Reactive Oxygen Species Scavengers Attenuate Endotoxin-induced Impairment of Hypoxic Pulmonary Vasoconstriction in Mice

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Background: Sepsis and endotoxemia attenuate hypoxic pulmonary vasoconstriction (HPV), thereby impairing systemic oxygenation. Reactive oxygen species (ROS) are implicated in the pathogenesis of sepsis-induced lung injury. The authors investigated whether treatment with scavengers of ROS prevents impairment of HPV in mice challenged with endotoxin.

Methods: The pulmonary vascular response to hypoxia in mice was studied after left mainstem bronchus occlusion (LMBO) was performed. Intraperitoneal administration of N-acetylcysteine or EUK-8, a scavenger of active oxygen species, was performed in endotoxin-challenged mice. The effects of these antioxidants were measured in lung tissues.

Results: The LMBO increased left pulmonary vascular resistance by 56 ± 24% in saline-challenged control mice but was only 53 ± 12% (P < 0.05) in endotoxin-challenged mice. Intraperitoneal administration of N-acetylcysteine or EUK-8 increased the hypoxic pulmonary vasoconstriction by 58 ± 6% and 68 ± 10%, respectively, in endotoxin-challenged mice. Intraperitoneal administration of ROS scavengers 1 h after endotoxin challenge was equally effective but required lower doses than systemic treatment. Admiration of the ROS scavengers 2 h after endotoxin challenge did not restore HPV.

Conclusions: Administration of N-acetylcysteine or EUK-8 increased hypoxic pulmonary vasoconstriction in mice and prevented the impairment of HPV after LMBO. Early therapy with ROS scavengers, either systemically or by inhalation, may provide a means to preserve HPV in sepsis-associated acute lung injury.

Hypoxic pulmonary vasoconstriction (HPV) is characterized by vasoconstriction of pulmonary vessels in poorly ventilated or atelectatic hypoxic lung regions, thus optimizing the matching of ventilation and perfusion and preserving systemic oxygenation. Despite intensive investigation, a comprehensive understanding of the cellular mechanisms that underlie HPV remains elusive.

Hypoxic pulmonary vasoconstriction is markedly impaired in patients with clinical sepsis. Experimental endotoxemia has been shown to impair HPV in several animal species. Various inflammatory mediators, including prostaglandins, thromboxanes, platelet-activating factor, and cytokines, have all been implicated in the sepsis-induced attenuation of HPV.

Sepsis and endotoxemia induce the production of reactive oxygen species (ROS). ROS have been suggested to play a role in the induction of many proinflammatory cytokines and mediators in producing the acute inflammatory responses associated with sepsis. Whereas a variety of endogenous substances can protect cells from exposure to ROS, endotoxemia and sepsis are associated with a reduced endogenous antioxidant capacity, thereby resulting in an oxidant-antioxidant imbalance. Along these lines, a number of studies have demonstrated beneficial effects with antioxidant therapy, including improved systemic oxygenation and lung compliance in animal models of acute lung injury and in patients with acute respiratory distress syndrome (ARDS).

Given the prominent putative roles of ROS in acute lung injury, we hypothesized that ROS produced by endotoxin-induced inflammation would contribute to the impairment of HPV. To test this hypothesis, we studied whether administration of two different ROS scavengers, N-acetylcysteine and EUK-8, can prevent the loss of HPV in endotoxin-challenged mice. N-acetylcysteine is a precursor of glutathione and scavengers of HO, H$_2$O$_2$, HOCl, and peroxynitrite. EUK-8, a novel nonselective manganese-containing ROS scavenger, has been shown to neutralize O$_2$ and H$_2$O$_2$, and to scavenge peroxynitrite and prevent protein tyrosine nitrosylation. We measured pulmonary blood flow redistribution in response to unilateral hypoxia produced by left mainstem bronchus occlusion (LMBO) in anesthetized mice 24 h after endotoxin challenge. We also studied the effects of lung-selective delivery of these antioxidants. We report that administration of either N-acetylcysteine or EUK-8 can prevent the impairment of HPV in endotoxin-challenged mice.

