Evidence for extracellular superoxide dismutase as a mediator of hemorrhage-induced lung injury

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Bowler, Russell P., John Arcaroli, Edward Abraham, Manisha Patel, Ling-Yi Chang, and James D. Crapo. Evidence for extracellular superoxide dismutase as a mediator of hemorrhage-induced lung injury. Am J Physiol Lung Cell Mol Physiol 284: L680–L687, 2003. First published January 10, 2003; 10.1152/ajplung.00191.2002.—Hemorrhage is often associated with severe acute lung injury (35). The ischemia and reperfusion that occur with hemorrhage result in production of reactive oxygen species (ROS) that are thought to cause some of the lung injury that follows hemorrhage (25). ROS directly injure lung proteins, lipids, and DNA, but may also potentiate lung injury by recruiting and activating leukocytes. ROS-mediated recruitment of leukocytes occurs by induction of adhesion molecules such as P-selectin (4). ROS also cause activation of transcription factors cyclic adenosine 5'-monophosphate response element binding protein and nuclear factor (NF)-κB that potentiate the release of proinflammatory cytokines (1, 20).

Superoxide is one of the reactive oxygen species thought to mediate lung injury after hemorrhage. For instance, inhibition of xanthine oxidase, which produces superoxide, attenuates hemorrhage-induced lung injury (25, 39, 40, 45). The family of superoxide dismutase (SOD) enzymes is a major enzymatic pathway that lowers superoxide concentrations. Exogenously administered, SOD improves blood pressure (42) and survival (36, 45, 46) after hemorrhage. Despite many studies that implicate a role for superoxide in hemorrhage-induced lung injury, the role of extracellular (EC)-SOD has not been defined.

Three observations suggest that EC-SOD may be particularly important in mediating hemorrhage-induced lung injury. First, EC-SOD is abundant in the lung and immunolocalizes to the alveolar septum and blood vessels (13, 30). Second, EC-SOD is upregulated by inflammatory cytokines that are activated during hemorrhagic shock (24, 44). Third, overexpression of EC-SOD can protect the lungs from hemorrhage-induced injury (7). Several questions remain unanswered. For instance, it is not known whether hemorrhage-induced lung injury is worse in EC-SOD-deficient mice, nor is it known whether SOD mimetics are capable of attenuating hemorrhage-induced lung injury.

To answer these questions, we have used two different techniques to manipulate superoxide dismutase activity in a mouse model of hemorrhage. First, the effects of hemorrhage-induced lung injury in EC-SOD knockout mice were studied. Second, the SOD mimetic manganese(III) mesotetakis (di-N-ethylimidazole) porphyrin (AEOL 10150) was administered to mice before hemorrhage to determine whether this class of antioxidant reduces hemorrhage-induced lung injury. AEOL 10150 has potent in vitro SOD activity and can protect lipids, proteins, and DNA from in vitro oxidative damage (33). Metalloporphyrin SOD mimetics augment natural antioxidant defenses and have been shown to attenuate lung injury in animal models of pulmonary fibrosis (31), paraquat toxicity (11), and stroke-induced brain injury (23). In the present experiments, we found that EC-SOD-deficient mice had increased neutrophil accumulation and hemorrhage-induced lung injury. Although AEOL 10150 pretreatment reduced markers of oxidative stress, it did not appear to effect neutrophil accumulation.
MATERIALS AND METHODS

Reagents. The SOD mimic AEOL 10150 [manganese(III)] mesotetrakis(di-N-ethylimidazolyl) porphyrin] was provided by Incara Pharmaceuticals (Research Triangle Park, NC). AEOL 10150 (Fig. 1) has a +5 charge with a SOD activity of ~8,500 U/mg and catalase activity of ~1% of purified bovine catalase (wt/wt basis). All other reagents were supplied by Sigma Chemical (St. Louis, MO) unless otherwise noted.

Animals. All experiments were conducted in accordance with institutional review board-approved protocols. The derivation of EC-SOD knockout mice has been previously described and has been shown not to result in induction of other SOD or antioxidant enzymes (10). Mice were bred into a C57BL/6 strain (Harlan, Indianapolis, IN) for >10 generations. Max-Bax testing (Charles River Laboratories, Wilmington, MA) revealed 100% homology to the C57BL/6 strain.

