Role of ROS in ischemia-induced lung angiogenesis

Julie Nijmeh, Aigul Moldobaeva, and Elizabeth M. Wagner

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

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Nijmeh J, Moldobaeva A, Wagner EM. Role of ROS in ischemia-induced lung angiogenesis. Am J Physiol Lung Cell Mol Physiol 299: L535–L541, 2010. First published August 6, 2010; doi:10.1152/ajplung.00002.2010.—Pulmonary artery obstruction and subsequent lung ischemia have been shown to induce systemic angiogenesis despite preservation of normoxia. The underlying mechanisms, however, remain poorly understood. In a mouse model of lung ischemia induced by left pulmonary artery ligation (LPAL), we showed previously, the formation of a new systemic vasculature to the ischemic lung. We hypothesize that LPAL in the mouse increases reactive oxygen species (ROS) production, and these molecules play an initiating role in subsequent lung neovascularization. We used oxidant-sensitive dyes (DHE and H2DCF-DA) to quantify ROS and measured the antioxidant-reduced glutathione (GSH) and its oxidized form (GSSG) as indicators of ROS levels after LPAL. The magnitude of systemic neovascularization was determined by measuring systemic blood flow to the left lung with radiolabeled microspheres 14 days after LPAL. An increase in ROS was observed early (30 min: 55% increase in H2DCF-DA) after LPAL, with a return to baseline by 24 h. GSH/GSSG was decreased (~50%) 4 h after LPAL, suggesting earlier ROS upregulation. Mice treated with the antioxidant N-acetylcysteine showed attenuated angiogenesis (62% of wild-type LPAL), and mice lacking Nrf2, a transcription factor important for antioxidant synthesis, resulted in increased neovascularization (207% of wild-type LPAL). Overall, GSH/GSSG was inversely associated with the magnitude of neovascularization. These results demonstrate that LPAL induces an early and transient ROS upregulation, and ROS appear to play a role in promoting ischemia-induced angiogenesis.

ischemic lung; blood flow; reactive oxygen species; N-acetylcysteine; Nrf2

ANGIOGENESIS, the process whereby new blood vessels form from a preexisting vasculature, is fundamental for many physiological processes including embryonic development and tissue repair as well as many pathological conditions. In the lung, obstruction of one pulmonary artery has been associated with systemic neovascularization by bronchial and intercostal arteries in all studied mammals (9, 13, 19, 38, 41). Systemic vascularization is also evident in several pulmonary pathologies, including chronic pulmonary thromboembolism (20) and primary lung tumors (10, 16, 24), where, in addition to bronchial arteries, new vessels arising from the thoracic wall to the lung have been observed. The new circulation appears essential in supporting ischemic lung tissue, where systemic blood flow to the lung can increase up to 50% of the cardiac output in some models (23). Our laboratory previously developed a mouse model of angiogenesis in response to left pulmonary artery ligation (LPAL) (25). This model differs from the previous ones in that the mouse has a poorly developed bronchial vasculature (37). Accordingly, ischemia-induced neovascularization in the mouse appears to arise exclusively from the intercostal arteries, rather than bronchial vessels. A unique attribute of lung ischemia that sets it apart from other organs is that it is not associated with tissue hypoxia, due to continuous ventilation.

The mechanisms responsible for neovascularization, however, are unknown. In an isolated lung model, non-hypoxic ischemia associated with cessation of perfusate flow results in an early production of reactive oxygen species (ROS) (1). ROS, such as superoxide anion (O2·-) and hydrogen peroxide (H2O2), are known to be involved in physiological and pathophysiological processes. In general, high levels of ROS are considered to be toxic, causing cell damage and cell death (36), whereas low amounts of ROS can serve as signaling molecules to induce proliferation and migration of endothelial cells (29, 43). The importance of ROS in promoting angiogenesis has been documented in several in vivo studies where a correlation between ROS production and angiogenesis was observed in diabetic eyes (12), in balloon-injured arteries (28), in response to myocardial ischemia (34), and hindlimb ischemia (33). Furthermore, antioxidants such as N-acetylcysteine (NAC) have been shown to inhibit angiogenesis both in vitro and in vivo (7, 8).

In the present study, we hypothesized that ROS released at the onset of pulmonary ischemia initiates a series of events that lead to systemic angiogenesis. Most studies of ischemia-induced angiogenesis in other organs occur in models where ischemia causes tissue hypoxia, which leads to the generation of hypoxia-inducible growth factors. Our model of complete left pulmonary artery obstruction in ventilated mice provides a unique opportunity to study the role of ROS in ischemia-induced angiogenesis, independent of a hypoxic environment. Our results demonstrate an early production of ROS, the magnitude of which is positively associated with the magnitude of systemic angiogenesis in the ventilated mouse lung.

