Effects of pulmonary ischemia on lung morphology

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PULMONARY ISCHEMIA RESULTING FROM CHRONIC PULMONARY EMBOLISM LEADS TO PROLIFERATION OF THE SYSTEMIC CIRCULATION WITHIN AND SURROUNDING THE LUNG. We previously developed a mouse model of lung angiogenesis and quantified new systemic perfusion of the lung following left pulmonary artery ligation (LPAL). Although we found no subarcinal bronchial vasculature in the mouse, rapid neovascularization and systemic perfusion of the lung from adjacent intercostal arteries were measured by 5 days after LPAL (10). However, it is not clear how well alveolar tissue is sustained during the time of complete pulmonary ischemia. Since the lung is ventilated, it is unlike other organs in that pulmonary ischemia is not accompanied by tissue hypoxia. Yet, the lack of endothelial shear stress and nutrient flow to alveolar epithelium, as well as the trapping of inflammatory cells, might all contribute directly or indirectly, to alveolar destruction during pulmonary ischemia. Our laboratory has shown that numerous tissue proteases are activated during the period of tissue ischemia (18) as well as increased caspase-3 levels after LPAL (22). Whether these enzymes are involved specifically in the alteration of lung structure is not clear. Early work by Strawbridge (19) first described a form of emphysema as ischemic atrophy of lung tissue after particulate embolization of the pulmonary vasculature. The lack of nutrient flow to alveolar epithelium during pulmonary ischemia also might be viewed in context with work on severe calorie restriction, where a change in alveolar size with variable effects on lung volume and compliance has often been reported (6, 12, 14). We have also shown an early increase in lavaged inflammatory cells as well as an increase in C-X-C chemokines early after pulmonary ischemia (7, 18). Interestingly, others have confirmed the participation of C-X-C chemokines in the loss of normal alveolar architecture in other models (4, 23).

Methods

Experimental design. All animal work was approved by the Johns Hopkins Animal Care and Use Committee. Seven- to eight-week-old male C57BL/6 (Charles River, Wilmington, MA) mice were randomly assigned to each of four surgical groups: two experimental groups with LPAL with 3 or 14 days postoperative survival (n = 15), and two control groups, with sham surgery and dissection at 3 or 14 days postoperative survival (n = 12). Age-matched naïve animals were also studied (n = 7). Littermates were randomly assigned to each of the five groups. Animals were provided food and water ad libitum for 3 or 14 days after their surgical procedure (sham or LPAL) and then underwent pulmonary function testing and histological analysis.

LPAL. Mice were anesthetized with 2% isoflurane in oxygen, intubated, and ventilated at a rate of 120 breaths/min with a tidal volume of 0.2 ml. A left thoracotomy was performed at the third intercostal space to expose the left lung. The left pulmonary artery was identified, separated from the airway, and ligated using a 6-0 silk suture. The thoracotomy was closed by suture around the third and fourth ribs while the animal was placed on 1 cmH2O positive end-expiratory pressure. Lidocaine was applied to the thoracotomy site, and the skin incision was closed using acrylamide adhesive (Future Glue; Pacer Technologies, Rancho Cucamonga, CA). The animal was removed from the ventilator, extubated, and allowed to recover. Sham surgical control mice were treated the same as the experimental mice.
in all respects except for LPAL. Naïve mice had no surgical procedures.

**Pulmonary function testing.** Quasistatic P-V relationships were determined according to procedures previously described (8, 16, 20). Briefly, the mice (n = 6/experimental group) were anesthetized with pentobarbital, the trachea was cannulated, and the animal was ventilated (as described above) with 100% oxygen for 5 min. The tracheal cannula was then occluded, which led to complete degassing of the lungs before in situ testing. The carina was then exposed through careful dissection. A vascular clip (Roboz Surgical Instruments, Gaithersburg, MD) was placed on the right mainstem bronchus to allow measurement of a P-V curve for the left lung. Placement of a vascular clip on the left mainstem bronchus and removal of the clip on the right mainstem bronchus then allowed measurement of the P-V relationship for the right lung. Airway pressure and volume were recorded on a PowerLab digital data acquisition system running Chart v5.3 software (ADInstruments, Castle Hill, Australia). The limits of inflation and deflation were 35 and −10 cmH2O, respectively. Residual volume, total lung capacity, and specific compliance were determined. Residual volume is defined as the volume in the lung at a pressure of −10 cmH2O during the first deflation. Total lung capacity (for each lung) is defined as the volume in the left lung at a pressure of 35 cmH2O on the third inflation. Specific compliance of the respiratory system was computed from the P-V relationships as the slope of the deflation limb from 5 to 0 cmH2O divided by the lung volume at 35 cmH2O.

