 5, 8, 37). These reports suggested that a supply of oxygen might be needed to prevent ischemia-reperfusion lung injury. In contrast, in some studies, oxygen supply was found to worsen ischemia-reperfusion lung injury (2, 9, 11, 20). For example, ischemia-reperfusion lung injury was related to oxygen ventilation during ischemia, and the injury could be prevented by administration of superoxide dismutase or ventilation with 100% nitrogen (20). However, it is unlikely that oxygen stress derived from alveolar oxygen concentration influenced ischemia-reperfusion lung injury in the present rabbit study because there was no difference among lung injury indexes in the experiments with 100% oxygen, air, and 100% nitrogen. The lack of effect of alveolar oxygen concentration in this study is consistent with the results in the isolated rabbit (12, 15, 16) and rat (25) lung experiments.

Because the inflation of the ischemic lung prevented the reperfusion lung injury, we carried out the additional experiments to examine the potential protective effect of hypothermia. We hypothesized that the injury during warm ischemia with the lung deflated may have occurred in part because of active reorganization of the endothelial cytoskeleton, resulting in endothelial gaps that developed during deflation. If this were the case, then cooling of the lung would prevent active reorgani-
zation of the endothelial cytoskeleton. Therefore, the ischemic deflated lung was exposed to hypothermia for 4 h. Interestingly, the injury did not develop in the ischemic lungs that had been cooled throughout the 4 h of deflation. Thus hypothermia protected the ischemic lungs from the reperfusion injury that would have occurred in the presence of warm ischemia. The protective effect of hypothermia is consistent with other studies of lung injury (3, 4, 6, 14). Cytotoxicity for alveolar epithelial type II cells from adult rat lungs was decreased with storage at 5°C compared with 37°C (23). These data are consistent with our previous studies in which alveolar epithelial fluid transport was preserved after rewarming after severe hypothermia (29, 31). Thus the results indicate that either hypothermia or inflation protects against ischemia-reperfusion injury in this rabbit model.

What is the common protective mechanism? As described above, one hypothesis to explain the protective effect of hypothermia or lung inflation is that deflation of the lung in the presence of warm ischemia induces active reorganization of the endothelial cytoskeleton, resulting in a marked increase in the gaps between endothelial cell junctions. This hypothesis is supported by the finding that hypothermia or lung inflation with any gas prevents the injury; also, hypothermia would prevent the active reorganization of the endothelial cytoskeleton that occurs in the deflated, normothermic lung. This explanation is also supported by the studies in which deflation alone with persistent pulmonary perfusion for 4 h did not cause lung injury, perhaps because persistent perfusion prevented formation of endothelial gaps. This explanation is also consistent with the observation that the injury does not depend on neutrophils (see below). The mechanism for protection of the alveolar epithelium is less certain. Alveolar type I cells are very susceptible to injury under a variety of pathological conditions, but the mechanisms that mediate their injury have not been worked out well.

We determined whether neutrophils played a significant role in ischemia-reperfusion lung injury. There has been controversy regarding the role of neutrophils in reperfusion lung injury. Accumulation of white blood cells in ischemia-reperfused lungs provides circumstantial evidence for neutrophil-dependent reperfusion injury. Inhibition of neutrophil adhesion during reperfusion inhibited reperfusion injury in some experimental studies (18–21, 39). Similarly, an increase in IL-8 was found in bronchoalveolar lavage fluid in sheep (39), and treatment with anti-IL-8 monoclonal antibody inhibited the reperfusion injury in a 2-h model of reperfusion lung injury in rabbits (17, 32). In contrast, neutrophils were not necessary for induction of ischemia-reperfusion lung injury in another study (7). Also, depletion of neutrophils had no protective effect against early microvascular permeability in the in vivo rat lung (10, 22). In the rabbits with depletion of neutrophils in the present study, ischemia-reperfusion lung injury occurred when the ischemic lungs were deflated but did not occur when the ischemic lungs were inflated. These results indicate that neutrophils did not play a major role in ischemia-reperfusion lung injury in this study, perhaps because the predominant mechanism for edema formation resulted from structural alterations in the pulmonary circulation that occurred in the deflated lung over 4 h before reperfusion. On the other hand, it is possible that neutrophils might be activated after a period of reperfusion through the lung, resulting in subsequent amplification of the lung injury.

What are the potential implications of these experiments for the clinical problem of reperfusion pulmonary edema in the transplanted lung? Many transplant centers cool the donor human lungs. Our study supports hypothermia as a central method of protection of the donor lung because the ischemia-reperfusion lung injury did not occur in the normothermic, deflated ischemic lungs. Even if part of the isolated donor lungs is not well inflated, hypothermia should prevent the reperfusion injury. On the other hand, it may be important also to inflate the donor lung. If a portion of the lung is not adequately cooled, inflation of the lung should prevent lung injury. Therefore, ideally, inflation and cooling of the donor lung should maximally protect the lung before it is reimplanted and reperfused.

In summary, we have developed an in vivo rabbit model of severe reperfusion pulmonary edema for determining the mechanisms responsible for ischemia-reperfusion lung injury. Inflation of the ischemic lung with an airway pressure of 30 cmH₂O prevented the ischemia-reperfusion lung injury. Because inflation of the ischemic lungs with 100% oxygen, air, or 100% nitrogen prevented the reperfusion lung injury, protection of the ischemic lung depends on lung inflation, not on the alveolar gas composition. Hypothermia at 6–8°C abolished the reperfusion lung injury, even if the ischemic lungs were deflated. Although a contribution of neutrophils was not completely excluded in this study, neutrophils did not play a major role in the ischemia-reperfusion injury in this model. Because either hypothermia or lung inflation prevented the injury, we hypothesize that reorganization of the actin cytoskeleton in the endothelial cells occurred in the deflated ischemic lung, resulting in a marked increase in lung endothelial permeability with the onset of reperfusion. The alveolar epithelial injury is also prevented by inflation or by hypothermia, but the mechanism for this protection is uncertain. Because inflation with any gas composition protects the ischemic lung, the protective effect on the endothelium and the alveolar epithelium depends on some stretch or inflation of the lung. Further work may be needed to determine the molecular basis for this remarkable finding.

This study was supported in part by National Institutes of Health Grants HL-51854, HL-51856, HL-30542, and AI-29103 and by a grant for Collaborative Research from Kanazawa Medical University (C98–6).

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