Protective effect of lung inflation in reperfusion-induced lung microvascular injury

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Srivasan, Hari B., Stephen M. Vogel, Dharmapuri Vidyasagar, and Asrar B. Malik. Protective effect of lung inflation in reperfusion-induced lung microvascular injury. Am. J. Physiol. Heart Circ. Physiol. 278: H951–H957, 2000.—We used the isolated-perfused rat lung model to study the influence of pulmonary ventilation and surfactant instillation on the development of postreperfusion lung microvascular injury. We hypothesized that the state of lung inflation during ischemia contributes to the development of the injury during reperfusion. Pulmonary microvascular injury was assessed by continuously monitoring the wet lung weight and measuring the vessel wall 125I-labeled albumin (125I-albumin) permeability-surface area product (PS). Sprague-Dawley rats (n = 24) were divided into one control group and five experimental groups (n = 4 rats per group). Control lungs were continuously ventilated with 20% O₂ and perfused for 120 min. All lung preparations were ventilated with 20% O₂ before the ischemia period and during the reperfusion period. The various groups differed only in the ventilatory gas mixtures used during the flow cessation: group I, ventilated with 20% O₂; group II, ventilated with 100% N₂; group III, lungs remained collapsed and unventilated; group IV, same as group III but pretreated with surfactant (4 ml/kg) instilled into the airway; and group V, same as group III but saline (4 ml/kg) was instilled into the airway. Control lungs remained isogravimetric with baseline 125I-albumin PS value of 4.9 ± 0.3 × 10⁻³ ml·min⁻¹·g wet lung wt⁻¹. Lung wet weight in group III increased by 1.45 ± 0.35 g and albumin PS increased to 17.7 ± 2.3 × 10⁻³, indicating development of vascular injury during the reperfusion period. Lung wet weight and albumin PS did not increase in groups I and II, indicating that ventilation by either 20% O₂ or 100% N₂ prevented vascular injury. Pretreatment of collapsed lungs with surfactant before cessation of flow also prevented the vascular injury, whereas pretreatment with saline vehicle had no effect. These results indicate that the state of lung inflation during ischemia (irrespective of gas mixture used) and supplementation of surfactant prevent reperfusion-induced lung microvascular injury.

PULMONARY MICROVASCULAR INJURY is a characteristic feature of reperfusion of lungs after a period of ischemia (1); however, the mechanisms mediating reperfusion-induced lung microvascular injury remain unclear. Sequestration of polymorphonuclear leukocytes (PMN) subsequent to the expression of vascular endothelial adhesion molecules and the activation of PMN in the pulmonary vascular bed may contribute to the pathogenesis of injury (14). However, their presence may not be obligatory, because vascular injury occurred in lung preparations perfused with a cell-free buffer and subjected to unventilated ischemia followed by reperfusion and reinstatement of ventilation (3, 16, 19). It has also been suggested that humoral factors such as arachidonic acid metabolites, reactive oxygen species, nitric oxide metabolites, and cytokines, released by the lung and blood cells during ischemia and reperfusion, are involved in the mechanism of vascular endothelial injury; however, reactive oxygen species are generated primarily during ventilated ischemia in proportion to the oxygen concentration of the inspired gas (see Ref. 6). Because the role of these mediators remains uncertain, we evaluated, in the present study, the possibility that reperfusion-induced lung vascular injury could occur under experimental conditions that minimized the contribution of humoral factors and activated PMN. We addressed the hypothesis that the absence of ventilation during ischemia is an essential causative factor mediating the development of vascular injury during reperfusion.

