VEGF, fetal liver kinase-1, and permeability increase during unilateral lung ischemia

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Kazi, Armina A., Won S. Lee, Elizabeth Wagner, and Patrice M. Becker. VEGF, fetal liver kinase-1, and permeability increase during unilateral lung ischemia. Am J Physiol Lung Cell Mol Physiol 279: L460–L467, 2000.—Vascular endothelial growth factor (VEGF) is a potent mediator of increased vascular permeability and an endothelial cell mitogen. Because VEGF is upregulated during ventilated ischemia of isolated lungs and may lead to both increased vascular permeability and neovascularization, we hypothesized that VEGF and kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/flk-1) expression would increase acutely after unilateral pulmonary arterial (PA) ischemia in vivo in association with evidence of endothelial cell barrier dysfunction. To test this hypothesis, VEGF and KDR/flk-1 mRNA and protein expression were measured after 4, 8, and 24 h of left PA ligation in mice. Permeability was assessed at the same time points by measurement of bronchoalveolar lavage protein concentration and lung wet-to-dry weight ratios. Results were compared with those from uninstrumented and sham-operated mice. VEGF and KDR/flk-1 protein in the left lung both increased by 4 h and then returned to baseline, whereas increased VEGF and KDR/flk-1 mRNA expression was sustained throughout 24 h of unilateral ischemia. Bronchoalveolar lavage protein concentration increased transiently during ischemia, whereas wet-to-dry weight ratio of the left lung increased more slowly and remained elevated after 24 h of left PA ligation. These results suggest that increased expression of VEGF and KDR/flk-1 during unilateral PA occlusion in mice may contribute to the development of acute lung injury in this model.

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increased vascular permeability. To test this hypothesis, we measured VEGF and KDR/flk-1 mRNA and protein during 24 h of unilateral PA ischemia. We then compared the time course of VEGF and KDR/flk-1 expression with two measures of endothelial permeability, lung wet-to-dry weight ratio and bronchoalveolar lavage (BAL) protein concentration, in this model.

METHODS

Preparation

Five-week-old male C57/B6 mice (weight range 14.6–23.1 g) were anesthetized with an intraperitoneal injection of 0.03 ml of acepromazine-ketamine (10:1). After endotracheal intubation, ventilation was maintained with compressed oxygen at a rate of 120 breaths/min with a pressure-controlled ventilator. A left thoracotomy was performed in the second intercostal space, and the left hilum was exposed. The left pulmonary artery was isolated and ligated with 6-0 suture, the lungs were hyperinflated to reverse atelectasis, and a positive end-expiratory pressure of 3 cmH2O was added. The thoracotomy incision was then closed, and the mice were allowed to recover from the anesthesia. After durations of left thoracotomy incision was then closed, and the mice were harvested the lungs or kill the animals. In addition, neither respiratory resistance nor dynamic compliance was altered after PA ligation and again when the chest was opened to visually confirm before closure of the thoracotomy incision. Inflation and deflation of the left lung with ventilation was performed by intratracheal instillation of 0.5 ml of saline immediately after the animal was anesthetized, with aspiration of fluid via the endotracheal tube and careful recording of the amount of fluid recovered (8). Protein concentration in lung lavage fluid, which is indicative of protein leak from the vascular to the alveolar space. Lavage protein concentration in each sample was normalized to the total protein concentration (Bio-Rad, Hercules, CA) or rabbit polyclonal anti-mouse antibodies against the constitutive protein β-actin (Santa Cruz Biotechnology).

Protocol II. In separate groups of mice exposed to the same durations of left PA ligation (n = 5 mice/time point), both left and right lungs were excised for measurement of wet-to-dry lung weight ratio, and results were compared with those from sham (n = 6) and control (n = 4) animals.

Protocol III. In a third series of experiments, BAL was performed by intratracheal instillation of 0.5 ml of saline after 4, 8, or 24 h (n = 5 mice/time point) of left PA ligation, and results were compared with those from sham (n = 6) and control (n = 4) animals.