METHODS

LPAL

Animal protocols were reviewed and approved by the Johns Hopkins Medical Institutions Animal Care and Use Committee and were conducted using national guidelines for the care and protection of animals. Mice used in this study were 6- to 8-wk-old C57Bl/6 male mice (Jackson Laboratories, Bar Harbor, ME) and male Nrf2 null mice on a CD1 background (provided by Drs. Shyam Biswal and Sekhar Reddy, Johns Hopkins Bloomberg School of Public Health). Mice were anesthetized (2% isoflurane), intubated, and then mechanically ventilated (120 breaths/min; 0.2-ml tidal volume) using the same anesthetic/room air mixture. As previously described, a left lateral thoracotomy was performed at the third intercostal space and the left pulmonary artery was separated from the airway and ligated (40). To prevent the development of pneumothorax, the chest was evacuated by placing the animal on positive end-expiratory pressure (1 cmH2O) after which the thoracotomy was closed with a silk suture. Lidocaine (2%) was applied to the thoracotomy site for analgesia, and the skin incision was closed using methyl acrylamide adhesive. Mice were removed from the ventilator, extubated, and allowed to recover.
Sham control mice were anesthetized, intubated, ventilated, and underwent thoracotomy, but the left pulmonary artery was not ligated. Sham control mice and 30-min LPAL mice were not allowed to recover, and left lungs were harvested after surgery.

**NAC Treatment**

The antioxidant NAC was reconstituted in saline, and its pH was adjusted to 6.8–7.2 using 1 N NaOH and was administered to mice intraperitoneally (1 mg/g body wt, 0.3 ml). An injection was given 24 and 12 h before LPAL and at the time of surgery (4, 27).

**Angiogenesis Index**

To determine the extent of neovascularization, systemic blood flow to the left lung was measured 14 days after LPAL using 10-μm radiolabeled microspheres as previously described (40). The 14-day time point was chosen based on previous work that demonstrated a stable, functional perfusing vasculature at this time (39). The carotid artery was obstructed and cannulated (PE 10) for retrograde infusion (0.04 ml/min; Harvard Apparatus, Holliston, MA) of 150,000 microspheres. Mice were killed by exsanguination, and the left lung was excised. Gamma emissions from lodged radiolabeled microspheres in individual organs were immediately counted in the Hidex Triathlab (Bioscan, Washington, DC). Left lung activity was normalized to whole body activity counted in a Capintec counter (Capintec Products, Ramsey, NJ), which had been calibrated to the Bioscan instrument. For blood flow in sham mice, a suture was tied around the left pulmonary artery immediately before microsphere injection to prevent recirculation of microspheres into the left lung. The radioactivity of the whole mouse is an indication of total systemic perfusion and represents cardiac output. Accordingly, the fraction of radioactivity of left lung compared with that of the whole mouse represents the percentage of cardiac output that reaches the left lung through the systemic circulation and thus serves as a functional index of angiogenesis.

**Quantification of ROS**

The following three experimental approaches were used to quantify ROS early after LPAL.

**Dihydroethidium staining.** Dihydroethidium (DHE) is a lipophilic cell-permeable dye that can undergo oxidation to ethidium bromide or a structurally similar product in the presence of superoxide, and, to a lesser extent, hydrogen peroxide and hydroxyl radicals. Ethidium then binds irreversibly to the double-stranded DNA, causing amplification of a red fluorescent signal, and appears as punctate nuclear staining indicative of ROS production (6). In pilot studies for this series, we examined a few left lungs of naïve mice (anesthesia only), sham 30-min (anesthesia, ventilation, thoracotomy), and sham mice at later time points that were allowed to recover from surgery (4 and 24 h: anesthesia, ventilation, thoracotomy, recovery). The DHE fluorescence values of these control lungs were within the margin of error of the measurement. Therefore, in an effort to simplify the experiments and reduce the number of controls, we elected to use sham controls from an early time point (30 min). Left lungs were collected from sham mice or following various durations of LPAL and were placed in optimum cutting temperature (OCT) formulation made of water-soluble glycols and resins (Sakura, Torrance, California) and were immediately frozen on dry ice. Lungs were sectioned (10 μm; −10°C) using a Reichert-Jung cryostat (model 2800 Frigocut; Arnsberg, Germany) and placed on slides before being incubated with DHE (10 μmol/l) in PBS in a light-protected, humidified chamber (37°C, 30 min; Ref. 42). Fluorescent images of five to seven fields/section were obtained with a microscope (Olympus IX-51) equipped with appropriate narrow band filter set with an excitation of 488 nm and an emission range of 574–595 nm. Fields were randomly chosen, avoiding the edges of the sections where autofluorescence was observed. Images were captured using a Sensicam High Performance camera (Cooke, Auburn Hills, MI), and pixel densities of the digitized fluorescent images were quantified using Image-pro Plus (version 5; Media Cybernetics, Silver Spring, MD). A few representative sections were also stained with FITC-labeled anti-CD31 antibody (BD Pharmingen, San Jose, CA) to mark pulmonary endothelium. Colocalization of DHE and CD31 was evaluated using an inverted confocal microscope (Zeiss LSM-510).