METHODS

The experimental protocol was approved by the Animal Care Committee of University of Illinois at Chicago. Sprague-Dawley rats weighing between 300 and 350 g were anesthetized with 3% halothane in 20% O₂ (balance N₂) using a vaporizer, which delivered the gas into a bell jar for induction and then into a specially designed face mask (flow rate 2 l/min) for maintenance. The trachea was cannulated, and lungs were ventilated with a small animal ventilator at 60 cycles/min, 3 ml tidal volume, and 2 cmH₂O expiratory pressure with continued administration of the anesthetic gas mixture. A thoracotomy was performed, and heparin (1,000 U/ml) was injected into the right ventricle. The pulmonary artery was cannulated and perfused with modified Krebs-Henseleit solution (composition in mM: 118 NaCl, 4.7 KCl, 1.0 CaCl₂, 0.5 MgCl₂, 4.45 HEPES Na⁺, 5.55 HEPES, 11 glucose, and 0.025 EDTA) supplemented with 5 g/100 ml BSA (Sigma Chemical, St. Louis, MO) using a peristaltic pump (Gilson, Minipuls 2) at a rate of 0.03 ml·g body wt⁻¹·min⁻¹. A cannula was placed in the left atrium to drain the venous effluent. The length of the cannula was kept constant at 4 cm in all the

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ventilated with 20% O2 (balance N2) and suspended from a calibrated beam balance that continuously monitored the wet weight gain of lung. The initial wet weight of lung preparations was obtained for 15 min, and subsequently, the different experimental protocols were implemented.

Experimental protocols. The experiments lasted for 2 h, which included a 15-min period of equilibration followed by 75 min of ischemia and 30 min of reperfusion (Fig. 1). At the end of reperfusion, the vascular permeability to 125I-labeled albumin (125I-albumin) was measured. Six groups of lung preparations were studied, including a control group and five experimental groups. The control lung preparations were continuously ventilated with 20% O2 and perfused without interruption for 120 min (n = 4). The experimental groups were ventilated with 20% O2 (balance N2) before ischemia and during the reperfusion phase. These groups differed only on the basis of the ventilatory gas mixtures used during the phase of ischemia: group I, lungs ventilated with 20% O2 (n = 4); group II, lungs ventilated with 100% N2 (n = 4); group III, lungs remaining collapsed and ventilated (n = 4); group IV, lungs similar to group III but instilled with 4 ml/kg bovine surfactant containing 25 mg/ml of phospholipid (SURVANTA, Ross Abbott Laboratories, Columbus, OH) before ischemia (n = 4); and group V, lungs similar to group III but instilled with 4 ml/kg saline before ischemia (n = 4). Two additional lung preparations were treated identically to group III lung preparations, except that the postischemic rise in Ppa (PS) was not permitted to exceed 7.5 mmHg above the preischemia baseline by means of clamp-circuit limiting Ppa to a preset maximum level.

Measurement of vessel wall 125I-albumin permeability-surface area product. BSA was labeled with Na 125I (New England Nuclear, Boston, MA) using the standard chloramine T method (2). Free 125I-labeled Krebs solution was prepared by adding 80,000 counts/ml of 125I-albumin plus a small quantity of Evans blue dye (4 mg/g albumin) as a vascular marker to 50 ml of Krebs solution; any preparation exhibiting an obviously nonuniform distribution of dye was discarded. At the end of 30 min of reperfusion, 125I-albumin-labeled Krebs solution was perfused through the lungs for a period of 3 min followed by a washout period of 6 min with Krebs solution containing no label. The lungs were detached from the perfusion apparatus and cut into 12 samples. The samples were weighed and placed in separate containers. A 0.5-ml sample of the 125I-albumin-labeled Krebs solution was also placed in one of the containers. The number of counts due to labeled albumin in each of the containers was measured by a gamma counter (Minaxi Auto Gamma 5000 Series Gamma Counter; Packard Instrument, Downers Grove, IL). The 125I-albumin permeability-surface area product (PS) was then calculated according to the formula (10): A/(C * t), where A represents measured tissue counts per gram of lung tissue, t is the duration of exposure (in minutes), and C is the concentration of labeled albumin in the perfusing liquid (counts/ml).

Statistical analysis. All values are expressed as means ± SE. Statistical comparisons between group means were made by Student’s t-test. The significance level was set at P < 0.05.