Measurements

mRNA expression. Lung tissue was snap-frozen in liquid nitrogen and homogenized in STAT-60 RNA lysis buffer (Tel-test, Friendswood, TX); then total RNA was isolated for Northern blot analysis. RNA was fractionated by 1% agarose gel electrophoresis and then transferred to Gene Screen Plus membrane filters with 10× saline-sodium citrate. Membranes were then hybridized with 32P-labeled cDNA encoding a 363-bp fragment of human VEGF121 (obtained from Augustine Choi (Yale University School of Medicine, New Haven, CT)). Hybridization and wash conditions were as previously described (6). Autoradiogram signals were quantified by densitometric analysis (Molecular Dynamics, Sunnyvale, CA). Lactate dehydrogenase (LDH) activity was also analyzed for VEGF with the Quantikine M VEGF immunoassay kit (R&D Systems, Minneapolis, MN). Samples were diluted 1:5 and 1:10 in assay diluent and incubated for 2 h. After the assay diluent was washed away, mouse VEGF conjugate was incubated with the protein samples. Plates were then developed, and optical density was measured at 450 and 570 nm to quantitate VEGF protein spectrophotometrically from a standard curve in accordance with the manufacturer’s instructions. VEGF protein concentration in each sample was normalized to the total protein concentration (Bio-Rad, Hercules, CA) in the tissue homogenate.

Pulmonary Vascular Permeability

BAL protein concentration. Pulmonary vascular permeability was first evaluated by measurement of the protein concentration in lung lavage fluid, which is indicative of protein leak from the vascular to the alveolar space. Lavage was performed by intratracheal instillation of 0.5 ml of saline immediately after the animal was anesthetized, with aspiration of fluid via the endotracheal tube and careful recording of the amount of fluid recovered (8). Protein concentration was measured in undiluted lavage fluid (5) (Bio-Rad).

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determined for both left and right lungs. The lungs were excised separately, and each lung was rapidly weighed on prepared dishes for determination of wet lung weight. The samples were then dried in an oven (Fisher isotemp, 65°C) for 3–4 days and weighed daily to establish dry lung weight. The wet-to-dry lung weight ratio would be expected to increase with an increase in extravascular lung water, increased blood volume, or both.

Statistical Analysis

Differences in mRNA or protein expression, protein concentrations, and wet-to-dry lung weight ratios between groups were compared with one-way analysis of variance. When significant variance ratios were obtained, least significant differences were calculated to allow comparison of individual group means (42). Differences were considered significant at P ≤ 0.05.

RESULTS

VEGF Expression

Representative VEGF mRNA expression in the left lung, measured by Northern blot analysis, is shown in Fig. 1, top. The predominant band labeled with human VEGF cDNA was at ~3.9 kb, consistent with the transcript for VEGF165. VEGF mRNA increased after PA ligation (Fig. 1, top, lanes 3–5) when compared with both control (lane 1) and sham (lane 2) animals. Mean relative expression for all experiments is shown in Fig. 1, bottom. VEGF expression began to increase 4 h after left PA ligation, with a further significant increase after 8 h of left PA occlusion, and remained elevated after 24 h of left lung ischemia compared with that in untreated and sham-operated control lungs. In contrast to mRNA, VEGF protein in left lung homogenates, measured both by Western blot analysis and ELISA (shown in Figs. 2 and 3), increased two- to threefold after 4 h of left PA occlusion when compared with levels in both untreated and sham-operated control animals, then returned toward control values. Both the timing and the magnitude of increased VEGF protein expression were similar in the two assays employed for measurement. VEGF mRNA and protein expression were also independently measured in the right lung in the same animals after 4, 8, and 24 h of left PA occlusion, and results were compared with measurements from control and sham groups. No differences were seen in either mRNA or protein expression in the right lung after contralateral lung ischemia (data not shown).

Flk-1 Expression

Expression of flk-1 mRNA and protein is shown in Figs. 4 and 5, respectively. Representative Northern blot analysis for flk-1 is shown in Fig. 4, top. The predominant band labeled with rat KDR/flk-1 cDNA was at ~5.5 kb. Similar to VEGF, flk-1 transcript in the left lung increased approximately twofold after all durations of left PA occlusion compared with the untreated and sham-operated control groups, although this increase achieved statistical significance only after 8 and 24 h of left lung ischemia (Fig. 4, bottom). Interestingly, flk-1 protein expression, shown in Fig. 5, paralleled VEGF protein expression, increasing by 4 and 8 h after left PA occlusion and then slowly decreasing toward control levels despite a sustained increase in mRNA. There was an unexplained small but significant increase in flk-1 protein expression in the sham-operated lungs. As with VEGF, no significant differences in either flk-1 mRNA or protein expression were detected in the right lung after left PA occlusion.