Methods

Animals and Experimental Groups

After institutional approval by the Massachusetts General Hospital Subcommittee on Research Animal Care, we studied SV129/B6F1 mice (the F1 generation prog-
Table 1. Experimental Groups

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<tr>
<th>Group</th>
<th>Challenge and Treatment</th>
<th>Number of Mice</th>
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<tr>
<td>1</td>
<td>Measurements of HPV 22 h after lipopolysaccharide or saline challenge and 21 h after intraperitoneal administration of scavengers</td>
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<td>Saline given intraperitoneally at t = 0 h and at t = 1 h</td>
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<td>Saline given intraperitoneally at t = 0 h, and 500 mg/kg N-acetylcysteine given intraperitoneally at t = 1 h</td>
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<td>Saline given intraperitoneally at t = 0 h, and 4 mg/kg EUK-8 given intraperitoneally at t = 1 h</td>
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<td>Lipopolysaccharide given intraperitoneally at t = 0 h, and 500 mg/kg N-acetylcysteine given intraperitoneally at t = 1 h</td>
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<td>2</td>
<td>Measurements of HPV 22 h after lipopolysaccharide or saline challenge and 21 h after intratracheal administration of scavengers</td>
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<td>Measurements of HPV 22 h after lipopolysaccharide or saline challenge and immediately after intravenous administration of EUK-8</td>
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<td>Saline given intraperitoneally at t = 0 h, and 30 mg/kg EUK-8 given intravenously at t = 22 h</td>
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<td>Lipopolysaccharide given intraperitoneally at t = 0 h, and vehicle given intravenously at t = 22 h</td>
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HPV = hypoxic pulmonary vasoconstriction.

eny of SV129 and C57 BL/6 mice obtained from Jackson Laboratory, Bar Harbor, ME) of both sexes weighing 18–30 g and aged 2–4 months (table 1). Endotoxin-challenged mice received 10 mg/kg body weight (bw) *Escherichia coli* 0111:B4 lipopolysaccharide (Difco Laboratories, Detroit, MI) intraperitoneally. Saline-challenged control mice received an intraperitoneal administration of equal amount of normal saline (0.01 ml/g bw).

**Drug Administration**

N-acetylcysteine (Sigma Chemical, St. Louis, MO) was dissolved in saline solution, and the pH of the solution was adjusted to 7.3 immediately before administration. EUK-8 (supplied by Eukarion, Bedford, MA) was dissolved in distilled water. Both solutions were passed through a 0.2-μm filter. Intraperitoneal drug administration was performed in awake mice 1 h after endotoxin or saline challenge. Intraperitoneal drug doses were based on the results of our previous study. For intratracheal administration of drugs, mice were anesthetized with an intraperitoneal injection of 0.1 mg/g bw ketamine and 6 μg/g bw xylazine, and the trachea was intubated with a 20-gauge Angiocath 1 h after endotoxin or saline challenge. Correct endotracheal tube positioning was confirmed by transient volume-controlled ventilation with observation of chest movements and monitoring of airway pressure. An intratracheal aerosolizer (IA-IC Microsprayer; Penn Century, Philadelphia, PA) was introduced into the endotracheal tube, and the study drug was aerosolized in a volume of 25 μl during spontaneous breathing. The mice were then extubated and allowed to recover.

**Hemodynamic Measurements**

Twenty-two hours after endotoxin or saline challenge, we anesthetized mice with 0.1 mg/g bw ketamine and 6 μg/g bw xylazine and surgically prepared them for hemodynamic study as previously described. This time point was chosen based on pilot studies. Systemic artery pressure (SAP), pulmonary artery pressure (PAP), and left pulmonary artery blood flow (QLPA) were continuously recorded. To estimate the left lung pulmonary vascular resistance (LPVR), the inferior vena cava (IVC) was partially occluded with a circumferential 5–0 silk ligature to transiently reduce cardiac output until QLPA was reduced by approximately 50%. To calculate LPVR, the flow–pressure relationship was constructed by plotting approximately 50 consecutive digitized data points of linear parts of PAP and QLPA tracings during transient IVC occlusions. The best-fit line that describes the relationship between PAP and QLPA was obtained by linear regression analysis. The slope of this best-fit line represents incremental LPVR during the IVC occlusion.

