Changes in lung permeability after chronic pulmonary artery obstruction

Elizabeth M. Wagner, Gulnura Karagulova, John Jenkins, John Bishai, and Jessica McClintock

Departments of Medicine and Environmental Health Sciences, Johns Hopkins University, Baltimore, Maryland

Submitted 30 August 2005; accepted in final form 18 October 2005

Changes in lung permeability after chronic pulmonary artery obstruction. J Appl Physiol 100: 1224--1229, 2006. First published October 20, 2005; doi:10.1152/japplphysiol.01060.2005.—We have shown that left pulmonary artery ligation (LPAL) in mice causes a prompt angiogenic response, with new systemic vessels from intercostal arteries penetrating the pleura within 6 days. Because angiogenic vessels in other organs have been shown to exhibit increased permeability, we studied vascular permeability (Evans blue dye extravasation, lung wet weight-to-dry weight ratio, and lavaged protein) in naive C57BL/6 mice and 4 h, and 14 and 21 days after LPAL (4–6 mice/time point). We also measured radiolabel clearance as an index of functional perfusion after LPAL. Tracer clearance from the left lung was maximal by 6 days after LPAL and not different from right lungs. Thus a functional vasculature is established before 6 days of LPAL that results in normal tracer clearance. By 21 days after LPAL, Evans blue-albumin was significantly increased in the left lung relative to both 4 h (no vasculature) and 14 days after LPAL. Only after 21 days of LPAL was left lung wet weight-to-dry weight ratio significantly different from naive lungs. Additionally, lavaged protein was significantly increased both 4 h and 21 days after LPAL relative to control mice. Thus, using three different methods, results consistently demonstrated increased permeability to protein and water 21 days after LPAL. Although changes in surface area of perfusion might affect the interpretation of these results, blood flow measured with labeled microspheres indicated no change in left lung perfusion between 14 and 21 days of LPAL. Thus the vasculature, remodeled as a consequence of chronic pulmonary artery obstruction, demonstrates increased water and protein permeability.

angiogenesis; bronchoalveolar lavage; Evans blue dye; lung wet weight-to-dry weight ratio; mice; technetium-99m-labeled diethylene-triamine pentaacetic acid

CHRONIC PULMONARY ISCHEMIA leads to angiogenesis involving systemic arteries that proliferate and anastomose with the pulmonary vasculature. Neovascularization of the systemic circulation in the lung after chronic pulmonary artery obstruction has been demonstrated in the human (5), sheep (1), dog (10), pig (7), rabbit (18), guinea pig (17), rat (20), and mouse (13). In most models, there is rapid growth of the bronchial circulation as well as other systemic thoracic arteries. Bronchial blood flow in the sheep was shown to increase fivefold by 3 wk after left pulmonary artery ligation (LPAL) (1). After 4 mo of left pulmonary artery obstruction in dogs, systemic blood flow to the left lower lobe increased from 5 to 330 ml/min (11). Despite these dramatic changes in the perfusion of the lung, little information exists to establish that these new vascular networks function normally. In systemic organs as well as tumors, angiogenic vessels are known to exhibit increased vascular permeability (9). One of the few studies focused on determining vascular barrier properties after chronic pulmonary artery ligation demonstrated increased lung vascular permeability; however, changes were measured after reperfusion of the obstructed pulmonary artery (7). Our laboratory has shown previously that, after LPAL in the mouse, there is a rapid neovascularization of the lung from intrathoracic arteries (13). Histological assessment of the lung demonstrates the preponderance of large blood vessels invading the lung through the pleural surface within 4 days after LPAL (19). Using this model, we designed studies to test whether this angiogenic bed demonstrates normal permeability characteristics over the course of development. Specifically, we assessed vascular permeability to water and protein, confirmed blood flow, and estimated functional perfusion of the left lung by applying the classical technique of measuring soluble tracer removal from the lung. Our results suggest that ventilation is matched to new perfusion of the left lung, resulting in normal clearance by 6 days after LPAL. However, by 21 days of LPAL, barrier function of the new vasculature is compromised.