Pulmonary Vascular Permeability

To determine whether increased VEGF and flk-1 expression were linked with the physiological effects of VEGF, we evaluated pulmonary vascular permeability after left PA occlusion by two separate measures. Shown in Fig. 6 is the mean concentration of protein in BAL fluid after 4, 8, and 24 h of left lung ischemia compared with that in control and sham-operated animals. The mean percent of BAL fluid recovered was 57 ± 3, and percent recovery of fluid did not differ among groups. Minimal or no protein was present in the BAL fluid recovered from control animals, and protein concentration increased only minimally in the sham group (0.34 ± 0.20 μg/μl). In contrast, protein concentration more than doubled after 4 h of left lung ischemia (0.90 ± 0.24 μg/μl; P < 0.05), with a further four- to fivefold increase after 8 h of left PA occlusion.
(1.67 ± 0.39 μg/μl; \(P < 0.05\)), then returned toward control values by 24 h (0.36 ± 0.33 g/μl), indicating a transient alveolar capillary leak during this duration of ischemia.

In addition to measuring alveolar protein leak, we assessed pulmonary edema formation by measurement of wet-to-dry lung weight ratios of both left and right lungs as a function of ischemic duration. Data are shown in Table 1. Control values for wet-to-dry weight in left and right lungs differed slightly and were 4.41 ± 0.18 and 4.54 ± 0.09, respectively. The wet-to-dry weight ratio of the right lung did not change after left PA occlusion or in sham-operated animals. In contrast, the wet-to-dry weight ratio of the left lung increased after left PA occlusion (4.75 ± 0.10 and 4.79 ± 0.07 after 8 and 24 h, respectively; \(P < 0.05\)) when compared with untreated control lungs, as shown in Table 1. This difference was accounted for primarily by a trend toward increased mean lung wet weight (data not shown). Differences in left lung wet-to-dry weight ratios between sham-operated control lungs and 8- and 24-h PA occlusion groups approached but did not achieve statistical significance (\(P = 0.1\) and \(P = 0.06\), respectively). Sham thoracotomy, however, did not alter left lung wet-to-dry weight ratio when compared with untreated control animals (\(P = 0.25\)).

**DISCUSSION**

In this study, we sought to develop an in vivo model of PA ischemia to evaluate both the expression of VEGF and its endothelial cell receptor, KDR/flk-1, and to determine whether increased VEGF and KDR/flk-1 expression was linked with increased vascular permeability during acute ischemic lung injury. Our results suggest that VEGF mRNA expression in the left lung increased acutely during left PA occlusion and that increased levels of VEGF mRNA were sustained throughout the 24-h ischemic period studied. Becker et al. (4) made a similar observation in an isolated lung model subjected to a shorter duration of global ischemia. Similarly, others have shown upregulation of VEGF mRNA after myocardial (18, 31), cerebral (11, 23), and retinal (38, 39) ischemia.

In ischemic organs other than the lung, the primary stimulus for VEGF expression is thought to be tissue...
hypoxia. In vitro studies have suggested that increased VEGF mRNA in several cell lines exposed to hypoxia occurred because of both increased transcription (29) and posttranscriptional stabilization (28, 30, 40, 44). Because ventilation was not interrupted during left PA occlusion in our murine model, tissue hypoxia is not likely to explain increased expression of VEGF in our study. In fact, previous data (4) in isolated lungs have suggested that increased expression of VEGF during ventilated pulmonary ischemia is oxygen independent and that the mechanism of VEGF upregulation may differ during hypoxic and hyperoxic ischemia. Hypogly-

Fig. 4. Top: representative Northern blot analysis of kinase insert domain-containing receptor/fetal liver kinase-1 (KDR/flk-1) expression in the left lung after 4, 8, and 24 h of left PA occlusion (lanes 3, 4, and 5, respectively) compared with that in control (lane 1) and sham-operated (lane 2) lungs. Even loading of RNA was confirmed by ethidium bromide staining of gels and measurement of 18S RNA expression. Bottom: mean relative expression of KDR/flk-1 mRNA in left lung measured by Northern blot analysis after 4, 8, and 24 h (n = 5 mice/time point) of PA occlusion compared with that in sham-operated animals (n = 6). Data are expressed relative to KDR/flk-1 mRNA in the left lung of control (n = 4) animals.

