Effect of ischemia and reperfusion without airway occlusion on vascular barrier function in the in vivo mouse lung

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Dodd-o, Jeffrey M., Maria L. Hristopoulos, Nauder Faraday, and David B. Pearse. Effect of ischemia and reperfusion without airway occlusion on vascular barrier function in the in vivo mouse lung. J Appl Physiol 95: 1971–1978, 2003.—Ischemia-reperfusion (I/R) lung injury causes increased vascular permeability and edema. We developed an in vivo murine model of I/R allowing measurement of pulmonary vascular barrier function without airway occlusion. The left pulmonary artery (PA) was occluded with an exteriorized, slipknotted suture in anesthetized C57BL/6J mice. The effect of ischemic time was determined by subjecting mice to 5, 10, or 30 min of left lung ischemia followed by 150 min of reperfusion. The effect of reperfusion time was determined by subjecting mice to 30 min of left lung ischemia followed by 30 or 150 min of reperfusion. Changes in pulmonary vascular barrier function were measured with the Evans blue dye (EBD) technique, dual-isotope radiolabeled albumin (RA), bronchoalveolar lavage (BAL) protein concentration, and wet weight-to-dry weight ratio (WW/DW). Increasing left lung ischemia with constant reperfusion time or increasing left lung reperfusion time after constant ischemic time resulted in significant increases in left lung EBD content at all times compared with both right lung values and sham surgery mice. The effects of left lung ischemia on lung EBD were corroborated by RA but the effects of increasing reperfusion time differed, suggesting binding of EBD to lung tissue. An increase in WW/DW was only detected after 30 min of reperfusion, suggesting edema clearance. BAL protein concentrations were unaffected. We conclude that short periods of I/R, without airway occlusion, increase pulmonary vascular permeability in the in vivo mouse, providing a useful model to study molecular mechanisms of I/R lung injury.

pulmonary edema; vascular permeability; Evans blue dye; radiolabeled albumin; pulmonary artery; bronchial artery

ISCHEMIA-REPERFUSION (I/R) lung injury is a well-known clinical phenomenon complicating lung transplantation (15), pulmonary artery (PA) thromboendarterectomy (17), thrombolytic therapy for acute pulmonary thromboembolism (35), and cardiopulmonary bypass surgery (29). I/R injury is characterized by increased pulmonary vascular permeability, edema, and resistance to blood flow (23). Large-animal preparations have been used to examine in vivo I/R lung injury through the use of unilateral PA occlusion, cardiopulmonary bypass, or lung transplantation (23). The advantages of unilateral PA occlusion preparations include easier surgical methods with less manipulation of the lungs and the lack of any confounding effects of anticoagulation or extracorporeal perfusion (23). However, large-animal models are limited by the paucity of species-specific molecular reagents, the exorbitant expense of experiments, and the necessity for long ischemic times (12–48 h) (5, 16, 36) unless the airway is simultaneously occluded (10) or the bronchial circulation is separately interrupted (13, 20).

Our laboratory has been interested in the mechanisms behind the increased endothelial permeability resulting from ischemic (4, 22) and I/R (9, 24) lung injury. Moreover, our laboratory (22, 26) and others (30, 32) have demonstrated that ventilatory lung motion and oxygenation during ischemia have profound effects on the changes in endothelial barrier function. To further explore the mechanisms behind these observations, we sought to develop a model of I/R lung injury in the in vivo mouse that would allow measurement of transvascular protein clearance as an index of pulmonary vascular permeability without simultaneous occlusion of the airway and the confounding effects of lung atelectasis or alveolar hypoxia. I/R lung injury has been reported in anesthetized mechanically ventilated mice (19) in which ischemia was produced by clamping the entire left hilum, resulting in airway occlusion, cessation of ventilation, and atelectasis, factors that greatly exacerbate I/R lung injury (32).

Interestingly, mice lack a bronchial circulation below the level of the trachea (18). On the basis of the known protective effects of the bronchial circulation on I/R injury (25), we hypothesized that the mouse pulmonary vasculature would be sensitive to brief periods of ischemia and reperfusion despite continued ventilation and oxygenation of the lung during ischemia.