Genotyping. Mouse tail DNA was obtained after overnight digestion at 55°C in 400-ug/ml proteinase K. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1 ratio, saturated with Tris buffer, pH 8.0) and then precipitated from the aqueous phase with one volume of 7.5 M ammonium acetate plus two volumes of 100% ethanol. After being centrifuged for 5 min at 9,000 g, the pellet was washed with 70% ethanol and air-dried. DNA was resuspended in sterile H2O and then used for PCR with the primers amplifying both an 805-bp region of the mouse EC-SOD gene (forward CAG CCA TGT TGG CCT TCT TGT TCT A and reverse GGC GCC TGG TGT TGG CCT TCT TGT TCT A) and a 510-bp amplification product, AGA CAT CTA TGC GT) or a 510-bp amplification product, AGA CAT CTA TGC GT). PCR products were imaged after 2% agarose electrophoresis. The EC-SOD phenotype was confirmed in the lung by demonstrating the absence of EC-SOD by immunoblotting as previously described (7).

Hemorrhage model. Mice were pretreated with either saline or AEOL 10150 (24 mg/kg) in saline by subcutaneous injection 2 h before hemorrhage. Subcutaneous dosing results in maximum lung and serum concentrations after 2 h (unpublished observations communicated by Dr. Brian Day, Denver, CO). Hemorrhage was performed as previously described (3). Thirty percent of the blood volume, calculated by weight (0.55 ml/20-g mouse), was removed by cardiac puncture. Mice were allowed to recover and were then killed 1 h after hemorrhage.

Histopathology. Mouse lungs were inflation fixed with 10% formaldehyde at 20 cmH2O pressure and embedded in paraffin. Sections (6 μM) were stained with hematoxylin and eosin. With the use of a Nikon light microscope with a ×40 objective, the entire lung was imaged and qualitatively assessed for inflammation by an investigator blinded to the experimental group.

Acnotinase and fumarase activity. The acnotinase and fumarase activity of lung homogenates was measured as previously described (34). In brief, the assay measures acnotinase activity spectrophotometrically by monitoring the formation of cis-aconitase from isocitrate at 240 nm in 50 mM Tris-HCl (pH 7.4) containing 0.6 mM MnCl2 and 20 mM isocitrate at 25°C. Fumarase activity was measured by monitoring the increase in absorbance at 240 nm at 25°C in a 1-ml reaction mixture containing 30 mM potassium phosphate, 0.1 mM EDTA, and 5 mM L-malate (pH 7.4).

Myeloperoxidase. Myeloperoxidase (MPO) activity was assayed using a modification of Anderson et al. (5) and Parsey et al. (32). A lobe of the lung from each animal was homogenized for 30 s in 1.5 ml of 20 mM potassium phosphate, pH 7.4, and centrifuged at 4°C for 30 min at 40,000 g. The pellet was resuspended in 1.5 ml of 40 mM potassium phosphate, pH 6.0, containing 0.5% hexacyclotrimethyl ammonium bromide, sonicated for 90 s, incubated at 60°C for 2 h, and centrifuged. The supernatant was assayed for peroxidase activity and corrected to lung weight.

EMSA analysis of NF-κB. Nuclear extracts were prepared as previously described (19, 21). Activation of the transcriptional factor NF-κB was determined by EMSA analysis (26, 39, 40).

Determination of lipid peroxidation. The lipid fraction was extracted from homogenized tissue using a 1:1 ratio of chloroform and methanol, partially purified by solid phase extraction and then derivatized to the pentafluorobenzyl ester trimethylsilyl ethers. F2-isoprostanes were measured by negative ion chemical ionization gas chromatography/mass spectrometry analysis as described by Waugh and colleagues (48).