Fig. 5. Top: representative Western blot analysis of KDR/flk-1 protein expression in the left lung after 4, 8, and 24 h of left PA occlusion (lanes 3, 4, and 5, respectively) compared with that in control (lane 1) and sham-operated (lane 2) lungs. Even loading of protein samples was confirmed by Coomassie blue staining of gels and measurement of β-actin expression. Bottom: mean relative expression of KDR/flk-1 protein in left lung measured by Western blot analysis after 4, 8, and 24 h (n = 5 mice/time point) of PA occlusion compared with that in sham-operated animals (n = 6). Data are expressed relative to KDR/flk-1 protein in the left lung of control (n = 4) animals.
cemia, another consequence of tissue ischemia, has also been shown to increase steady-state VEGF mRNA and transcription rate in vitro (41, 44). Increased VEGF mRNA expression has also been demonstrated in response to oxygen radicals (10, 24) and various cytokines (17) in several cell types, all stimuli that may be relevant in the ischemic lung.

As with VEGF mRNA, increased VEGF protein has been shown in response to hypoxic ischemia in both the brain and the retina (11, 27, 39). The previous study of Becker et al. (4) in isolated ventilated ferret lungs also demonstrated increased VEGF protein during 180 min of either hypoxic or hyperoxic ischemia. In the present study, VEGF protein concentration increased only in the lung subjected to PA occlusion, not in the contralateral lung, in a time course consistent with that seen in isolated lungs exposed to global ischemia. The PA ligation model has the advantage of allowing more sustained periods of ischemia than an isolated lung model, and our results suggest that elevated VEGF protein was transient during 24 h of pulmonary ischemia. The differential time course of VEGF mRNA and protein expression suggests that VEGF expression during pulmonary ischemia in vivo is regulated, at least in part, posttranscriptionally (3, 43).

KDR/flk-1 expression was upregulated coordinately with VEGF in response to 24 h of left PA occlusion in mice, with rapid and sustained upregulation of KDR/flk-1 mRNA and transient increases in KDR/flk-1 protein concentration in the ischemic tissue. Similar observations linking VEGF expression with expression of one or both of its receptors have been made in other models of nonpulmonary ischemia (23, 27, 31, 45) and in lungs exposed to hypoxia (9, 46). These earlier data, in conjunction with our results, suggest that similar stimuli regulate both VEGF and KDR/flk-1 expression. Our findings suggest that the stimulus for this regulation is not necessarily hypoxia, as ventilation was maintained throughout ischemia in this murine model. In addition, expression of VEGF and KDR/flk-1 during pulmonary ischemia is not likely due to the elaboration of humoral factors, as neither VEGF or KDR/flk-1 expression was increased in the contralateral lung after unilateral PA occlusion in these experiments.

Although there is evidence of a causal relationship between VEGF (2, 50) and KDR/flk-1 (34, 35) upregulation and neovascularization, studies of the role of VEGF and KDR/flk-1 in vascular barrier dysfunction are more limited. To determine whether vascular barrier dysfunction occurred in this model in a time course consistent with increased VEGF and flk-1 expression, we performed two independent assays to evaluate vascular permeability. Both BAL protein concentration and left lung wet-to-dry weight ratios increased after left PA ligation, which is consistent with the development of endothelial leak. Previous studies have demonstrated morphological evidence of pulmonary capillary endothelial cell injury and alveolar barrier dysfunction after both days (20) and hours (21) of unilateral pulmonary occlusion. Increased extravascular lung water has also been reported (26) within hours after unilateral PA embolization in the embolized, but not in the contralateral, lung. The observed increase in both BAL protein concentration and left lung wet-to-dry weight ratio occurred after left PA occlusion in the present study suggests a link between early transient increased VEGF and KDR/flk-1 expression and vascular permeability during relatively brief periods of unilateral pulmonary ischemia. This hypothesis is supported by previous data (4) linking increased VEGF expression and pulmonary vascular permeability after 3 h of global ischemia in an isolated lung model, as well as by a recent report (47) that inhibition of VEGF attenuated brain edema after transient cortical ischemia.