**Histological analysis.** To quantify alterations of air space dimensions, we used procedures previously described to assess the MCL between septal walls (16, 17). Following completion of pulmonary function testing, the lungs were inflated with 1% low-melt agarose at 25–30 cmH2O. Pressure was maintained for ~1 min, by which time the agarose began to gel substantially. The lungs were then sealed with a stopcock, and the whole animal was placed in a refrigerator for at least 1.5 h. The mice were then removed, and the stiffened, inflated lungs were excised and placed in 10% phosphate buffered formalin for at least 48 h. The left lung was dissected, and the inferior and superior 3 mm along the long axis of the lung was removed and discarded. The remaining tissue was cut into three 2-mm-thick sections, dehydrated in ethanol, and embedded in glycol methacrylate (Polysciences, Warrington, PA). Three-micrometer-thick sections were cut with the microtome and stained with toluidine blue. Three sections were randomly selected, one from each of the upper, middle, and lower regions of the left lung. Images were acquired using a Nikon Digital Camera DXM1200 (Nikon, Tokyo, Japan) at ×40 magnification. The entire cross-sectional area of each section was photographed, yielding approximately five to six images per section. MCLs were measured using ImageJ software (National Institutes of Health, Bethesda, MD) with sampling grid lines 17 μm apart ensuring one to two chords per alveolus. Furthermore, a lower and upper cutoff of 8 μm and 250 μm, respectively, was applied making certain that no capillaries, arteries, veins, or bronchioles were included. Overall, this technique enabled the measurement of ~50,000 chords/mouse, which were averaged to obtain the air space MCL of each animal.

**Statistical analysis.** All data are presented as means ± SE. The MCL data were analyzed using one-way ANOVA to compare the MCL of each group. The P-V and compliance data were analyzed using one-way ANOVA to evaluate differences in the lung capacities and lung volume distributions. Bonferroni multiple comparisons tests were performed between all groups to identify specific differences between groups. A P value ≤0.05 was accepted as significant.

**RESULTS**

**Unilateral P-V measurements.** Statistically significant changes in both the left lung capacity and the percentage of volume present in the left lung were observed in mice after LPAL (P < 0.0001). The left lung capacity was not altered either 3 or 14 days following sham surgery compared with the naïve group (Table 1). The left lung capacity was significantly decreased 3 days following LPAL with an average decline in volume from 0.375 ml in the naïve animals to 0.218 ml following LPAL. No changes were noted in the right lung capacity in any group (data not shown; P > 0.05). This change in left lung capacity in the 3-day LPAL group

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**Table 1. Lung volume and body weight**

<table>
<thead>
<tr>
<th>Group</th>
<th>Left Lung Capacity</th>
<th>Left Lung Capacity/Total Lung Capacity</th>
<th>Weight at Time of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (ml)</td>
<td>SE</td>
<td>Mean (%)</td>
</tr>
<tr>
<td>Naïve</td>
<td>0.37</td>
<td>0.016</td>
<td>34</td>
</tr>
<tr>
<td>3-Day sham</td>
<td>0.38</td>
<td>0.012</td>
<td>32</td>
</tr>
<tr>
<td>3-Day LPAL</td>
<td><strong>0.22</strong></td>
<td>0.013</td>
<td><strong>22</strong></td>
</tr>
<tr>
<td>14-Day sham</td>
<td>0.32</td>
<td>0.022</td>
<td>32</td>
</tr>
<tr>
<td>14-Day LPAL</td>
<td>0.29</td>
<td>0.015</td>
<td>27</td>
</tr>
</tbody>
</table>

One-way ANOVA demonstrated statistically significant decreases in left lung capacity (volume at 35 cmH2O) between the 3-day left pulmonary artery ligation (LPAL) group and all other groups (n = 6 mice/group). A statistically significant decrease in the percentage of total lung capacity present in the left lung was also demonstrated. The mean weight of the animals at the time of death was also significantly decreased between the 3-day LPAL animals and all other groups (bold type = P < 0.05).