RESULTS

Ventilation during ischemia prevents vascular injury after reperfusion. During a 120-min perfusion, control lung preparations did not gain weight but rather showed a slight weight loss, probably secondary to evaporative loss from the surface (Table 1). The 125I-albumin PS value calculated at the end of 120 min of perfusion was 4.9 ± 0.3 x 10⁻³ ml·min⁻¹·g⁻¹ (Table 1; control group). The lung preparations in groups I and II also did not develop edema, and the albumin PS values were similar to those of control lungs (Table 1). The ventilated lung preparations (group III) showed significant (P < 0.05) vascular injury as evidenced by

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**Table 1. Effects of ventilation and surfactant administration on reperfusion-induced injury of isolated rat lung preparations**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Wet Weight Gain, g</th>
<th>Albumin PS Product, ml·min⁻¹·g⁻¹·10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>-0.10 ± 0.07</td>
<td>4.91 ± 0.31</td>
</tr>
<tr>
<td>Experimental groups</td>
<td></td>
<td></td>
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<tr>
<td>Group I</td>
<td>-0.12 ± 0.04</td>
<td>5.39 ± 0.71</td>
</tr>
<tr>
<td>Group II</td>
<td>-0.20 ± 0.04</td>
<td>5.05 ± 0.39</td>
</tr>
<tr>
<td>Group III</td>
<td>+1.45 ± 0.35*</td>
<td>17.70 ± 2.31*</td>
</tr>
<tr>
<td>Group IV</td>
<td>-0.15 ± 0.12</td>
<td>5.68 ± 0.81</td>
</tr>
<tr>
<td>Group V</td>
<td>+4.00 ± 0.24*†</td>
<td>72.20 ± 4.40*†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Weight gain of lung preparations given as the change from baseline wet weight after 120 min of perfusion (control group) or after the reperfusion period (groups I–V; see Fig. 1). Negative values indicate decrease in wet weight. Permeability-surface area product (PS) of 125I-labeled albumin measured after 120 min of perfusion (control lung preparations) or after reperfusion period (groups I–V). Control group, no ischemia; group I, 20% O2 ventilation; group II, N2 ventilation; group III, no ventilation; group IV, no ventilation and surfactant treatment; group V, no ventilation and saline vehicle. * Statistical significance (P < 0.05) compared with control; † statistical significance between groups III and V.
marked increases in wet weight of the lung (1.45 ± 0.35 g) and \( ^{125}\text{I} \)-albumin PS (17.7 ± 2.3 × 10\(^{-2}\)) compared with controls (Table 1).

Figure 2, A and B, shows the typical changes in lung weight in an injured preparation (i.e., group III) and an uninjured preparation (group I), respectively. The results show a marked gain in lung weight during reperfusion and reventilation (Fig. 2A) compared with the lung ventilated with 20% O\(_2\) during the ischemic period (Fig. 2B). These results indicate that the lung vascular injury observed during the reperfusion period occurs only if the lung remained unventilated during the period of no flow.

Effects of intratracheal surfactant administration on reperfusion-induced lung vascular injury. Pretreatment with 4 ml/kg of surfactant before ischemia (group IV) prevented the reperfusion injury as evidenced by the lack of significant changes in the lung wet weight and albumin PS compared with control (Table 1). Instillation of 4 ml/kg of saline (group V) caused a significant (P < 0.05) increase in both lung wet weight (4.00 ± 0.24) and albumin PS (72.2 ± 4.4 × 10\(^{-2}\)) compared with group IV (Table 1).

Changes in P\(_{pa}\). The baseline P\(_{pa}\) at the end of the equilibration period (see Fig. 1) was 5–8 mmHg in all groups. In all experimental groups, during the 30-min reperfusion period, there was a marked but transient increase in P\(_{pa}\) that declined toward the normal baseline value. Figure 3 shows a typical recording for a group III lung preparation. For purposes of analyzing the data, we defined peak ΔP as the difference between the peak pressure attained on reperfusion and the baseline P\(_{pa}\), and we defined 30-min ΔP as the difference between the P\(_{pa}\) at the end of the 30-min reperfusion period and the baseline P\(_{pa}\) (see Fig. 3). Peak ΔP significantly increased (P < 0.05) over the values for the protected lungs (group I) only in lungs of groups III and V (Fig. 4), which also showed evidence of reperfusion injury (Table 1). There was no significant difference in the 30-min ΔP among any of the experimental groups (see Fig. 4). To determine whether peak ΔP was related to reperfusion-induced lung injury, we evaluated the correlations between the magnitude of peak ΔP and either lung wet weight gain or albumin PS values at the end of the reperfusion period. These correlations were absent for lungs from groups I, II, and IV in which there was also no evidence of lung injury (Fig. 5, A and B). In contrast, the peak ΔP and wet lung weight gain values (Fig. 5A; \( r = 0.78, P < 0.05 \)) and peak ΔP and albumin PS values (Fig. 5B; \( r = 0.61, P < 0.05 \)) were significantly correlated in groups III and V (Fig. 5, A and B).