METHODS

Preparation

The protocols in this study were approved by The Johns Hopkins Institutions Animal Care and Use Committee. Female C57BL/6J (Jackson Laboratories, Bar Harbor, ME)
mice weighing 20–22 g were anesthetized (2 mg/ml etomidate, 0.3 ml ip), intubated (20-gauge Angiocath), and mechanically ventilated (inspired O2 fraction 1.0, tidal volume 0.25 ml, respiratory rate 120 breaths/min, no positive end-expiratory pressure) with a Harvard mouse ventilator (model 687, Holliston, MA). The left neck was cleaned (70% alcohol) and shaved, and an incision was made to expose the left internal jugular vein for injection of tracers. The left thorax was cleaned (70% alcohol) and shaved, and a left thoracotomy was performed at the third intercostal space. The apex of the left lung was retracted caudally, exposing the left main PA was performed at the third intercostal space. The PA and bronchus were separated by blunt dissection. The ability to reinflate atelectatic portions of the left lung was visually confirmed adequate occlusion of the left PA. An 8-0 Prolene suture was placed around the PA, and the PA was occluded at that moment with a slipknot. One end of the 8-0 Prolene suture was exteriorized through a hole in the left thorax made with a 30-gauge needle for later release of the PA to allow reperfusion.

In all animals, a 5-0 silk suture was utilized to close the thoracotomy in three layers (a single ligature around the third and fourth ribs, a continuous suture closing the muscle layer, and a continuous suture closing the skin). Just before closure of the ribs, the expiratory limb of the ventilatory circuit was transiently occluded until complete inflation of the left lung was visually verified. On completion of the three-layer closure, the lungs were hyperinflated for two breaths while the animal was extubated (~10–15 min after initiation of anesthesia). The total duration of mechanical ventilation was ~30 min with extubation 10–15 min after left PA occlusion. Left lung reperfusion was performed after chest closure but while the animal was on mechanical ventilation for ischemic times <10 min, whereas left lung reperfusion occurred after chest closure and extubation for ischemic times >15 min.

Assessment of Barrier Function

Extravascular albumin extravasation with EBD. In 45 mice, pulmonary vascular barrier function was determined by the Evans blue dye (EBD) technique (21). Before the closure of the above-described left thoracotomy but after occlusion of the left PA, 30 mg/kg EBD (Sigma Chemical, St. Louis, MO) dissolved in 250 μl normal saline solution was injected into the left internal jugular vein. A 5-0 silk continuous suture was used to close the left neck skin incision. The left lung was again visualized, and its continued white appearance confirmed adequate occlusion of the left PA. The left thoracotomy was then closed, and the animals were extubated as described above. After the desired reperfusion period, the mice were anesthetized with etomidate and intubated, and mechanical ventilation was resumed. A midline sternotomy was performed, and the lungs were hyperinflated for two breaths to remove any atelectasis. The right atrium was opened and the beating right ventricle was slowly injected with 6–10 ml normal saline solution to remove extravascular EBD. Death occurred by exsanguination during this procedure.

The heart and lungs were removed en bloc, and the right and left lungs were excised and separately weighed. The lungs were homogenized in formamide (1 ml formamide/100 mg lung, Sigma Chemical) and incubated for 24 h at 37°C. In some experiments, the left upper and left lower lobes were separated and processed separately to determine whether the surgical manipulation of the left upper lobe during placement of the PA ligature resulted in nonspecific injury. The samples were centrifuged (5,000 rpm × 30 min), and 200-μl aliquots of supernatant were placed in 96-well plates. Optical density was measured by using a microplate reader (Organon Teknika, Durham, NC). We confirmed that the relationship of EBD concentration and optical density at 620 nm was linear (R = 0.999) through the range of absorbance values encompassed by our lung samples by constructing a seven-point standard curve as previously described (8).

To determine whether unbound EBD was present in the mice after injection, three additional mice were injected with EBD as described above, and blood was removed by cardiac puncture 15 min later. The plasma was immediately separated by centrifugation, and a 200-μl plasma sample was further centrifuged (30 min at 13,000 rpm) in a Microcon centrifugal filter (Millipore, Bedford, MA), which excluded proteins of >10,000 molecular weight. The optical density at 620 nm of plasma and filtrate was determined. The plasma concentration of EBD 15 min after injection of 30 mg/kg in control mice (n = 3) was 0.964 ± 0.136 mg/ml. Of this, 98.9 ± 0.1% was protein bound.