Left lung alveolar hypoxia was induced by reversibly occluding the LMBO with a microvascular clip. Complete collapse of the left lung was visually observed within about 1 min and confirmed by transient overinflation of the right lung. Transient IVC occlusion was repeated three times before and during LMBO in each animal, and the average of three slopes each was reported as LPVR at baseline and during LMBO. The per-
cent increase in LPVR induced by LMBO (ΔLPVR) was obtained by calculating the percent change of the mean value of the slope in each mouse.25

Myeloperoxidase Assay
Polymorphonuclear leukocyte (PMN) infiltration into lung tissue was estimated by measuring myeloperoxidase (MPO) activity in endotoxin-challenged mice treated with intratracheal EUK-8 or saline solution as previously described.26 We chose to study MPO activity 7 h after endotoxin challenge because MPO activity was greatest 7 h after endotoxin challenge in a pilot study. We did not study effects of N-acetylcytstein on lung MPO activity because N-acetylcytstein inhibits MPO activity in vitro (data not shown).

RNA Blot Hybridization
RNA was extracted from the lungs of endotoxin-challenged mice treated with intratracheal EUK-8 or saline solution using the guanidium isothiocyanate-cesium chloride method. RNA, 15 μg, was fractionated in formaldehyde-agarose gels containing ethidium bromide, photographed, and transferred to nylon membranes. Membranes were hybridized with a 32P-labeled 0.3-kb mouse-inducible NO synthase-2 (NOS2) cDNA probe and subsequently with a 15-fold excess of a 32P-labeled oligonucleotide complementary to rat 18S RNA. Autoradiograms and photographs were scanned using a color image scanner (Seiko Epson, Shimosuwa Japan) and NIH Image 1.44 software (NIH, Bethesda, MD). To estimate pulmonary NOS2 mRNA concentrations, the NOS2 mRNA:18S RNA ratio was determined by dividing the absorbance corresponding to the NOS2 cDNA probe hybridization by the absorbance corresponding to the 18S RNA probe hybridization on autoradiographs.25 Levels were measured 7 h after the intraperitoneal administration of endotoxin or saline solution followed by aerosolized saline or EUK-8. This time point was chosen because previous studies had shown that pulmonary NOS2 mRNA levels were maximal 6-8 h after mice were challenged with endotoxin.

Statistical Analysis
The LMBO-induced increase in LPVR was expressed as the percentage increase from the baseline LPVR before LMBO. Differences between groups were determined using a two-way analysis of variance (ANOVA) with repeated measures. When significant differences were detected by ANOVA, a post hoc Newman–Keuls test was used (Statistica for Windows, version 5.0; StatSoft, Tulsa, OK). A P value < 0.05 was considered a significant difference. All data are expressed as mean ± SD.

Results
Effects of Unilateral Alveolar Hypoxia on Pulmonary Blood Flow
Intraperitoneal administration of N-acetylcytstein or EUK-8 partially preserves HPV 22 h after an endotoxin challenge. LMBO increased LPVR (106 ± 24%) without affecting SAP and PAP in saline-challenged mice (table 2, fig. 1A). The hemodynamic effects of LMBO were similar in saline-challenged mice treated with intraperitoneal N-acetylcytstein or EUK-8. In contrast, LMBO increased
LPVR in endotoxin-challenged mice by only 23 ± 12% (P < 0.001 vs. saline-challenged mice). In mice treated with 30 mg/kg intraperitoneal EUK-8 1 h after endotoxin challenge, the LMBO-induced increase in LPVR was partially preserved (68 ± 10%; P < 0.01 vs. endotoxin-challenged mice, P < 0.05 vs. saline-challenged mice).