METHODS

LPAL. Our protocol was approved by the Johns Hopkins Animal Care and Use Committee. Six- to 8-wk-old male C57BL/6 mice (Charles River Wilmington, MA) were studied 4 h to 21 days after LPAL. Mice were anesthetized (2% isoflurane in oxygen), intubated, and ventilated at 120 breaths/min, 0.2 ml/breath with the anesthetic-gas mixture. A left thoracotomy was performed at the third intercostal space to expose the left lung, and the left pulmonary artery was located, separated from the airway, and ligated using 6-0 silk suture. The thoracotomy was closed with a suture while the animal was placed on positive end-expiratory pressure (1 cmH2O). A drop of 2% lidocaine was applied for analgesia, and the skin incision was closed using methyl acrylamide adhesive. The animal was removed from the ventilator, extubated, and allowed to recover. Sham surgical control mice were treated the same as experimental mice in all respects except for LPAL. No surgical procedures were performed on naive mice. After specified times after LPAL and unless otherwise described, anesthetized mice were killed by dripping saturated KCl on the heart.

Technetium-99m-labeled diethylenetriamine pentaacetic acid. Each animal was anesthetized with an intraperitoneal injection of ketamine-acepromazine (10:1 at 1.0 mg/kg body wt) and intubated. A microsprayer syringe (PennCentury, Philadelphia, PA) was guided into the airway with sprayer tip extending slightly beyond the end of the endotracheal tube. The microsprayer, filled with 25 μl of technetium-99m-labeled diethylenetriamine pentaacetic acid (99mTc-DPTA; activity: ~10 μCi), delivered a plume (mass mean diameter of 16–22 μm) directly into the lungs. The mouse was placed on an imaging table, and two-dimensional, ventral images (XSPECT/CT; Gamma Medica, Northridge, CA) were acquired every 4 min over a 60-min

Address for reprint requests and other correspondence: E. M. Wagner, Johns Hopkins Asthma and Allergy Center, Div. of Pulmonary and Critical Care Medicine, 5501 Hopkins Bayview Circle, Baltimore, MD 21224 (e-mail: wagnerem@jhmi.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
days, and 14 days after LPAL (activity). Naive mice were studied as well as mice 2 days, 4 days, 6 days, and 14 days after LPAL (n = 4–6 mice/time point).

Bronchoalveolar lavage and protein determination. Immediately after death, the right lung was ligated and the left lung was washed (3 × 0.3 ml PBS). Cells were removed by centrifugation (5,000 g for 10 min), and lavaged protein was determined by bicinchoninic acid assay (BCA protein assay kit, Pierce, Rockford, IL). Mice were studied 4 h after sham surgery and 4 h, 14 days, and 21 days after LPAL (n = 5 mice/time point).

Evans blue dye albumin. Evans blue dye (50 mg/kg) in 250 μl of 0.9% saline was infused into the left internal jugular vein in anesthetized and ventilated mice (as described above) and allowed to circulate for 30 min. To remove intravascular Evans blue dye, the systemic vasculature was flushed (3 ml saline) through a cannula placed in the aorta. Left lungs were removed, weighed, and homogenized; and the dye was extracted with formamide (1.5 ml), quantified spectrophotometrically (absorbance 620 nm), and expressed as micrograms per milligram lung weight. Mice were studied 4 h, 14 days, and 21 days after LPAL (n = 6 mice/time point). The 4-h time point was considered a control because a new systemic vasculature is not present at this early time point (13).

Lung wet weight-to-dry weight ratio. Left lungs were excised separately and rapidly weighed. Samples were oven dried (65°C; Fisher Isotemp, Fisher Scientific, Pittsburgh, PA) for 72 h to a stable dry lung weight. Data are presented as the ratio of left lung wet weight-to-dry weight of naive mice and 4 h, 14 days, 21 days (n = 5/time point), and 28 days (n = 2 mice) after LPAL.

Blood flow determination. To determine the extent of neovascularization, systemic blood flow to the left lung was measured 14 days and 21 days after LPAL (n = 7 mice/time point) using radiolabeled microspheres. DTPA-coated polystyrene microspheres (10-μm diameter; Kisker Products, Steinfurt, Germany) were bound to 99mTc radioligand (Cardinal Health, Dublin, OH). At the specified time point after LPAL, mice were anesthetized and ventilated as described above. The carotid artery was cannulated (PE-10), and 150,000 microspheres (stock = 1.5 × 106 spheres/ml) were infused (0.1 ml at 0.04 ml/min: Harvard Apparatus, Holliston, MA). Mice were killed by exsanguination, and the left lung, kidneys, liver, heart, and brain were excised. Gamma emissions from lodged radiolabeled microspheres in individual organs were immediately counted in the Hidex Triathler (Bioscan, Washington, DC). Organ activity was normalized to whole body activity counted in a Capintec counter (Capintec Products, Ramsey, NJ), which had been calibrated to the Bioscan instrument. Organ blood flow was expressed as percentage of total measured blood flow (carcass + all organs), i.e., %cardiac output.