AEOL 10150 quantitation. Serum concentrations of AEOL 10150 were determined using high-performance liquid chromatography with electrochemical detection (18). For AEOL 10150 lung concentrations, one lobe of the lung was homogenized in water. Half of the lung homogenate was used for bicinchoninic acid protein concentration (Pierce), and half was used for the AEOL 10150 assay with the following modifications: standards were made by spiking wild-type serum or lung homogenates with known concentrations of AEOL 10150. Retention time peaks of 13.6 min from the −400-mV electrode were used for calibration curves and unknowns.

Statistical analysis. A one-way analysis of variance was used to determine whether the means were significantly different (P < 0.05). If means were significantly different, a Tukey-Kramer multiple group comparison test was used to compare individual groups. SE of the mean was indicated for each value by a bar. All values were calculated using Graph-
Table 1. AEOL 10150 drug levels after a single 24 mg/kg SQ dose

<table>
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<tr>
<th></th>
<th>Wild-type</th>
<th>EC-SOD KO</th>
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<tr>
<td>Serum 2 h after AEOL 10150 (at time of hemorrhage), µg/ml</td>
<td>4.06 ± 0.31</td>
<td>3.60 ± 0.88</td>
</tr>
<tr>
<td>Lungs 3 h after AEOL 10150 without hemorrhage, µg/lungs</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.04</td>
</tr>
<tr>
<td>Lung 3 h after AEOL 10150, 1 h after hemorrhage, µg/lungs</td>
<td>0.29 ± 0.09*</td>
<td>0.21 ± 0.03*</td>
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Values are means ± SE. *P = not significant for differences between wild-type and extracellular superoxide dismutase (EC-SOD) knockout (KO) for all 3 measurements. *P < 0.001 for difference between drug levels in mice that had no hemorrhage vs. hemorrhage.

RESULTS

AEOL 10150 concentrations. After a single subcutaneous injection, concentrations of AEOL in the serum and lung were similar for wild-type and EC-SOD lungs (Table 1). There was a significant increase in AEOL 10150 in the lungs of hemorrhaged mice compared with unhemorrhaged mice in both the wild-type (4.8 ± 1.5-fold increase; P < 0.001) and EC-SOD-deficient (3.5 ± 0.5-fold increase; P < 0.001) mice.

Hemorrhage-induced lung lipid peroxidation. Hemorrhage has been shown to cause oxidative damage to lipids as measured by lipid peroxidation (9). ROS-specific lipid peroxidation can be detected by measuring isoprostanes. F₂-isoprostanes are free radical catalyzed prostaglandin isomers that increase in tissue during oxidative stress (6) and have previously been shown to be elevated in hemorrhage-induced lung injury (7). To determine the role of EC-SOD in modulating such oxidant-induced lung injury, we measured lung 8-epi-F₂-isoprostanes in wild-type and EC-SOD-deficient mice before and after hemorrhage (Fig. 2). In the wild-type mice, lung 8-epi-F₂-isoprostanes increased from 60 ± 6 ng/g of protein in unmanipulated mice to 91 ± 5 ng/g of protein after hemorrhage (P < 0.05). In EC-SOD-deficient mice, there was a significantly larger increase in lung 8-epi-F₂-isoprostanes (from 64 ± 2 ng/g of protein in unmanipulated mice to 107 ± 3 ng/g of protein after hemorrhage; P < 0.01).

To determine whether a SOD mimetic could decrease the lung lipid peroxidation, mice were pretreated with 24 mg/kg of AEOL 10150 2 h before hemorrhage. Pre-
treatment with AEOL 10150 attenuated hemorrhage-induced lipid peroxidation in both wild-type (52 ± 9% vs. 18 ± 10%; P < 0.05) and EC-SOD-deficient mice (79 ± 5% vs. 46 ± 21%; P < 0.05).