Intratracheal Administration of Aerosolized N-acetylcysteine or EUK-8 Partially Preserves HPV 22 h after an Endotoxin Challenge

Left mainstem bronchus occlusion 22 h after aerosolized saline administration markedly increased LPVR (109 ± 22%) without affecting SAP and PAP (table 3, fig. 1B). These findings were similar to those observed in saline-challenged mice treated with aerosolized N-acetylcysteine or EUK-8. In endotoxin-challenged mice, there was a significant impairment of LPVR increase in response to LMBO (22 ± 10%; P < 0.001 vs. saline-challenged mice). In mice treated with 100 mg/kg N-acetylcysteine via aerosol 1 h after endotoxin challenge, the LMBO-induced increase in LPVR was partially preserved (67 ± 13%; P < 0.05 vs. endotoxin-challenged mice, P < 0.05 vs. saline-challenged mice). Similarly, in mice treated with 4 mg/kg EUK-8 via aerosol, the LMBO-induced increase in LPVR was partially preserved (69 ± 12%; P < 0.01 vs. endotoxin-challenged mice, P < 0.05 vs. saline-challenged mice).

Intravenous Bolus Administration of EUK-8 Does Not Restore HPV when Given 22 h after Endotoxin Challenge

When given as an intravenous bolus to saline-challenged mice during LMBO, 30 mg/kg EUK-8 acutely decreased LPVR reversing HPV (from 201 ± 74 to 94 ± 19 mmHg · ml⁻¹ · min⁻¹ · g bw⁻¹; P < 0.05; fig. 2). Intravenous EUK-8 also tended to decrease the total pulmonary vascular resistance (from 26 ± 3 to 21 ± 3 mmHg · ml⁻¹ · min⁻¹ · g bw⁻¹; P = 0.07) and total systemic vascular resistance (from 75 ± 10 to 60 ± 13 mmHg · ml⁻¹ · min⁻¹ · g bw⁻¹; P = 0.08) in saline-challenged mice. Bolus intravenous administration of EUK-8 to mice 22 h after endotoxin challenge during LMBO did not affect LPVR (from 139 ± 75 to 89 ± 21 mmHg · ml⁻¹ · min⁻¹ · g bw⁻¹; P = NS), demonstrating that EUK-8 did not restore HPV (fig. 2). Bolus intravenous administration of vehicle of EUK-8 did not affect LPVR in mice challenged with saline or endotoxin 22 h earlier (fig. 2).

Myeloperoxidase Activity

Lung MPO activity was more than 10-fold higher in endotoxin-challenged mice than in saline-challenged mice (P < 0.001; fig. 3). Treatment with aerosolized EUK-8 attenuated the increase in lung MPO activity in endotoxin-challenged mice (P < 0.01 vs. endotoxin-challenged mice).
We measured pulmonary NOS2 mRNA levels to assess if the beneficial effects of EUK-8 on HPV were mediated by a reduction in the induction of NOS2 gene expression by endotoxin. NOS2 mRNA levels were not detectable in mouse lungs after saline challenge. In contrast, NOS2 gene expression was induced in the lungs of all mice challenged with endotoxin whether or not they were also treated with EUK-8. The NOS2 mRNA:18S ribosomal RNA ratio was not different between EUK-8-treated and -untreated endotoxin-challenged mice.

**Discussion**

In the current study, we have demonstrated that early treatment with a single dose of the antioxidants N-acetylcysteine, or EUK-8 intratracheally 1 h after saline challenge. Mice in the endotoxin group received intratracheal saline (lipopolysaccharide) 22 h before hemodynamic measurements. Control mice received saline, LPS (lipopolysaccharide)-acetylcysteine (lipopolysaccharide + saline), N-acetylcysteine (lipopolysaccharide + N-acetylcysteine) or EUK-8 (lipopolysaccharide + EUK-8) 1 h after endotoxin challenge.

All values at baseline and LMBO were compared between saline-challenged mice and endotoxin-challenged mice: * P < 0.05, † P < 0.01, ‡ P < 0.001 versus corresponding control; § P < 0.05 versus endotoxin. The effect of LMBO on each parameter was analyzed in each group: || P < 0.05, ‖ P < 0.01, ‡‡ P < 0.001 versus baseline.

HR = heart rate; SAP = mean systemic arterial pressure; PAP = mean pulmonary artery pressure; QLPA = flow through left pulmonary artery; bw = body weight; LPVR = left pulmonary vascular resistance.