**H2DCF-DA.** The relative levels of ROS generated in the lungs were monitored by a flurometric assay using H2DCF-DA. This non-fluorescent molecule can be passively loaded into whole tissue since it is freely diffusible across cell membranes. Once internalized, esterases cleave the diacetate group. The product is then converted into the highly fluorescent DCF in the presence of ROS (14). Due to the difficulty of delivering this dye in vivo, since in our model the circulation to the left lung is blocked, the dye was added to the lungs ex vivo. After 30 min of in vivo LPAL or sham surgery, left lungs were removed, quickly frozen, and homogenized by polytron (40 mM ice-cold Tris-HCl and 0.1% Tween buffer, pH 7.4). The homogenate was divided into two equal fractions, one loaded with H2DCF-DA (final concentration of 5 μM) and the other served as a blank (equal volume of DMSO). All samples were incubated (45 min, 37°C), and then fluorescence was measured (Hitachi F2500 Fluorescence Spectrophotometer; 504-nm excitation and 529-nm emission; 5-nm slit).

Blank readings were subtracted from loaded sample readings, and values were presented as fluorescence units per milligram of tissue (FU/mg). The LPAL lungs were normalized to the average FU/mg of sham lungs on a given day to correct for small day-to-day variability.

**GSH/GSSG ratio.** Glutathione (GSH) is one of the most important non-enzymatic oxidant defenses within the body. GSH exists in two forms: the more abundant (mM) reduced sulfhydryl form (GSH) and the oxidized disulfide form (GSSG). ROS are neutralized by GSH through a cascade of detoxification mechanisms, to form GSSG. Upon exposure to increased ROS levels, the ratio of GSH/GSSG will decrease as a consequence of GSSG accumulation. Therefore, GSH/GSSG ratio is frequently measured in physiological and pathophysiological conditions as a dynamic indicator of ROS levels. A Bioxytech GSH/GSSG–412 kit (Oxis Health Products, Portland, OR) was used to measure reduced and oxidized glutathione in the lung homogenates as per the manufacturer’s instructions. Briefly, the left lungs from sham mice or those subjected to varying durations of LPAL were divided longitudinally and quick-frozen.

One-half of each left lung was used for measuring GSH levels; for that purpose it was homogenized (750 μl of 5% MPA in PBS). The other half was used to measure GSSG levels and was thus homogenized in the same volume of buffer, with the addition of the provided scavenger 1-methyl-2-vinylpyridinium trifluoromethanesulfonate. The homogenates were sonicated and centrifuged. The supernatants were added to the 96-well plates, along with the provided buffers, chromogen (DTNB) and enzyme (GR). The substrate NADPH was promptly added to the reaction, and the change of absorbance at 412 nm was monitored every 6 s for 3 min. The calculation of GSH/GSSG ratio required four steps: 1) determination of the reaction rate, 2) construction of calibration curves, 3) calculations of the analyte concentrations, and 4) calculation of the GSH/GSSG ratio.

**Statistical Analysis**

All data sets are expressed as means ± SE. Control groups were tested for normality using Shapiro-Wilk Normality test. All groups showed a normal distribution. Accordingly, statistical significance was evaluated with student’s unpaired or one-sample t-test or ANOVA with Newman-Keuls posttest, using Prism software for Macintosh. Differences were considered to be statistically significant when P ≤ 0.05.