Extravascular albumin extravasation with radiolabeled albumin. In a separate group of 20 mice, pulmonary vascular extravascular albumin extravasation was assessed by a dual-isotope technique using separate injections of radiiodine-labeled human serum albumin (RA) [125I-labeled albumin (125I-alb) and 131I-labeled albumin (131I-alb); Iso-Tex Diagnostics, Friendswood, TX). Both radiolabels were shown to have <1% unbound radiiodine by trichloroacetic acid precipitation. Approximately 2 μCi of each isotope were injected intravenously in 200 μl of 0.9% NaCl as described above for EBD. The 125I-alb injection was performed at the same time in the protocol as described above for EBD. The 131I-alb was injected 5 min before the end of the experiment to mark the intravascular space. A blood sample was obtained by cardiac puncture before the mice were killed by exsanguination. The lungs and kidneys were harvested and cleared of surface blood with a brief saline rinse. Excess surface fluid was removed by blotting dry with gauge. The right lung, left lower lobe, and kidneys were separated and processed separately to determine whether unbound EBD was present in the lungs after injection of the RA.

Blood samples were centrifuged (5,000 rpm × 10 min), and 200-μl aliquots of supernatant were placed in 96-well plates. Optical density was measured by using a microplate reader (Organon Teknika, Durham, NC). We confirmed that the relationship of albumin concentration and absorbance values at 660 nm was linear (R = 0.999) through the range of absorbance values encompassed by our blood samples (8).

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<th>Isotope</th>
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where 125I is the wet weight (WW) of the lung sample in grams, B is the blood sample weight in grams, and 125I-alb and 131I-alb are the lung (L) and blood (B) counts per minute of each designated RA. 125IWW is the wet weight (WW) of the lung sample in grams, and WWB is the blood sample weight in grams. The activity of 125I-alb in the blood was included in the denominator to adjust for differing amounts of injected 125I-alb between mice.

Lung water. After radioactivity was quantified, the lungs were dried at 58°C for 72 h and weighed to determine lung dry weight (DW) and allow calculation of WW-to-DW ratio (WW/DW).
Epithelial permeability. In a separate group of 16 mice, a lethal dose of anesthetic was injected after the desired reperfusion period, and the anterior thorax was removed to expose the heart and lungs. The right hilum and left upper lobe were occluded with smooth hemostats, and the trachea was cannulated with an 18-gauge Angiocath. Air (100 μl) was injected through the 18-gauge angiocath and inflation of only the left lower lobe confirmed exclusion of other lung fields from the subsequent lavage. The left lower lobe was lavaged with 130 μl of HBSS (without Ca²⁺, Mg²⁺, or phenol red), and the returned fluid (usually 50–100 μl) was collected. The right lung was unclamped, and the left hilum was occluded. Injection of 200 μl of air through the tracheal catheter verified left lung occlusion and the right lung was lavaged with 350 μl of HBSS (no Ca²⁺, no Mg²⁺, no phenol red), and the returned fluid (usually 150–250 μl) was collected. Bronchoalveolar lavage (BAL) protein concentration was determined in duplicate by the method of Bradford (7).

Protocol

Six groups of mice were studied with EBD to distinguish the effects of ischemic and reperfusion time on I/R injury. To determine the effect of ischemic time on transvascular protein clearance, mice were subjected to 5 (n = 7), 10 (n = 8), or 30 (n = 6) min of left lung ischemia followed by 150 min of reperfusion. The effect of reperfusion time was studied in additional groups of mice exposed to 30 min of left lung ischemia followed by either 30 (n = 6) or 150 (n = 7) min of reperfusion. The effect of surgery was determined in sham-operated controls (Sham). In Sham lungs (n = 8), the left PA was isolated, and the left PA was occluded for ~10 s while EBD was injected. The thoracotomy was closed, and the mice were allowed to recover. The lungs were removed for analysis after 150 min of reperfusion. In EBD I/R mice the entire left lungs were processed for determination of EBD content, whereas in Sham mice, the left upper and lower lobes were processed separately to determine whether physical contact with the left upper lobe during surgery caused a nonspecific injury. To allow the appropriate comparison with the EBD I/R lungs, the weighted average of the left upper and lower lobes EBD absorbance was used for the Sham group in the statistical analysis.