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**Table 2. Lung compliance and specific compliance**

<table>
<thead>
<tr>
<th>Group</th>
<th>Left Lung Compliance</th>
<th>Right Lung Compliance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Compliance</td>
<td>Specific Compliance</td>
</tr>
<tr>
<td>Naïve</td>
<td>0.021</td>
<td>0.057</td>
</tr>
<tr>
<td>3-Day sham</td>
<td>0.020</td>
<td>0.052</td>
</tr>
<tr>
<td>3-Day LPAL</td>
<td><strong>0.008</strong></td>
<td><strong>0.035</strong></td>
</tr>
<tr>
<td>14-Day sham</td>
<td>0.016</td>
<td>0.049</td>
</tr>
<tr>
<td>14-Day LPAL</td>
<td>0.015</td>
<td>0.051</td>
</tr>
</tbody>
</table>

Absolute compliance (ml/cmH2O) and specific compliance (ml/cmH2O−1·ml−1) are both significantly (bold type = P < 0.05) decreased in left lungs of animals 3 days following LPAL compared with all other groups.

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**Fig. 1.** Distribution of inflated volume between left and right lungs is depicted as the percentage of volume present in the left lung during the 3rd deflation in a pressure-volume maneuver. Left lung volume was statistically significantly decreased in the 3-day left pulmonary artery ligation (LPAL) group compared with the 3-day sham at pressures of 20 cmH2O and greater (P < 0.05). Only mean data are presented for the sake of clarity. The ratio of the SE to the mean was a maximum of 7%.
was significantly decreased compared with the 3-day sham group \( (P < 0.05) \).

The distribution of volume between the two lungs was also determined. In naïve mice and in both 3- and 14-day sham groups, the left lung contained 32–34% of the total lung capacity (Fig. 1). Three days following LPAL, the volume in the left lung at 35 cmH\(_2\)O was significantly decreased compared with all other groups both in terms of the absolute volume (Table 1) and the percent of total lung volume (Fig. 1; \( P < 0.001 \)). Recovery of lung volume occurred over time following LPAL, and by 14 days, lung volume was not different from naïve or sham controls.

Compliance changes of the left lung following LPAL are consistent with a smaller volume. However, there were also significant differences between the specific compliance measurements in the left lung at 3 days following LPAL (Table 2; \( P < 0.05 \)). The specific compliance in the left lung decreased significantly following LPAL from 0.052 ml·cmH\(_2\)O\(^{-1}\)·ml\(^{-1}\) in the 3-day sham animals to 0.035 ml·cmH\(_2\)O\(^{-1}\)·ml\(^{-1}\) in mice 3 days after LPAL. At 14 days, the specific compliance in the LPAL-tied lungs was not significantly different from that in the sham-operated mice. No changes in specific compliance were identified in the right lung.

**Morphometry.** Representative histological sections of the left lung of mice from each of the study groups are shown in Fig. 2. Each section shows normal parenchymal morphology. The results from the MCL analysis are presented in Fig. 3 with each symbol representing an individual animal. The average MCL for naïve mice lungs was 43.9 ± 1.8 \( \mu \)m. No significant differences among groups were observed \( (P = 0.18) \).

**Weight.** As summarized in Table 1, only the 3-day LPAL animals demonstrated a significant decrease in body weight (mean 20.3 g) at the time of death compared with all other
DISCUSSION

The present report describes changes in lung function following ligation of the left pulmonary artery. This model induces an ischemic injury locally in the left lung of mice, and we have previously shown that a new systemic vasculature arising from the intercostal arteries connects to the pulmonary capillaries by 5–7 days after LPAL. This new blood flow continues to increase for the next week or more, whereupon it finally stabilizes (10, 21). In the present study, we questioned whether the severe ischemia in the early days after LPAL would be sufficient to cause altered lung structure and function. Three days following LPAL, we observed a decrease in total lung capacity, a decrease in the fraction of the total lung volume in the left lung, a decrease in the compliance of the left lung, and no change in mean linear intercept in the left lung. The functional changes showed recovery at the 14-day time point.