**RESULTS**

**Angiogenesis After LPAL**

Systemic neovascularization as quantified by blood flow to the ischemic left lung 14 days after LPAL is shown in Fig. 1. In mice subjected to sham surgery alone, systemic blood flow...
Systemic blood flow to the left lung was negligible and represented experimental background \((n = 1005)\) to the left lung was markedly increased with an average perfusion of 1.44% \((\pm 0.18\% ; n = 7)\) of cardiac output. This significant \((P = 0.002)\) increase in systemic blood flow confirms previous observations that LPAL induces systemic angiogenesis after LPAL in mice \((25)\).

**Early Ischemia-Induced ROS Production**

Given the low steady-state concentrations and the short half-life of ROS under physiological conditions, several known methodologies were applied to confirm early ROS detection following ischemia. Results showing DHE fluorescence early after LPAL \((1, 4, 24) h\) in left lung sections are presented in Fig. 2. The average fluorescence intensity from five to seven fields in three to six sections/lung \((n = 2–5 mice/time point)\) is summarized in Fig. 2A. DHE fluorescence intensity trended upward after 1- and 4-h LPAL. Fluorescence 24 h after LPAL was not different from lungs of mice after sham surgery alone. Due to variability with this histological approach, changes did not reach statistical significance \((P = 0.26)\). Figure 2B shows a vessel from the left lung 4 h after LPAL treated with DHE and CD31, which stains pulmonary endothelium. The merged image confirms the colocalization of DHE and CD31 of a pulmonary vessel. However, this image also confirms that other intravascular and lung parenchymal cells are also DHE-positive and contribute to ROS release.

Based on the trends observed with DHE staining, an earlier time point was used for experiments using the DCF fluorescence indicator. Additionally, sham controls were studied on every experimental day, and results served as a reference value. As seen in Fig. 3, DCF fluorescence was significantly increased by 52% in lungs subjected to 30 min of LPAL compared with those subjected to 30 min of sham surgery alone \((n = 9 mice/group, P = 0.03)\). This ex vivo measurement demonstrates an upregulation of ROS production 30 min after LPAL and the onset of ischemia. To confirm that DCF fluorescence was due to the production of ROS in the lungs, homogenates were preincubated with apocynin \((2 mM)\). Results demonstrated that apocynin completely abolished any DCF fluorescence in both sham \((98\% \text{ reduction})\) and LPAL lungs \((99\% \text{ reduction})\). Given that apocynin is an antioxidant as well as an inhibitor of NADPH oxidase, these results confirmed that measured DCF fluorescence was due to the presence of ROS, with the possibility of NADPH oxidase contributing to measured ROS.

The third method to confirm the presence of ROS relied on the conversion of GSH to GSSG as a dynamic indicator of the lung tissue response to ROS generation. Figure 4 shows the time course of changes in GSH/GSSG after LPAL compared with time-matched sham controls \((n = 4–8 mice/group; P < 0.01)\). No differences in GSH/GSSG were detected among sham lungs at any time point after surgery. However, GSH/GSSG was significantly reduced in left lungs 4 h after LPAL compared with 4-h sham lungs, as well as compared with 0.5-h LPAL lungs \((P < 0.01)\). This change represents a 48% decrease in GSH/GSSG compared with the average of all sham lungs. GSH/GSSG of lungs 24 h after LPAL was not different from 24-h sham lungs. The significant decrease in GSH/GSSG 4 h after LPAL is suggestive of earlier ROS upregulation and consistent with the timing suggested by results using DHE and H₂DCF-DA fluorescent dyes.

**Effects of ROS Manipulation on Angiogenesis After LPAL**

To determine whether the transient increase in ROS levels seen in the early periods following LPAL played a role in promoting angiogenesis in response to ischemia in this model, we studied the effects of ROS downregulation or upregulation on systemic blood flow to the left lung 14 days after LPAL. Figure 5 shows that blood flow in mice pretreated with NAC averaged 0.89% \((\pm 0.15\% ; n = 7)\) of cardiac output and was significantly lower than the saline-treated group, with a blood flow of 1.43% \((\pm 0.11\% ; n = 6)\) of cardiac output \((P = 0.03)\). Saline-treated mice, however, had a similar systemic blood flow as untreated mice after 14 days of LPAL. The significant 55% decrease in blood flow associated with NAC treatment suggested that ROS produced at the early time points play an important role in promoting ischemia-induced angiogenesis.