To confirm the EBD results, additional studies using the RA technique for assessing vascular protein extravasation were performed in mice subjected to either 5 (n = 5) or 30 (n = 6) min of left lung ischemia followed by 150 min of reperfusion. An additional group of mice were studied after 30 min of ischemia and 30 min or reperfusion (n = 4). These groups were compared with sham surgery mice (n = 5). In all of the RA groups, the right lungs and left lower lobes and the kidneys were processed separately to assess changes in pulmonary vascular permeability and nonspecific effects of the surgery, respectively. After measurement of radioactivity, WW/DW was successfully determined in a subset of these lungs.

To evaluate changes in epithelial permeability, lung lavage was performed in separate groups of Sham lungs (n = 5) and lungs exposed to 30 min of ischemia and 150 min of reperfusion (n = 11).

Statistics

The lung data were analyzed with a two-factor (group, lung), split-plot ANOVA (33). All other measurements were analyzed by randomized one-factor ANOVA. The difference in EBD content between the Sham right lung and left lower lobe was analyzed by Student’s paired t-test. The groups of lungs subjected to varying periods of ischemia, varying periods of reperfusion, and sham surgery were analyzed separately with the exception that the Sham lungs were included in each of these ANOVAs because this group underwent brief PA occlusion and 150 min of reperfusion. When a significant ANOVA interaction variance ratio indicated that the change in EBD content as a function of ischemic or reperfusion time was different between experimental groups, the least significant difference was calculated to allow comparison of individual means between groups. When a significant column effect (right vs. left lung) occurred, Student’s paired t-tests were performed to compare corresponding right and left lungs. The relationship between ischemic time and left lung EBD content was determined by least squares linear regression. Values presented in the text are means ± SE. Differences were considered significant when P ≤ 0.05.

RESULTS

Protein Permeability Indexes: Effect of Ischemic Time

Figure 1 shows the effect of increasing left lung ischemia on EBD content measured after a fixed reperfusion period. The ANOVA interaction term demonstrated that I/R increased left lung EBD content compared with ipsilateral Sham lungs with the exception of the group subjected to 5 min of ischemia and 150 min reperfusion. The left lung EBD contents differed between all three I/R groups despite the identical exposure time of the pulmonary vasculature to EBD in these and Sham lungs. For example, left lung EBD content increased from 0.276 ± 0.046 absorption units (AU) in Sham lungs to 0.681 ± 0.084 and 1.13 ± 0.18 AU after 10 and 30 min of ischemia (150 min reperfu-

![Fig. 1. Evans blue dye content in lungs from mice undergoing sham surgery with brief left pulmonary artery occlusion (Sham; n = 6) and ischemia-reperfusion (I/R) mice subjected to 5 (I/R 5-150; n = 6), 10 (I/R 10-150; n = 8), or 30 (I/R 30-150; n = 6) min of left lung ischemia followed by 150 min of reperfusion. Inset: relationship between left lung Evans blue dye content and ischemic time. The contribution of left lower lobe Evans blue dye content is shown for the Sham group. Values are means ± SE. *P < 0.05 vs. all ipsilateral I/R lungs (excludes Sham) by ANOVA interaction. †P < 0.05 vs. ipsilateral 5 min ischemic group by ANOVA interaction. §P < 0.05 vs. ipsilateral Sham lung by ANOVA interaction. ¶P < 0.05 vs. corresponding contralateral lung by ANOVA column (right and left lung) effect.](http://jap.physiology.org/doi/10.1152/jappl.00233.2003)
The ANOVA also indicated that left lung EBD content differed from right lung EBD content in all groups, including the Sham lungs, when comparing the EBD content of the entire left lung. The increasing EBD content in the Sham group appeared to be explained entirely by the inclusion of the surgically manipulated left upper lobe because there was no difference between the Sham right lung EBD content and the separately analyzed left lower lobe (0.199 ± 0.06 vs. 0.199 ± 0.04 AU, respectively). The right lung EBD content differed by ANOVA interaction in that 30 min of ischemia with 150 min of reperfusion increased right lung EBD content compared with ipsilateral values in Sham and 5-min I/R groups. Ischemic time and left lung EBD content after 150 min of reperfusion appeared to be linearly related with a regression coefficient of 0.98 (Fig. 1, inset).

The effects of increasing left lung ischemic time on pulmonary vascular barrier function were corroborated by the RA experiments. As shown in Fig. 2, the left lung extravascular albumin index was increased after 30 min of ischemia and 150 min of reperfusion compared with both the 5-min ischemia/150-min reperfusion and Sham group left lungs and the contralateral right lung. The 5-min ischemic left lung had a significantly increased albumin escape index compared with its contralateral right lung, whereas there was no difference between Sham lungs.