**Pulmonary NOS2 Gene Expression after Endotoxin Challenge**

We measured pulmonary NOS2 mRNA levels to assess if the beneficial effects of EUK-8 on HPV were mediated by a reduction in the induction of NOS2 gene expression by endotoxin. NOS2 mRNA levels were not detectable in mouse lungs after saline challenge. In contrast, NOS2 gene expression was induced in the lungs of all mice challenged with endotoxin whether or not they were also treated with EUK-8. The NOS2 mRNA:18S ribosomal RNA ratio was not different between EUK-8-treated and -untreated endotoxin-challenged mice.

**Discussion**

In the current study, we have demonstrated that early treatment with a single dose of the antioxidants N-acetyl-...
tylcysteine or EUK-8 in mice partially attenuates the endotoxin-induced impairment of HPV (fig. 1), whereas treatment with EUK-8 22 h after endotoxin challenge did not restore HPV. Aerosolization of these ROS scavengers 1 h after endotoxin challenge confers a similar degree of protection but requires lower drug doses than systemic administration. The protective effects of EUK-8 may be partly mediated by inhibiting pulmonary leukocyte sequestration because EUK-8 decreased the endotoxin-induced increase of MPO activity measured in the lung 7 h after endotoxin challenge. However, EUK-8 did not affect endotoxin-induced NOS2 gene expression, suggesting that the protective effects of EUK-8 were not mediated by decreasing NO levels in the lung.

It has been suggested that changes in cytosolic redox state act as signals for HPV. However, the precise roles of ROS in HPV signaling are controversial with some investigators reporting that hypoxia reduced ROS production and others reporting that hypoxia increased ROS production. Similarly, the administration of antioxidants has been shown to either enhance or inhibit HPV. In the current study, the bolus intravenous administration of EUK-8 to healthy anesthetized mice acutely induced nonspecific pulmonary and systemic vasodilation impairing HPV for more than 10 min (fig. 2). Although our results may support an important role of ROS in vasomotor regulation, our results are limited because we only tested one dose of a drug that scavenges many species of ROS. Potential roles of ROS in HPV signaling warrant further investigation.

Although ROS appear to modulate pulmonary vascular tone, it is known that the excessive production of ROS damages cellular constituents and impairs pulmonary contractile vascular function. Therefore, in the presence of an oxidative stress such as in endotoxemia, antioxidants may inhibit the actions of ROS either by opposing their vasomodulatory function (vasoconstriction or vasodilation) or by preventing the cytotoxic effects of ROS. The beneficial effects of antioxidants in this study appear to be mediated by the latter mechanism because only early (1 h after endotoxin) but not late (22 h after endotoxin) administration of EUK-8 partially preserved HPV. The observation that intravenous administration of EUK-8 induced nonspecific vasodilation 2 h after endotoxin challenge does not support the possibility that EUK-8 acutely preserved HPV by vasoconstriction. Our findings are consistent with those of Archer et al., who reported that pretreatment with exogenous catalase and superoxide dismutase preserved HPV in isolated-perfused rat lungs in the presence of oxidative stress generated by xanthine and xanthine oxidase. We have recently reported that early, but not late, treatment with antioxidants prevented endotoxin-induced impairment of pulmonary vasodilation to inhaled NO. Taken together, these observations suggest that ROS impair pulmonary vascular function early in the course of en-
traperitoneal doses of 500 mg/kg N-acetylcysteine or 30 mg/kg EUK-8 were required to achieve a similar degree of protection, the total effective dose ratio of intratracheal versus intraperitoneal administration of N-acetylcysteine and EUK-8 to prevent impairment of HPV would be 1:5.6, and 1:8.3 (i.e., only 18% and 12% of the intraperitoneal doses of N-acetylcysteine and EUK-8, respectively, are necessary to produce the same protection when intratracheally administered).

In conclusion, a single dose of N-acetylcysteine and EUK-8 when administered early in endotoxemia partially prevented the impairment of HPV measured 22 h after endotoxin challenge. Administration of these antioxidants via the intratracheal route was as effective as intraperitoneal administration, but lower drug doses are required. The efficacy of a single dose of antioxidant agents in this murine model of endotoxin-induced impairment of HPV underlines the importance of oxidative stress in the pathologic process leading to impaired HPV. Antioxidant therapy early in the course of endotoxemia may prove to be a useful strategy for preserving HPV and systemic arterial oxygenation in patients with acute lung injury.

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References