Statistics. All data are presented as means ± SE. The time course of change in each parameter was evaluated using Student’s t-test (blood flow), one-way ANOVA (lavaged protein, Evans blue-albumin, wet weight-to-dry weight ratio). A two-way ANOVA was used to compare retained 99mTc-DTPA. Group comparisons of statistically significant differences were made using Bonferroni’s test of multiple comparisons. A P value <0.05 was accepted as a significant difference.

RESULTS

99mTc-DTPA clearance. The first series of experiments provided information concerning early functional perfusion of the left lung and right lungs. Figure 1 shows a representative example of a dynamic series of in vivo images of the left and right lungs of a mouse 4 days after LPAL. Color intensity reflects the level of 99mTc-DTPA retained within each lung. In Fig. 1, bottom, each small frame represents 4-min cumulative counts of radioactivity. Note the gradual disappearance of radiation intensity in the right lung over the course of the 15 frames (60 min). By comparison, the left lung retained much greater radiation intensity over the same period of time. The group analysis of these experiments is provided in Fig. 2. The amount of 99mTc-DTPA retained in each lung 60 min after deposition in naive mice and groups of mice at four time points after LPAL is presented as a percentage of the initial deposited activity. A significant increase in the retained label was observed in left lungs only after 2 and 4 days of LPAL relative to
naive left lungs (P < 0.001 and P < 0.05, respectively) as well as compared with paired right lungs (P < 0.001). By 6 days after LPAL, there was no difference in the amount of retained tracer compared with naive left lungs (2 days: P < 0.001, 4 days: P < 0.05).

Percent retained tracer at 60 min in right lungs did not differ at any time point. The mean transit times for left and right lungs for each group are presented in Table 1. These results assessing the entire time course of clearance of the radioactive tracer are consistent with the end point results presented in Fig. 2.

Vascular permeability. To assess vascular barrier function of the systemic circulation to the lung after LPAL, three different, complementary strategies were applied. In the first series of experiments, the protein concentration in the bronchoalveolar lavage fluid of the left lung was determined in mice 4 h after sham surgery and 4 h, 14 days, and 21 days after LPAL. Results are presented in Fig. 3 and demonstrate overall significant changes in lavaged protein (P < 0.0001). Specifically, 4 h and 21 days after LPAL, there was a substantial and significant increase in lavaged protein (P < 0.001) compared with sham lungs. Fourteen days after LPAL, lavaged protein was not different from the sham control. Additionally, the 21-day lavaged protein was significantly different from the value at 4 h and 14 days (P < 0.001). Another method used to assess protein extravasation in another series of mice relied on the in vivo binding of Evans blue dye to circulating albumin. These results are presented in Fig. 4. Evans blue dye-albumin extracted from the left lung interstitium at each of the three time points after LPAL was significantly different from each of the other time points (P < 0.001). In a third series of mice, the left lung wet weight-to-dry weight ratio was used to assess overall lung fluid balance after LPAL (Fig. 5). Twenty-one days after LPAL, the left lung wet weight-to-dry weight ratio was significantly greater than after 4 h of LPAL (P < 0.01). The average left lung wet weight-to-dry weight ratio in two additional mice at 28 days after LPAL (5.14) was similar to that at 21 days (5.15 ± 0.20). Right lungs of naive and sham mice were not different from right lungs from mice after LPAL (4.32 ± 0.04 vs. 4.36 ± 0.08; P > 0.05). Only after 21 days LPAL were left

Table 1. Mean transit times for $^{99m}$Tc-DTPA clearance from left and right lungs

<table>
<thead>
<tr>
<th>Time after LPAL</th>
<th>$^{99m}$Tc-DTPA Retained (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>27.6 ± 0.8*</td>
</tr>
<tr>
<td>2 days of LPAL</td>
<td>24.0 ± 0.6*</td>
</tr>
<tr>
<td>4 days of LPAL</td>
<td>25.4 ± 0.9</td>
</tr>
<tr>
<td>6 days of LPAL</td>
<td>24.0 ± 0.6</td>
</tr>
<tr>
<td>14 days of LPAL</td>
<td>26.4 ± 0.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE. LPAL, left pulmonary artery ligation; $^{99m}$Tc-DTPA, technetium-99m-labeled diethylenetriamine pentaacetic acid. *P < 0.001 compared with right lung.
lungs significantly different from paired right lungs ($P < 0.001$).