Nrf2-deficient mice, on the other hand, showed an exaggerated angiogenic response 14 days after LPAL compared with their CD1 wild-type counterparts. CD1 mice demonstrated a less robust angiogenic phenotype than C57Bl/6 mice as indicated by left lung blood flow \((0.74\% \text{ cardiac output} \pm 0.12; n = 12)\). However, the Nrf2-deficient mice showed increased angiogenesis with blood flow averaging 1.53% \((\pm 0.31, n = 9)\) of cardiac output \((P = 0.03)\). This significant 107% increase further suggests the importance of oxidants/antioxidants in regulating ischemia-induced angiogenesis \((P = 0.017)\).

To determine whether the changes observed in blood flow were associated with early differences in ROS production, GSH/GSSG was measured in NAC-treated mice and in Nrf2-deficient mice 4 h after LPAL as an indicator of increased ROS levels. This time point was selected because of the significant changes observed in the initial time course evaluation. Additionally, because we confirmed there were no time-dependent changes in sham lungs \((P = 0.26)\), GSH/GSSG values for NAC-treated mice and Nrf2−/− mice were compared with strain-specific sham lungs \((0.5 h)\) within each replicate assay. Figure 7 shows the fold-change in GSH/GSSG in NAC-treated C57Bl/6 mice, CD1 mice, and Nrf2−/− mice \((P = 0.017)\) on a CD1 background.
Significant reductions in GSH/GSSG relative to sham were observed 4 h after LPAL in wild-type mice and in Nrf2−/− mice compared with CD1 shams (P = 0.001; n = 6–7 mice/group). The fold change in GSH/GSSG of NAC-treated mice and CD1 mice did not differ from a value of 1, i.e., their strain-specific sham values. Thus, in these two mouse strains, on average, lower GSH/GSSG ratios 4 h after LPAL were associated with greater neovascularization as measured by left lung perfusion (Figs. 5 and 6).

**DISCUSSION**

Numerous models of angiogenesis have been associated with ischemia and the inherent tissue hypoxia that results from lack of perfusion. These models have helped define crucial angiogenic mechanisms and growth factors essential for neovascularization. Models of ischemia-induced angiogenesis in the lung, such as the LPAL mouse model used in the current study, offer the unique advantage of studying angiogenesis induced by ischemia during ventilated normoxia. In this established model of ischemia-induced systemic neovascularization, we have shown, with the use of oxidant-sensitive fluorophores, an early and transient increase in ROS production following occlusion of the left pulmonary artery. To address whether changes in the oxidant response to LPAL altered angiogenesis, we studied mice treated with the antioxidant NAC and mice lacking the antioxidant transcription factor Nrf2. We found that neovascularization was decreased when early ROS release was limited. On the other hand, when ROS release was increased due to decreased antioxidants, neovascularization was enhanced. Collectively, these results suggest that ROS play a
central role in initiating the sequelae of events that leads to systemic neovascularization during lung ischemia.

The overall goals of the present study were to measure changes in the oxidant status of the lung early after the onset of pulmonary ischemia and link them with subsequent angiogenesis. The experiments presented technical challenges due to the inherent nature of the in vivo model with no perfusion and the expected short half-life of ROS. Consequently, several approaches were employed to capture information on ROS levels and lung redox status, which provided complementary information. Sham lungs provided relevant tissue comparisons for all measurements. Results examining lung tissue stained for DHE demonstrated trends toward a transient increase in ROS production as early as 1 h after the onset of ischemia and a return to sham control level by 24 h after LPAL (Fig. 2). Furthermore, examination of the lung sections demonstrated that pulmonary vessels as well as the capillary network showed prominent fluorescent staining in addition to some intravascular and parenchymal cells. When quantifying fluorescence intensity, however, these measurements were sufficiently variable that trends were not statistically significant. Subsequent experiments using the two other methods to assess redox status included same-day sham lungs to use for reference comparisons. Also, based on the results using DHE, we applied the second technique for determining ROS at an earlier time point after LPAL (30 min). Although loading lung cells with H2DCF-DA in vivo would have been preferable, due to the inability to perfuse the lung during ischemia, we studied sham and experimental lungs ex vivo. This approach has been used previously to study ex vivo ROS levels in the lung (26), as well as muscle (5), kidney (18), and brain (11). Our results confirm enhanced ROS production in lungs after LPAL compared with time-matched sham control lungs (Fig. 3).