Hemorrhage-induced inactivation of aconitase. On the basis of previous reports describing EC-SOD as a primarily extracellular antioxidant, we suspected that EC-SOD deficiency would have minimal impact on hemorrhage-induced intracellular oxidative stress. Additionally, although the cellular distribution of AEOL 10150 is not known, we suspected that its +5 charge would limit its efficacy as an in vivo intracellular antioxidant. To assess intracellular oxidative stress, we measured aconitase activity per gram of protein in lung homogenates (Fig. 3). Aconitase is an intracellular iron-sulfur-containing dehydratase that is sensitive to superoxide-mediated inactivation (14). Compared with the control group, hemorrhage resulted in a 45 ± 7% decrease in lung aconitase activity of the wild-type mice (P < 0.01) and a 58 ± 11% decrease in the EC-SOD-deficient mice (P < 0.05). The decreases in aconitase activity in wild-type mice were not statistically different from the decreases in the EC-SOD-deficient mice. Additionally, the decreases in aconitase activity after pretreatment with AEOL 10150 were not statistically different in either wild-type or EC-SOD-deficient mice. This study was not powered to detect small differences in aconitase activity (<20%), thus it cannot be excluded that EC-SOD-deficient mice have slightly more intracellular oxidative stress compared with that of wild-type mice.

To confirm that oxidative stress was likely the cause of decreased aconitase activity, we measured the activity of fumarase, an intracellular enzyme that is not known to be sensitive to superoxide. There were no significant differences in fumarase activity after hemorrhage in either the wild-type (17 ± 20% increase) or the EC-SOD-deficient mice (10 ± 20% decrease).

Neutrophilic inflammation after hemorrhage. Because hemorrhage-induced neutrophil accumulation mediates much of hemorrhage-induced lung injury (2), we suspected that EC-SOD-deficient mice would have increased lung neutrophil accumulation, and mice treated with AEOL 10150 would have decreased neutrophil accumulation. With the use of light microscopy, we found that hemorrhage results in neutrophil accumulation in both wild-type and EC-SOD-deficient mouse lungs (Fig. 4). One hour after hemorrhage, the measured aconitase activity per gram of protein in lung homogenates (Fig. 3). Aconitase is an intracellular iron-sulfur-containing dehydratase that is sensitive to superoxide-mediated inactivation (14). Compared with the control group, hemorrhage resulted in a 45 ± 7% decrease in lung aconitase activity of the wild-type mice (P < 0.01) and a 58 ± 11% decrease in the EC-SOD-deficient mice (P < 0.05). The decreases in aconitase activity in wild-type mice were not statistically different from the decreases in the EC-SOD-deficient mice. Additionally, the decreases in aconitase activity after pretreatment with AEOL 10150 were not statistically different in either wild-type or EC-SOD-deficient mice. This study was not powered to detect small differences in aconitase activity (<20%), thus it cannot be excluded that EC-SOD-deficient mice have slightly more intracellular oxidative stress compared with that of wild-type mice.

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Neutrophilic inflammation after hemorrhage. Because hemorrhage-induced neutrophil accumulation mediates much of hemorrhage-induced lung injury (2), we suspected that EC-SOD-deficient mice would have increased lung neutrophil accumulation, and mice pre-treated with AEOL 10150 would have decreased neutrophil accumulation. With the use of light microscopy, we found that hemorrhage results in neutrophil accumulation in both wild-type and EC-SOD-deficient mouse lungs (Fig. 4). One hour after hemorrhage, the
EC-SOD-deficient mouse lungs had qualitatively more neutrophilic infiltration in the lung interstitium compared with wild-type mouse lungs. Pretreatment with AEOL 10150 did not appear to change histological evidence of neutrophil accumulation in either the wild-type or EC-SOD-deficient mouse lungs (data not shown).

To confirm and quantify the role of EC-SOD in modulating neutrophil accumulation, we measured MPO in wild-type and EC-SOD-deficient mice 1 h after hemorrhage (Fig. 5). Hemorrhage resulted in an increase in MPO from 1.10 ± 0.35 U/g lung to 8.85 ± 1.02 U/g lung (P < 0.05), similar to previously reported increases in C57BL/6 mice (2, 7). In mice lacking EC-SOD, there was an increase in neutrophil accumulation after hemorrhage, from 1.69 ± 0.52 U/g lung to 34.12 ± 6.26 U/g lung. There was a significantly larger increase in MPO activity in EC-SOD-deficient mice compared with wild-type mice (34.12 ± 6.26 vs. 8.85 ± 1.02 U/g lung; P < 0.001).