**Protein Permeability Indexes: Effect of Reperfusion Time**

Figure 3 shows the effect of increasing left lung reperfusion duration on EBD content measured after a fixed ischemic period. The ANOVA interaction term demonstrated that I/R increased left lung EBD content compared with ipsilateral Sham lungs and that left lung EBD content also differed between both I/R groups. The ANOVA also determined that within each group, left lung EBD content differed from right lung EBD content. The right lung EBD content was not different between the three groups.

The effect of varying reperfusion time was different in the RA experiments. As shown in Fig. 4, the left lungs of both I/R groups had an increased extravascular albumin index compared with the ipsilateral Sham lung and their contralateral right lungs, but, unlike the results with EBD, they did not differ from each other. The renal extravascular albumin index was not different between any of the four experimental groups, averaging 0.08 ± 0.01 (P > 0.05; data not shown).

![Fig. 2. Extravascular albumin index in lungs from Sham (n = 5), I/R 5-150 (n = 5), and I/R 30-150 (n = 6) mice. Values are means ± SE. *P < 0.05 vs all ipsilateral I/R lungs (excludes Sham) by ANOVA interaction. **P < 0.05 vs. ipsilateral Sham lung by ANOVA interaction. + P < 0.05 vs. corresponding contralateral lung by ANOVA column effect.](image)

![Fig. 3. Evans blue dye content in lungs from Sham mice (n = 6), I/R mice subjected to 30 min of left lung ischemia followed by 30 min of reperfusion (I/R 30-30; n = 6), and I/R 30-150 mice (n = 7). Values are means ± SE. *P < 0.05 vs. I/R 30-30 group by ANOVA interaction. **P < 0.05 vs. ipsilateral Sham lung by ANOVA interaction. + P < 0.05 vs. corresponding contralateral lung by ANOVA column effect.](image)

![Fig. 4. Extravascular albumin index in lungs from Sham (n = 5), I/R 30-30 (n = 4), and I/R 30-150 (n = 6) mice. Values are means ± SE. *P < 0.05 vs. ipsilateral Sham lung by ANOVA interaction. **P < 0.05 vs. corresponding contralateral lung by ANOVA column effect.](image)
**BAL Protein and WW/DW**

In contrast to the changes in endothelial barrier function, 30 min of ischemia and 150 min of reperfusion had no effect on BAL protein concentration compared with Sham values (Fig. 5). WW/DW did not differ between the Sham and I/R lungs by ANOVA interaction. Rather, a significant column effect was found, indicating a difference between left and right lungs that was present only in lungs subjected to 30 min of ischemia and 30 min of reperfusion (Fig. 6).

**DISCUSSION**

We have developed an in vivo mouse model of I/R lung injury in which the effect of ischemia and reperfusion on pulmonary vascular barrier function can be measured without the confounding effects of prolonged mechanical ventilation, continuous anesthesia, anticoagulation, extracorporeal perfusion, atelectasis, or hypoxemia. This preparation has the further advantages of a contralateral control lung and the ability to utilize the molecular reagents and genetic manipulations available for murine experiments.

We utilized lung EBD content to allow comparisons of lung protein clearance, a process that depends in part on pulmonary vascular protein permeability. EBD remains tightly bound to albumin and is a nonradioactive but easily measured index of albumin movement across the pulmonary endothelium (21). Moreover, it does not independently alter pulmonary vascular reactivity or barrier function (8, 21). Many investigators have shown that changes in lung EBD content tightly correlate with changes in lung radiolabeled protein (8, 34), indicating that EBD content can be used to compare the extravascular clearance of vascular protein between experimental groups. Dallal and Chang (8) also showed, however, that EBD can apparently dissociate from albumin and bind to lung tissue proteins, thus causing an overestimation of the absolute permeability-surface area product of albumin. Because the effect of tissue injury on this phenomenon has not been directly studied, we compared the EBD results with measurements of RA extravasation in separate experimental groups.