In the third method, we assessed the redox potential of lung tissue at time points after LPAL. GSH, through a cascade of detoxification mechanisms, neutralizes ROS to form GSSG. The ratio of GSH to GSSG is therefore frequently used as a dynamic indicator of tissue response to ROS generation. In this study, the GSH/GSSG ratio was significantly reduced at the
4-h time point in C57Bl/6 mice and the Nrf2−/− mice after LPAL but not in lungs after sham surgery, or in NAC-treated mice or CD1 mice after LPAL. The time course of change of this ratio is consistent with early changes in ROS. Furthermore, the levels are, on average, predictive of the magnitude of neovascularization. Thus, the three methods used provide complementary information that there are measurable, transient increases in ROS release in the lung following cessation of pulmonary perfusion. Furthermore, as shown by blood flow determination, this increase in ROS is associated with the magnitude of systemic neovascularization.

The cellular sources for ROS production in the lung during ischemia are not completely known. However, the endothelium lining the pulmonary vasculature likely provides the greatest source. In the current study, DHE staining showed consistent pulmonary vascular ROS production. Furthermore, studies by Al-Mehdi and colleagues (1, 2), using an isolated perfused lung preparation, showed the earliest change (1 min) following cessation of pulmonary flow was depolarization of the endothelial cell membrane followed by ROS generation as indicated by intravital imaging of subpleural microvascular endothelial cells. These cells are known to express the Nox2 subunit of NADPH oxidase, a major ROS-releasing enzyme, and are thought to be the first affected by ischemia, by sensing the lack of shear stress associated with stopping blood flow (17).

However, numerous trapped inflammatory cells within the lung after LPAL could also contribute to the ROS signal, and Fig. 2B shows that other cells besides pulmonary endothelium likely contribute to the measured ROS. Others have shown in a hindlimb ischemia model that recruited inflammatory cells were the source of ROS production seen at 3 days, and the neovascular endothelial cells were responsible for upregulated ROS at 7 days postischemia (33). Further studies are needed to determine which cells are predominantly responsible for ROS release in this model. Additionally, mechanisms accounting for the reversal of the ROS signal by 24 h after the onset of ischemia require further investigation.

How the early release of ROS after the onset of pulmonary ischemia initiates an in vivo process that results subsequently in systemic angiogenesis is not clear. However, that the two are linked was confirmed in the present study when we intervened with NAC treatment and when Nrf2−/− mice were studied. Functional angiogenesis, as assessed by systemic perfusion to the left lung, was predictably altered based on a decrease or increase in oxidant burden. A much earlier marker of angiogenesis would be preferable; however, functional perfusion measured 14 days after LPAL has provided a reliable method to quantify the magnitude of neovascularization. Although we believe it to be unlikely, small changes in vasoreactivity of the new vasculature could contribute to the measured increase (vasodilation) and decrease (vasoconstriction) in angiogenesis. Historical approaches also might be used at earlier time points after the onset of ischemia. However, these require the ability to differentiate existing pulmonary vessels from new systemic vessels. Currently, we know of no labels that can differentiate new from existing components/cells within the lung.

However, the physiological importance of ROS is consistent with a large body of literature implicating oxidant regulation of angiogenesis (21). In addition to in vitro evidence showing the direct effect of ROS on endothelial cell migration, proliferation, and tube formation (31, 43), the use of many in vivo models of angiogenesis, associated with tumorogenesis or following ischemia, have further confirmed the importance of ROS (3, 15, 33, 35). Despite the strong evidence of ROS involvement, the exact mechanisms by which ROS promote the angiogenic response seem to be stimulus and environment specific and are not fully resolved. Although the mechanisms by which ROS activate a proangiogenic series of events were not studied, we speculate, based on the previous work of others. Turning on angiogenesis seems to be mainly under the regulation of two master switches, namely the transcription factors HIF-1α and NF-κB (32). HIF-1α is activated in hypoxic environments and in the presence of growth factors via the PI3-kinase-AKT and MAPK-ERK pathways to mediate angiogenesis, mainly via the upregulation of VEGF expression (32). As for NF-κB, it is also under strict regulation and could become activated by the same signaling pathways as HIF-1α, in addition to a change in the redox status of the tissue, due to ROS production (32). NF-κB translocation to the nucleus could subsequently promote genes responsible for cell proliferation and angiogenesis, such as the CXC chemokines (22). Given that prior work in this model shows no evidence for changes in HIF-1α and VEGF, yet shows early upregulation of the ELR+ CXC chemokines, it is more likely that the latter NF-κB pathway is more important in promoting ischemia-induced angiogenesis in this model (30).

Thus, in summary, we have shown an early increase in ROS in the ischemic lung. Inhibition of ROS release or enhancement
of ROS production lead to respective decreases or increases in systemic angiogenesis. Results of this study suggest that early ROS release initiates a series of events that lead to lung neovascularization.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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