**Systemic perfusion.** Results using labeled microspheres to assess systemic perfusion of the left lung after LPAL are presented in Fig. 6. Perfusion to the left lung, presented as %cardiac output (left lung microspheres/total body microspheres), 14 days after LPAL did not differ from that measured 21 days after LPAL ($P = 0.348$).

**DISCUSSION**

The purpose of the described series of experiments was to evaluate the physiological integrity of the new systemic vasculature established after left pulmonary artery ligation in the mouse. On the basis of previous studies characterizing this model of lung angiogenesis, forward flow through the lung vasculature could be minimally discerned by $\sim 6$ days after LPAL (13). The time course of neovascularization in this initial series was quantified by the measurement of systemic perfusion using 15-μm labeled microspheres. Lung histology demonstrated a preponderance of large blood vessels located within the visceral pleura by at least 4 days after LPAL (19). We questioned whether the microsphere technique was sufficiently sensitive to assess functional perfusion at very early time points after LPAL. Thus the first series of experiments in the present study was designed to assess functional perfusion within the left lung by examining the removal from the lung of a soluble tracer deposited on the epithelial surface. The technique measuring $\text{99mTc-DTPA}$ clearance from the lung has been a classical approach to measure epithelial barrier properties (8, 14), but it also serves as a measure of functional perfusion of the lung in this model. We reasoned that, with increased vascularization after LPAL, the uptake of $\text{99mTc-DTPA}$ would provide a sensitive estimate of capillary perfusion of the ventilated alveolar epithelial surface. Our results indicate that, by 6 days after LPAL, the clearance of the soluble tracer was essentially normal and not different from the clearance that occurred in the right lung. Furthermore, forward flow was obvious by 4 days after LPAL. Whether this forward flow was significant at even earlier time points was not further evaluated because the first time point measured was 2 days after LPAL. The finding that label retention at this earliest measured time point was not 100% might suggest even earlier capillary perfusion, although, based on results from other vascular beds, this seems unlikely (2, 4, 6). A more plausible explanation relates to the fact that the left pulmonary vein was not obstructed in this model of pulmonary ischemia, and there may have been mixing of pulmonary venous blood due to cardiac and ventilatory motion, leading to a small amount of label removal from the left lung. Despite this caveat, the method allowed for earlier detection of forward flow through the lung and capillary perfusion matching with ventilated surfaces.

The second goal was to determine barrier properties of the new systemic vasculature to the lung after LPAL. Several studies have shown that neovascularization is accompanied by increased permeability (9, 12). But whether this would occur in our model where new systemic blood vessels perfuse existing pulmonary capillaries was unclear. We found that all three estimates of barrier function showed significant increases in vascular permeability by 21 days after LPAL. Lavaged protein, Evans blue-albumin, and left lung wet weight-to-dry weight ratio all showed an increase by 21 days after LPAL over control responses. The need to use multiple techniques to assess vascular permeability was based largely on the assumption that the perfused surface area of the vasculature, assessed by microsphere flow, might be increasing over the time course studied (13). However, we performed an additional series of experiments in a larger group of mice and confirmed with microsphere flow that no significant changes in perfusion occurred during the time period between 14 and 21 days. This confirmation allows us to conclude that the changes in permeability observed 21 days after LPAL accurately reflect an
increase in protein and water flux. The Evans blue-albumin results, as well as the lavaged protein, demonstrated a significant difference between 14- and 21-day LPAL. Although the wet weight-to-dry weight ratio at 21 days was not statistically greater than that at 14 days, the mean value was statistically greater than the naive and sham left lungs and trended toward a higher level than the 14-day LPAL lungs. Thus it appears that water flux as well as protein extravasation are not completely normal in the newly established vasculature at the later time points. Although unlikely, it is possible that the measured blood flow was insensitive to small changes between the 14- and 21-day time points, and the observed differences in protein flux by 21 days merely reflect an increased perfusion surface area. Despite this explanation, both lavaged protein and lung weight are statistically greater than the left lung from sham mice with a normal pulmonary circulation. Thus the systemic neovasculature supplying the left lung during chronic left pulmonary artery obstruction results in abnormal barrier properties within the lung vasculature at 21 days after LPAL.