Because transcription of neutrophil inflammatory genes may also mediate hemorrhage-induced lung injury, we measured NF-κB activity. Neutrophils are the primary source of activated NF-κB in hemorrhage-induced lung injury (40). To confirm a role for EC-SOD in modulating hemorrhage-induced NF-κB activation, we measured lung NF-κB activity in wild-type and EC-SOD-deficient mice after hemorrhage (Fig. 6). Hemorrhage resulted in a 1.97 ± 0.38-fold increase in NF-κB activity compared with controls (P < 0.05), similar to previously reported increases (7). EC-SOD-deficient mice demonstrated larger increases in NF-κB activation (2.86 ± 0.40-fold) compared with unhemorrhaged controls (P < 0.01). Thus EC-SOD-deficient mice had a 1.45-fold increase in NF-κB activity compared with similarly hemorrhaged wild-type mice (P < 0.05). Pretreatment with AEOL 10150 2 h before hemorrhage attenuated the increase in NF-κB activity in both the wild-type (P < 0.05) and EC-SOD-deficient mice (P < 0.05; Fig. 6B). These results suggest that one possible mechanism by which EC-SOD and AEOL 10150 protect the lung after hemorrhage is by attenuating the induction of inflammatory genes.

**DISCUSSION**

Ischemia and reperfusion cause oxidative stress that results in tissue injury in a variety of diseases such as myocardial infarction, stroke, and ischemic bowel. Unlike these organ-specific diseases, hemorrhage results in whole body ischemia with production of systemic ROS (22). Much of the lung injury that occurs after hemorrhage is mediated by superoxide (25, 28, 36, 42, 45, 46). Although it is well established that the lung is sensitive to oxidative stress-mediated injury to proteins, lipids, and DNA (6, 16, 27, 49), the mechanisms by which this injury occurs remain unclear. Previous studies have suggested that decreasing superoxide, either by inhibiting superoxide-generating enzymes such as xanthine oxidase (40) or increasing SOD enzymatic activity (36), can lead to a reduction of hemorrhage-induced lung injury. However, these studies did not distinguish between the types of SODs. The present experiments suggest that EC-SOD deficiency worsens hemorrhage-induced lung injury.

There are two possible mechanisms by which EC-SOD activity might reduce the severity of hemorrhage-induced lung injury. First, EC-SOD could prevent direct ROS-mediated injury through its actions as an antioxidant. Second, EC-SOD could act indirectly by attenuating recruitment or activation of inflammatory cells to the lung. The data presented in these experiments suggest that both mechanisms may be important after hemorrhage.

In the present experiments, hemorrhage resulted in a rapid, moderate increase in pulmonary lipid peroxidation (F_2-isoprostanes) that was significantly increased in EC-SOD-deficient mice. The SOD mimetic AEOL 10150 also significantly decreased lipid peroxidation in both the wild-type and EC-SOD-deficient mice. F_2-isoprostanes are prostaglandin-like compounds formed during ROS-mediated attack of arachi-

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**Fig. 5.** EC-SOD deficiency worsens hemorrhage-induced increases in lung myeloperoxidase (MPO) activity. Lung MPO activity was measured in wild-type control mice (n = 7) or mice that had 30% of blood volume removed 1 h previously (n = 10) and EC-SOD-deficient (KO) mice controls (n = 7) or mice that had 30% of blood volume removed 1 h previously (n = 8). Compared with control, hemorrhage increased MPO activity in both the wild-type (*P < 0.05) and EC-SOD KO mice (***P < 0.001). The EC-SOD KO mice had a significantly greater increase in MPO activity compared with the wild-type mice (†P < 0.001).
EC-SOD IN HEMORRHAGE