Our data are also supported by a study (16) demonstrating that overexpression of VEGF in avian embryos during periods of limb bud morphogenesis was associated with both increased expression of KDR/flk-1 and increased vascular permeability. Several in vitro studies provide additional evidence that VEGF causes vascular barrier dysfunction and that these effects may be mediated by activation of KDR/flk-1. Increased permeability in response to VEGF in vitro has been demonstrated by measurement of macromolecular flux across endothelial cell monolayers (19, 22, 48) as well as by assessment of morphological changes, including the development of fenestrae and the modification of caveolae (13) in endothelial cells derived from various

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**Table 1. Effects of left pulmonary arterial occlusion on left lung wet-to-dry weight ratio**

<table>
<thead>
<tr>
<th>Group</th>
<th>Left Lung Wet-to-Dry Weight Ratio</th>
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<tbody>
<tr>
<td>Control</td>
<td>4.41 ± 0.18</td>
</tr>
<tr>
<td>Sham</td>
<td>4.56 ± 0.04</td>
</tr>
<tr>
<td>4-h PA occlusion</td>
<td>4.66 ± 0.05</td>
</tr>
<tr>
<td>8-h PA occlusion</td>
<td>4.75 ± 0.10e</td>
</tr>
<tr>
<td>24-h PA occlusion</td>
<td>4.79 ± 0.07*</td>
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</tbody>
</table>

Values are means ± SE. PA, pulmonary arterial. *P < 0.05 vs. control.

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**Fig. 6. Bronchoalveolar (BAL) protein concentration after 4, 8, and 24 h (n = 5 mice/time point) of left PA occlusion compared with that in control (n = 4) and sham (n = 6) animals.**
tissues, although there are few published in vitro data in endothelial cells derived from the lung.

Interestingly, our measurements to assess increased permeability in vivo in this murine model were notable for a discrepancy in the time course of increased BAL protein and lung wet-to-dry weight ratios. BAL protein concentration increased after 4 and 8 h of left PA ligation, then returned to control values after 24 h, whereas left lung wet-to-dry weight ratios increased after 8 h and remained elevated through 24 h of PA occlusion. Lung wet-to-dry weight can increase because of increased lung water and/or lung blood volume; thus we cannot exclude the possibility that the sustained increase in left lung wet-to-dry weight ratios after 24 h of left PA occlusion was due to neovascularization rather than to increased permeability. Other studies (7, 33, 49) have demonstrated physiological and morphological evidence of neovascularization after days to weeks of unilateral PA occlusion. Interestingly, this phenomenon may occur earlier than previously demonstrated, with histological evidence of newly forming blood vessels at the left pleural surface after 24 h of left PA ligation in mice (36).

In summary, we have shown that upregulation of VEGF and KDR/flk-1 mRNA expression occurred during 24 h of unilateral PA ischemia in vivo. VEGF and KDR/flk-1 protein also increased after left PA occlusion, although, unlike mRNA, the increases in protein concentration were transient. Finally, increased BAL protein concentration and left lung wet-to-dry weight ratios during 24 h of PA ischemia in vivo suggest that vascular barrier dysfunction occurred as a consequence of increased VEGF and KDR/flk-1 expression. Because acute exposure (2–8 h) of the lungs to global ischemia occurs with lung preservation for transplantation and pulmonary edema is a common early complication of lung transplantation, our results suggest VEGF as a potential mediator of early pulmonary allograft dysfunction. Further studies will explore whether VEGF and KDR/flk-1 expression are altered during longer durations of PA occlusion and whether VEGF and KDR/flk-1 are important mediators of angiogenesis that occurs after more chronic periods of pulmonary ischemia. Determination of the relationship between VEGF expression and both vascular permeability and neovascularization after ischemia may suggest potential therapeutic interventions for clinical conditions that involve acute ischemic lung injury and subsequent vascular remodeling.

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