In additional experiments (n = 2), lungs were treated identically to those in group III (i.e., no ventilation was allowed during the ischemic period) except that the peak ΔP, which normally averaged 20 mmHg over basal levels during the reperfusion period (Fig. 3), was kept to a maximum rise of 7.5 mmHg above baseline by

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**Fig. 2.** A: representative recording of increase in lung wet weight in an injured preparation (group III). During ischemia wet weight decreased by 0.6 g, and on reperfusion the value increased by 0.8 g relative to baseline weight (1.5 g), which was electronically nulled at time 0. B: representative recording of changes in lung wet weight in an uninjured preparation (i.e., group II lung ventilated with 100% N\(_2\) during no-flow period). During ischemia lung wet weight was decreased by 1.3 g. At end of 30 min of reperfusion, lung preparation showed no evidence of injury (i.e., lung wet weight was stable at −0.5 g below baseline value).
means of a pressure controller inserted in the perfusion circuit (see METHODS). This matched the typical increase in \( P_{pa} \) of the uninjured lungs. Lung weight gains of 1.1 and 1.5 g and PS of 40 and 50 \( \times 10^{-3} \) ml·min\(^{-1} \)·g\(^{-1} \) observed at the end of reperfusion were consistent with the values predicted from Fig. 5 for injured lungs experiencing the peak \( \Delta P \) of 7.5 mmHg.

**DISCUSSION**

The lung preparations in group II, which were in a state of anoxia (i.e., ventilated with 100% \( \text{N}_2 \)) during the cessation of perfusion, did not show evidence of vascular injury on reperfusion and ventilation with the oxygen in gas mixture. Reperfusion injury was evident only in lungs in groups III and V (both of which were unventilated) in agreement with other reports (8, 16). The results are consistent with the hypothesis that the state of lung inflation rather than anoxia followed by reintroduction of oxygen is a critical determinant of reperfusion-induced lung vascular injury.

Continuous recordings of \( P_{pa} \) showed a transient but marked rise immediately at the onset of reperfusion. The magnitude of the peak rise in \( P_{pa} \) in groups III and V lungs, which showed evidence of vascular injury, was significantly greater than that of \( P_{pa} \) values in other groups without vascular injury. Normoxic, perfused rabbit lungs exposed to similar high transmural pressures demonstrated injury of both capillary endothelium and alveolar epithelium (22). Vascular injury was evident even though the vessel wall was exposed to the high pressure for 1–5 min (12). The rapid onset of injury may be the result of stress failure because the capillaries were subjected to high wall stress during the episode of increased hydrostatic pressure (22). Fu et al. (7), using rabbit lungs exposed to high capillary transmural pressure, demonstrated that \( P_{pa} \) rise above a critical value (>32.5 cmH\(_2\)O) is required to produce capillary wall stress failure. However, the present results cannot be explained on this basis. In lung preparations showing evidence of reperfusion vascular injury (i.e., groups III and V), peak \( \Delta P \) during reperfusion was correlated with lung wet weight gain, consistent with greater liquid permeability in the injured lung preparations. In contrast, in lung preparations protected by either mechanical ventilation (groups I and II) or surfactant instillation (group IV) during the no-flow period, no such correlation was observed even though the rise in \( P_{pa} \) was similar (Fig. 5). Moreover, in