As shown in Fig. 1, interruption of PA blood flow to the left lung for either 10 or 30 min followed by 150 min of reperfusion resulted in a significant dose-response increase in left lung EBD content compared with both ipsilateral sham and contralateral lungs. These data suggest that PA ischemic periods as short as 10 min followed by 150 min of reperfusion in the intact mouse lung resulted in increased protein permeability as measured by EBD extravasation. Decreasing the ischemic time to 5 min was still associated with an increase in the reperfused lung EBD content compared with the right lung. However, this group did not differ from the Sham lungs, which also demonstrated increased left lung EBD. Because the Sham left lower lobe EBD content was identical to the right lung value (Fig. 1), the increase in the EBD content of the whole Sham left lung appeared to be due to the inclusion of the left upper lobe, which had to be manipulated during placement of the left PA tie.

The I/R protocol also affected EBD content of the nonischemic (right) lung. A significant increase in right lung EBD content was detected in mice subjected to 30 min of left lung ischemia and 150 min of reperfusion compared with right lung EBD content in mice subjected to Sham surgery and 5 min of left lung ischemia (Fig. 1). Because we injected EBD at the beginning of left lung ischemia, the right lungs were subjected to the entire cardiac output while the left lungs were ischemic. Furthermore, because the reperfusion period was kept constant while left lung ischemic time was changed, increasing left lung ischemic time also augmented the total duration of right lung EBD exposure. Thus the changes in right lung EBD content could have been caused by enhanced protein convection and diffusion resulting from the increased right lung perfusion.
pressure and EBD exposure as left lung ischemic time was increased. Alternatively, contralateral lung injury has been found in other in vivo models of unilateral I/R lung injury (6, 20). The mechanism of this contralateral injury remains poorly understood, although it appears to occur from a circulating mediator that arrives via the pulmonary circulation inasmuch as an intact bronchial circulation is not required (20).

To confirm the EBD results and specifically evaluate the effect of 5 min of PA ischemia, we repeated the protocol (excluding the 10-min ischemia group) and assessed protein permeability by using a double-indicator RA method to measure extravascular albumin accumulation as an index of vascular protein permeability. We calculated extravascular albumin accumulation as described by Kaner et al. (14), but in addition normalized the result to the blood content of the radiolabel to account for any differences in the amount of injected radiolabel between animals. We also limited the measurement to the left lower lobe to avoid the nonspecific surgical injury discovered in the EBD experiments. As shown in Fig. 2, the RA method confirmed the presence of I/R-dependent increase in protein permeability in the reperfused left lung that was detectable after just 5 min of left PA occlusion followed by 150 min of reperfusion. Unlike the EBD results, the right lungs in the RA experiments were not different from each other. This suggests that the increase in EBD content in the right lung from the 30-min ischemia/150-min reperfusion group in the first series of experiments was independent of albumin extravasation (8, 34) and exacerbated by either the duration of right lung exposure to EBD or duration of right lung exposure to the entire cardiac output. The mechanism of the EBD tissue uptake appears to have resulted from an interaction of albumin-bound EBD with lung tissue proteins rather than the presence of free EBD in the plasma because we found that 99% of the plasma EBD was protein bound. Whether this involves transfer of EBD from albumin to lung tissue EBD-binding sites or an EBD-induced enhancement of albumin binding to specific endothelial surface proteins, such as gp60 (31), remains to be determined.

When ischemic time was held constant and reperfusion time varied, we found that lung EBD content increased as a function of reperfusion duration (Fig. 3). This suggests that either 1) the change in endothelial barrier function persisted between 30 and 150 min of reperfusion (allowing a continuous accumulation of extravascular EBD-bound albumin), 2) an additional separate albumin leak occurred at the later time point, or 3) free EBD accumulated in the tissue (as suggested by the right lung results in Fig. 1). To assess this, we determined the effect of varying reperfusion time on our RA-derived index of extravascular albumin accumulation (Fig. 4). With this method, there was no difference in the reperfusion injury between the 30-min ischemia/30-min reperfusion and 30-min ischemia/150-min reperfusion groups. This result suggests that an increase in extravascular accumulation occurred within the first 30 min of reperfusion and then resolved. This result is consistent with previous work in an intact, anesthetized rat lung preparation where a biphasic reperfusion injury was found after 90 min of unilateral lung ischemia (10, 11). In these studies, endothelial barrier function decreased at 30 min of reperfusion secondary to products from activated macrophages but then recovered before a second neutrophil-mediated decrease in barrier function occurred at 4 h.