The mechanisms responsible for the abnormal barrier properties of the neovasculature in this model can only be speculated on at this time. Fadel and colleagues (7), as well as many other investigators, have shown that acute pulmonary ischemia followed by pulmonary reperfusion results in protein and water flux. These effects have been ascribed to a variety of mechanisms, including oxidant-induced injury, inflammation, and the release of edematogenic cytokines (3). The large increase in lavaged protein we observed 4 h after LPAL is consistent with a generalized lung injury due to acute ischemia and at a time before a new vasculature has developed.

Barrier properties of the lung vasculature after chronic pulmonary artery obstruction have not been widely studied. Fadel and colleagues (7) have shown, in a pig model, that the pulmonary vasculature, after a period of chronic pulmonary ischemia and subsequent reperfusion, is characterized by increased vascular pressures and fluid filtration. The extent to which these measurements partially reflect bronchial and systemic vessels, which had proliferated extensively and anastomosed with pulmonary capillaries, is difficult to deduce. The authors of this study state that, when the pulmonary artery ligation was removed, vigorous back-bleeding from bronchial vessels was observed, indicative of significant bronchial-to-pulmonary anastomotic flow. Thus the vastly expanded bronchial anastomosing network may have contributed to the estimate of filtration coefficient. We have been unable to find other reports of the permeability characteristics of the systemic circulation that proliferates and expands in patients and animals after chronic pulmonary artery obstruction. Animal models of the clinical situation of reperfusion edema after thromboendarterectomy have not reported specifically the barrier properties of the newly expanded systemic vasculature of the lung. Thus we believe our observations are important to understanding a relatively unstudied area of lung vascular pathology. Additionally, these results may have implications regarding the unexplained pathology of reperfusion edema.

Michel and Hakim (10) also have demonstrated extensive changes in vascular smooth muscle architecture of lung vessels during chronic pulmonary artery obstruction. Thus changes in the cellular components of the new systemic vessels that support pulmonary capillary perfusion might contribute overall to changes in perfusion pressure profiles and alterations in fluid and protein flux. At this time, we can only speculate with regard to the mechanisms of increased permeability; however, studies at later time points may clarify specific mechanisms.

Whether molecular changes within lung endothelial cells in this model contribute to increased fluid and protein flux after 21 days of LPAL requires further investigation. A recent study by Predescu and colleagues (15) confirmed that constitutive endothelial nitric oxide synthase in lung endothelium was critical to establishing barrier properties within the lung. Additionally, a recent study by Saadoun and colleagues (16) suggested the requirement for aquaporin-1, a water channel protein, for endothelial cell migration during tumor angiogenesis. Both of these proteins might be expected to impact fluid balance early in neovascularization. However, the time of increased permeability in our model was when angiogenesis and shear stress appeared to be reaching a plateau phase. Thus it seems more plausible to speculate that the observed increase in lung permeability is due to changing physical factors such as increased pressure or flow through pulmonary capillaries.

Consideration of one of the techniques used to assess permeability requires further comment. The technique for measuring extravasated protein by the infusion of Evans blue dye is a well-documented and extensively utilized technique. The observation that after 4-h LPAL, there was a measurable Evans blue dye signal in the left lung needs clarification. At this early time point, there is no neovascularization. The measured dye at this early time is likely due to the equilibration of this tracer in the pulmonary venous blood because the pulmonary vein was not obstructed. Furthermore, this method of evaluating protein extravasation requires that intravascular tracer be completely washed from the vasculature of the organ of interest. However, the method used for flushing the lung neovasculature was through the aorta and systemic circulation. As such, there was no forward flow at this early time point, and consequently, trapped label could not be removed from pulmonary veins. This was not the case after a forward-flowing systemic neovascularization was established by 14 and 21 days of LPAL, and the neovasculature was amply flushed by the 3 ml used to wash the intravascular compartment. Thus we believe that this measurement 4 h after LPAL is an artifact of incomplete removal of intravascular dye.

The systemic circulation to the lung that is established by 21 days after LPAL and/or the pulmonary capillaries demonstrates increased water and protein permeability. The abnormal barrier function may be related to perfusion pressures or alterations in endothelial junctions. Studies at even later time points may clarify specific mechanisms within the remodeled lung vasculature.

**ACKNOWLEDGMENTS**

We thank Dr. Martin Pomper and James Fox of the rodent imaging laboratory, Johns Hopkins University, for assisting in the design and implementation of studies of tracer clearance from the lungs.

**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute Grant HL-71605.
REFERENCES