![Fig. 3. Representative recording of pulmonary artery pressure (\( P_{pa} \)) in an injured preparation (group III). From a baseline pressure of 9 mmHg, peak pressure rose to 24 mmHg (i.e., peak \( \Delta P = 15 \) mmHg) at onset of reperfusion, and then pressure gradually declined to 8 mmHg at end of 30 min of reperfusion (i.e., 30-min \( \Delta P = 1 \) mmHg). Peak \( \Delta P \), difference between peak pressure attained on reperfusion and baseline \( P_{pa} \); 30-min \( \Delta P \), difference between \( P_{pa} \) at end of reperfusion period and baseline \( P_{pa} \).](image-url)

![Fig. 4. Changes in \( P_{pa} \) after reperfusion in all experimental groups. All values are represented as means ± SE. Baseline \( P_{pa} \) (before ischemia) varied between 5 and 8 mmHg in all groups. Note peak \( \Delta P \) was augmented significantly in unprotected lung preparations (groups III and V). Group I, 20% \( \text{O}_2 \) ventilation; group II, \( \text{N}_2 \) ventilation; group III, no ventilation; group IV, no ventilation and surfactant treatment; and group V, no ventilation and saline vehicle. *Significance (\( P < 0.05 \)) from group I.](image-url)
an experiment in which peak $\Delta P$ during reperfusion was regulated to a rise of only 7.5 mmHg, the increases in lung wet weight and 125I-albumin PS persisted during the reperfusion period. Thus these findings indicate that the increase of in hydrostatic pressure occurring during the reperfusion period cannot explain the development of lung vascular injury.

We observed that reperfusion injury occurred only when the lungs were both ischemic and collapsed (as in group III). Lung inflation or instillation of surfactant (in groups I, II, and IV) prevented the injury. Two forces are influenced by the state of lung inflation (7, 15) and thus may be involved in the mechanism of vascular injury. Both forces act at the alveolar-capillary wall: 1) surface tension of the alveolar lining layer (15) and 2) alveolar distending pressure (7). Both of these factors are discussed below.

With respect to alveolar surface tension, this force primarily depends on the amount of surfactant lining the alveolar epithelium. It is known that alveolar distension stimulates surfactant release. In studies on freshly excised dog lungs, Faridy (5) showed that surface tension and lecithin content of bronchoalveolar fluid were increased when the lobe was inflated with air, and the increases were directly related to the inflating pressure, indicating that lung distension enhanced the release of surfactant. In excised rat lungs, Hilderbren et al. (9) showed that air inflation to total lung capacity is a major physiological stimulus to the release of lung surfactant into the alveolar space. In studying the effects of atelectasis on surfactant production in rabbit lungs, Levine and Johnson (13) showed a variable but significant loss of surface activity with atelectasis. Moreover, surfactant synthesis is also known to be impaired during ischemia. Veldhuizen et al. (21) showed that phosphatidylglycerol and surfactant-associated protein A levels were decreased after 12 h of lung graft storage and 6 h of reperfusion in dogs. In a study on rat lung graft transplantation after warm ischemia of 60–120 min, Erasmus et al. (4) showed that the concentration of phosphatidylincholine decreased in association with severity of lung injury. Klepetko et al. (11) documented a steady decrease in dipalmitylphosphatidylincholine concentrations of lungs stored at 4°C and showed that this correlated with ischemic damage to type II pneumocytes. Hence, it can be concluded that combination of atelectasis and ischemic damage of type II alveolar epithelial cells during the phase of ischemia can result in decreased surfactant content in alveoli, thereby leading to an increase in surface tension and alveolar collapse. Moreover, alveolar collapse itself can influence the integrity of pulmonary capillaries (15) because at high transmural pressures, the capillaries bulge into the alveolar space, and the alveolar lining layer acts to support the bulging capillary wall by compressing the endothelial and epithelial cell layers (22). Thus lung microvessel injury may, in part, be attributed to stress failure of pulmonary microvessels secondary to the loss of surfactant and the resultant alveolar collapse. Our finding that surfactant instillation prevented the reperfusion-induced lung vascular injury in the collapsed lung supports this mechanism of lung vascular injury. In this regard, a possible explanation for the higher labeled-albumin PS product in group V (saline instilled) lungs compared with unventilated lungs of group III (with no instillation) could be dilution or washout of surfactant by saline (17).