The observed decreased volumes of the left lung 3 days after LPAL were unexpected and led to a significantly decreased lung compliance. This change in absolute, quasistatic compliance was not just a reflection of the smaller volume, since the specific compliance was also decreased significantly. These observations are consistent with measurements previously made of dynamic compliance and lung resistance of the whole lung (10). In this original description of the model, we found that during the first week following LPAL, there were small increases in resistance and decreases in compliance. Since the left lung of a mouse is normally only ~40% of the total lung volume (9), these changes in the whole lung are blunted by the lack of change in the right lung. Nevertheless, they are quite consistent with a smaller, stiffer left lung assessed by quasistatic P-V curves found here at 3 days after LPAL.

The observed decrease in lung compliance might be explained by acute lung inflammation. Previous studies in this model document early inflammatory changes that are largely resolved by 14 days. These include an increase in lavaged inflammatory cells (7), increased protein and water exudate (21), and increased inflammatory cytokines/chemokines (7, 18). A number of studies by others have shown decreased lung compliance due to products of inflammation in ischemia-reperfusion injury (24), after LPS-induced sepsis (5), and during Escherichia coli pneumonia (11). Thus the reversible decrease in compliance and the lack of lung structural changes might be predicted with acute inflammation that resolves as new systemic vessels perfuse pulmonary capillaries.

Destruction of alveolar units leading to emphysematous-like changes in lung mechanics, after complete unilateral pulmonary ischemia, was predicted based on the work of others. This model of pulmonary ischemia could be compared with particulate embolization of the pulmonary vasculature leading to lung atrophy (19), decreased nutrient flow with accompanying acute calorie restriction and air space enlargement (6, 14), and the participation of C-X-C chemokines in the loss of normal alveolar architecture (4, 23). Despite these previous studies, results of the present experiments did not confirm increases in air space size. It is perhaps worth noting, however, that there is considerable variability in the response of the lung to caloric restriction in rodents. While some studies do show emphysemalike changes with an increased lung volume and alveolar size (15), most show significant decreases in lung volume similar to what we observed here in the left lung (2, 3, 13).

For our present work, we chose the 3-day time point because it is known that a new vasculature is not functionally established until at least 5 days after LPAL. Thus the changes observed at 3 days reflect the lung’s acute response to ischemia, when the only possible source of vascular perfusion is retrograde pulsations from the left atrium. From this perspective, we conclude that retrograde perfusion must be sufficient to sustain the metabolic needs of the lung tissue during acute pulmonary ischemia, and the lung atrophy observed by Strawbridge (19) does not occur in the mouse. We unfortunately can say little about the mechanism underlying the decreased lung volume. Since the alveolar size doesn’t change, there must be fewer alveoli 3 days after LPAL. This could occur if some of the alveolar walls folded in on themselves during the severe ischemia (1). The reason for the folding may be related to changes in surfactant that could occur in the absence of normal vascular perfusion. These tissues would have to remain viable until the new perfusion occurred, whereupon they would then be reinflated normally. The alternative explanation, that alveolar walls are actually destroyed and rebuilt anew with reperfusion, seems much less likely. Verification of such septal pleating would require extensive visualization with electron microscopy.

The lack of statistically significant changes following the sham surgery at either time point compared with the naïve animals suggests that the signals associated with the physiological stress of surgery and decreased food and water intake peripheratively cause no lasting effect on lung morphology or function. In this regard, the acute weight loss of 13% noted in the animals 3 days following LPAL might possibly have some effect on the lung. However, since there was no significant change in right lung volume, it is unlikely that the body weight loss had a localized effect only on the left lung.

In conclusion, we have used a unique model that affords an opportunity to study lung structural responses during ischemia and subsequent recovery. Contrary to our expectations, we showed no changes in lung morphology despite complete unilateral pulmonary ischemia. However, we showed early decreases in lung volume and lung compliance that resolved at 14 days, a time when neovascularization is known to be well established. We speculate that the decreased pulmonary function was due to the acute lung inflammation during ischemia.