Fig. 6. EC-SOD deficiency increases and AEOL 10150 decreases hemorrhage-induced NF-kB activity in lungs. A: NF-kB activity in the wild-type control lungs (n = 8) increased after hemorrhage (n = 8; * P < 0.05). NF-kB activity in the EC-SOD KO control lungs (n = 7) also increased after hemorrhage (n = 5; * P < 0.05). There was a greater increase in activity in the EC-SOD KO compared with the wild type († P < 0.01). B: pretreating the mice with AEOL 10150 (24 mg/kg) 2 h before hemorrhage attenuated the increase in NF-kB activity in both the wild-type (n = 4; ‡ P < 0.05) and EC-SOD KO mice (n = 7; ‡ P < 0.05). C: a representative experiment is shown for both the wild-type and EC-SOD KO mice. CON, control; HEM, hemorrhage.

donic acid. Hemorrhage from aortic rupture results in elevated plasma F₂-isoprostanes in humans (22) and increased F₂-isoprostanes in the lungs of rats (9). Overexpression of EC-SOD in the lung has previously been shown to reduce increases in F₂-isoprostanes after hemorrhage (7). Although hemorrhage resulted in an inactivation of lung aconitase, in wild-type mice the amount of inactivation was not statistically different from either the EC-SOD-deficient mice or mice pre-
treated with AEOL 10150. This suggests that the protective effect of EC-SOD and AEOL 10150 is not due to a large reduction in intracellular superoxide concentration; however, there are alternative explanations that could account for these findings. First, both EC-SOD and the SOD mimetic AEOL 10150 might be attenuating the recruitment of cells that produce large amounts of ROS (e.g., neutrophils). Second, EC-SOD and AEOL 10150 could be inhibiting the in vivo enzymatic production of ROS by an undescribed mechanism. Neither EC-SOD nor AEOL 10150 is known to inhibit ROS-producing enzymes; however, ROS have been implicated in the recruitment and activation of neutrophils. For instance, ROS are capable of potentiating neutrophil recruitment to the lung (47) by increasing neutrophil adhesion to endothelial cells (29), presumably by enhancing the vascular expression of neutrophil adhesion molecules such as P-selectin (4) and E-selectin (37). Additionally, superoxide potentiates lung damage by inducing neutrophils to secrete proinflammatory cytokines such as interleukin-1β, macrophage inflammatory protein-2, and tumor necrosis factor-α (32, 39, 40).

The relationship between hemorrhage-induced superoxide production and neutrophil-mediated lung injury is complex and likely involves steps that include both the recruitment and activation of neutrophils. In the EC-SOD-deficient mice, there was increased recruitment of neutrophils both histologically and by measuring the activity of the neutrophil enzyme MPO. After hemorrhage, the EC-SOD-deficient mice also had increased NF-kB activation in the lungs, suggesting that EC-SOD plays a role in both recruitment and activation of neutrophils. Although pretreatment with the SOD mimetic AEOL 10150 attenuated isoprostane formation and NF-kB activity, there was only a mild decrease in these markers of oxidative stress, and there was no reduction in neutrophil recruitment to the lungs. This suggests that AEOL 10150 can attenuate markers of oxidative stress but may not reduce lung injury as measured by lung neutrophil accumulation. Several explanations could account for the differences between EC-SOD and AEOL 10150. First, AEOL 10150, but not EC-SOD, has slight catalase activity; however, catalase has previously been shown not to effect neutrophil activation in hemorrhage (41). Second, the distribution of EC-SOD and AEOL 10150 may be different. For instance, EC-SOD is highly expressed in pulmonary vasculature and thus may play a role in superoxide-mediated neutrophil recruitment to the lung. There are no data regarding the distribution of AEOL 10150; however, in this study there was a tripling of the lung concentration of AEOL 10150 after hemorrhage.

Although it is well accepted that EC-SOD attenuates lung damage during oxidative stress (7, 8, 10), the duality of EC-SOD as an antioxidant and anti-inflammatory enzyme remains controversial. Overexpression of EC-SOD has previously been shown to blunt the inflammatory response in lung from hyperoxia (12), oil fly ash (15), and influenza (43). EC-SOD-deficient mice...
show increased neutrophil inflammation after ozone exposure (17) but do not have statistically significant increased lung inflammation after lipopolysaccharide and yzmosan exposure (38). Thus the majority of pre-
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