An elevated alveolar distending pressure during inflation of previously collapsed alveolar units can also lead to lung microvascular injury (7). This can be appreciated by consideration of the Young-Laplace equation, $P = 2T/R$, where $P$ represents the distending pressure needed to expand the alveoli, $T$, the alveolar surface tension, and $R$, the radius of curvature. The pressure required to inflate the alveoli is greatly increased if the surface tension rises. Hence, decreased surfactant production (due to both collapse of alveoli during ischemia and ischemic damage of type II alveo-
lar epithelial cells) will require a significantly greater distending pressure to expand alveoli during the reperfusion phase. If the alveolar distending pressure is directly transmitted to the alveolar wall and leads to increased longitudinal tension in the capillary (22), this could be a mechanism of vascular injury in the reperfused lung. Therefore, the combined effects of increased alveolar surface tension and increased distending pressure transmitted to open alveoli could explain our findings that only unventilated and ischemic lung preparations demonstrated microvascular injury on reperfusion.

There is a possibility that the increase of $^{125}$I-albumin PS observed in injured lung preparations may have occurred as a result of changes in the lung vascular surface area. Because our experiments were made at constant flow (see METHODS) and the perfusion pressure (except for a brief period immediately after reperfusion) remained near control levels during the 30-min reperfusion phase, it is unlikely that the vascular surface area was greatly increased by ischemia-reperfusion. Also, we observed a threefold increase in albumin PS (measured in the final 3 min of reperfusion period). Such a large increase in albumin PS cannot be accounted for by a vascular surface area shift. Finally, Fig. 5 shows the relationship between peak $\Delta P$ and albumin PS. The slope of the line for lungs developing edema was significantly greater than for the protected lungs. The markedly greater increase in albumin PS product for a given change in pressure in the lungs developing edema suggests increased vascular permeability in these lungs. Moreover, in the experiments in which the rise in $P_{pa}$ was limited to 7.5 mmHg, we still observed an increase in albumin PS after reperfusion. The albumin PS increased markedly in these lungs even though vascular surface area increase (due to vascular distension and recruitment) would be expected to be less than in the lungs that experienced a marked increase in reperfusion $P_{pa}$.

It is also unlikely that vascular volume expansion contributes significantly to the measured increases of wet weight in the injured lungs. Vascular volume in the rat lung is $\sim 0.1$ ml/g lung wet wt when determined at a steady-state perfusion pressure of 10 cmH$_2$O (Vogel and Malik, unpublished observations) and consequently could not account for weight gains of several grams observed in injured lungs. Thus a doubling of vascular volume would account for only $\sim 3\%$ of the observed lung wet weight gain. It is also unlikely that vascular distension during reperfusion explains the weight gain in injured lungs (groups III and V). The peak rise in pulmonary arterial pressure was transient, and therefore any resulting increase in lung wet weight attributable to distension of microvessels would be short lived.

The observed protective effect of surfactant could conceivably be a result of its potential anti-inflammatory effects (23), because several groups have demonstrated that there is a population of resident neutrophils even in the buffer-perfused lung preparation (18). However, the anti-inflammatory effect of surfactant required several hours to develop (20) and consequently is not expected to be marked in the time frame of our experiments. Moreover, if inflammation is important in the development of vascular injury in this model, then we would have expected to see signs of barrier breakdown (lung wet weight gain and an increased albumin PS product) in group II lung preparations (ventilated with 100% N$_2$). Because this was not the case, the present results suggest that the reperfusion injury cannot be explained on the basis of a resident population of neutrophils.

In conclusion, the present findings suggest that alveolar collapse during the period of lung ischemia is a key determinant of reperfusion-induced lung vascular injury. The rise in pulmonary vascular hydrostatic pressure and increased vascular surface area cannot account for the marked increases in the vessel wall albumin PS product observed during reperfusion. The study raises the possibility that cellular and humoral factors may not be dominant, although they may contribute to the mechanism of the response. Alveolar collapse secondary to the loss of surfactant during ischemia may be an important factor in the mechanism of reperfusion injury, because ventilation of the lungs during ischemia or instillation of surfactant prevented the response. Mechanical factors resulting from the loss of alveolar tethering forces during alveolar collapse may injure the pulmonary capillaries on reperfusion.

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