The results in Fig. 4 also imply that the difference in left lung EBD content between the I/R groups shown in Fig. 3 was again due to an accumulation of unbound EBD to lung tissue proteins. This phenomenon was not just a result of EBD exposure time, because there was no difference in EBD content between the right lungs of these two I/R groups despite the 120-min difference in total EBD exposure. Thus it appears that the reperfusion injury contributed to this process perhaps because of enhanced transfer of EBD from albumin to lung tissue proteins altered by the reperfusion injury. These results suggest that caution must be used when using EBD to assess protein permeability in injured tissues because exposure time, vascular pressure, and tissue injury may enhance EBD tissue binding independent of albumin extravasation. On the basis of these considerations, tissue EBD content can still be used as an index of reperfusion injury in the left lung of our preparation as long as the lungs being compared have the same total exposure to EBD during the experiment or the injury is expressed as a left-to-right lung EBD ratio. Additionally, the results in Fig. 1 suggest that excluding the left upper lobe from the analysis further improves the correlation between the EBD and RA methods in quantifying reperfusion injury in this model. Even under these circumstances, however, it is possible that part of any injury-induced increase in tissue EBD is unrelated to albumin extravasation but still dependent on tissue injury.

Not surprisingly, the injury resulting from brief periods of PA ischemia without airway occlusion was relatively mild and limited to the vascular endothelium. Thus alveolar epithelial protein permeability, assessed by BAL fluid protein concentration, was not altered after 30 min of ischemia and 150 min of reperfusion (Fig. 5). The WW/DW was also a less sensitive indicator of lung injury, likely for several reasons. First, there was a trend toward an increased WW/DW in the left lower lobe from the Sham group, suggesting that the left-sided thoracotomy may have caused hydrostatic edema potentially obscuring the edema formation from I/R. Second, the I/R injury may have preferentially affected the pathways determining protein permeability as our laboratory previously reported in ischemic lung injury (26). Finally, there may have been significant edema clearance during the reperfusion phase after resolution of the increased vascular permeability, tending to return lung water back to baseline values (27). In support of this possibility, the group with the shortest reperfusion time (30-min ischemia/30-min reperfusion group) was the only group demonstrating an increase in left lung WW/DW.

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To our knowledge, our data are the first to demonstrate a decrease in pulmonary vascular barrier function during reperfusion after only 5 min of ischemia in an in vivo preparation of I/R without airway occlusion. One possible explanation for this marked sensitivity to an interruption of pulmonary blood flow may be the absence of a bronchial circulation below the trachea in mice (18). The maintenance of normal bronchial arterial flow markedly attenuates the increased pulmonary vascular permeability that results from ischemia and reperfusion of isolated sheep lungs (25). This may explain the increased sensitivity of isolated lungs to I/R injury compared with intact-animal models in which bronchial blood flow is not interrupted (23).

Although lung ischemia without reperfusion is capable of increasing pulmonary vascular permeability (4), several previous studies suggest that it is unlikely that the short ischemic times examined in the present study altered endothelial permeability independently of reperfusion (4, 24, 26, 28). The adverse effect of ischemia on endothelial permeability during reperfusion began between 10 s and 5 min after the onset of ischemia. The rapid onset of this effect was not consistent with substrate depletion or changes in lung energy state (12), suggesting that the sudden cessation of vascular distension (3, 32) or shear forces (12) may have been the triggering factor. For example, studies in isolated rat and mouse lungs perfused with specific fluorophores found that ischemia caused immediate endothelial depolarization (2), activation of an endothelial NADPH oxidase (1), and generation of reactive oxygen species (1, 2). Although pulmonary vascular permeability was not measured in these studies (2), we recently found that inhibition of NADPH oxidase completely prevented the increased protein and water permeability caused by I/R in an isolated sheep lung model (9), supporting the proposed link between NADPH oxidase and lung I/R injury.

In summary, we have developed an in vivo mouse model of I/R lung injury in which increased pulmonary endothelial barrier dysfunction can be detected after just 5 min of left lung ischemia followed by 150 min of reperfusion. Epithelial barrier function was not altered, suggesting a specific effect of I/R on endothelial junctions. Moreover, pulmonary artery ischemia was generated without the confounding effects of airway occlusion and atelectasis, allowing further investigation of the effects of ventilatory lung motion and alveolar gas content. We speculate that the mouse lung may be particularly sensitive to I/R injury because of the lack of a bronchial circulation below the carina. This preparation will greatly enhance the study of molecular mechanisms of I/R endothelial barrier dysfunction in the lung.

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DISCLOSURES

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REFERENCES


