Anesthesiology 2002; 97:1227–33

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

Hemanth A. Baboolal, M.D., Fumito Ichinose, M.D.,† Roman Ullrich, M.D.,* Noriko Kawai, M.D.,* Kenneth D. Bloch, M.D.,‡ Warren M. Zapol, M.D.§*

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 vasoconstrictor response to left mainstem bronchus occlusion (LMBO) was studied in anesthetized mice 22 h after an intraperitoneal challenge with saline solution or 10 mg/kg *Escherichia coli* endotoxin. In some mice, challenge with saline solution or endotoxin was followed after 1 h with intraperitoneal or intratracheal administration of the ROS scavengers *N*-acetylcysteine or EUK-8. Myeloperoxidase activity and nitric oxide synthase-2 gene expression were measured in lung tissues.**

Results: **The LMBO increased left pulmonary vascular resistance by 106 ± 24% in saline-challenged control mice but by only 23 ± 12% ($P < 0.05$) in endotoxin-challenged mice. Intraperitoneal administration of *N*-acetylcysteine or EUK-8 1 h after endotoxin challenge attenuated the endotoxin-induced impairment of HPV (58 ± 6% and 68 ± 10%, respectively; both $P < 0.05$ *vs.* endotoxin-challenged mice). Intratracheal administration of ROS scavengers 1 h after endotoxin challenge was equally effective but required lower doses than systemic treatment. Administration of the ROS scavengers 22 h after endotoxin challenge did not restore HPV.**

Conclusions: **Administration of *N*-acetylcysteine or EUK-8 1 h after endotoxin challenge in mice 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.[1]

Hypoxic pulmonary vasoconstriction is markedly impaired in patients with clinical sepsis.[2,3] Experimental endotoxemia has been shown to impair HPV in several animal species.[4,5] Various inflammatory mediators, including prostaglandins,[6] thromboxanes,[7] platelet-activating factor,[4] and cytokines,[8] have all been implicated in the sepsis-induced attenuation of HPV.

Sepsis and endotoxemia induce the production of reactive oxygen species (ROS).[9,10] ROS have been suggested to play a role in the induction of many proinflammatory cytokines and mediators important in producing the acute inflammatory responses associated with sepsis.[11] 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.[12] 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[13–16] and in patients with acute respiratory distress syndrome (ARDS).[12,17,18]

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, given early after an endotoxin challenge would prevent the loss of HPV in endotoxin-challenged mice. *N*-acetylcysteine is a precursor of glutathione and scavenges · HO, H_2O_2, HOCl, and peroxynitrite.[19–21] EUK-8, a novel nonselective manganese-containing ROS scavenger, has been shown to neutralize O_2^- and H_2O_2, and to scavenge peroxynitrite and prevent protein tyrosine nitrosylation.[16,22] We measured pulmonary blood flow redistribution in response to unilateral hypoxia produced by left mainstem bronchus occlusion (LMBO) in anesthetized mice 22 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-

* Research Fellow, † Assistant Professor of Anesthesia, § Reginald Jenny Professor of Anesthesia, Department of Anesthesia and Critical Care, ‡ Associate Professor of Medicine, Cardiovascular Research Center of the Department of Medicine. Drs. Baboolal and Ichinose have contributed equally to this investigation.

Received from the Department of Anesthesia and Critical Care, and the Cardiovascular Research Center and Cardiology Division of the Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts. Submitted for publication January 22, 2002. Accepted for publication July 15, 2002. Supported by US Public Health Service grants HL-42397 and HL-57172, National Institutes of Health, Bethesda, Maryland. Dr. Ullrich was supported by the Max Kade Foundation, Vienna, Austria. Dr. Bloch was an Established Investigator of the American Heart Association, Dallas, Texas. Presented in part at the annual meeting of the American Society of Anesthesiologists, New Orleans, Louisiana, October 13–17, 2001.

Address reprint requests to Dr. Ichinose: Department of Anesthesia and Critical Care, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114. Address electronic mail to: ichinose@etherdome.mgh.harvard.edu.

Table 1. Experimental Groups

Group	Challenge and Treatment	Number of Mice
1	Measurements of HPV 22 h after lipopolysaccharide or saline challenge and 21 h after intraperitoneal administration of scavengers	
	Saline given intraperitoneally at t = 0 h and at t = 1 h	7
	Saline given intraperitoneally at t = 0 h, and 500 mg/kg *N*-acetylcysteine given intraperitoneally at t = 1 h	4
	Saline given intraperitoneally at t = 0 h, and 30 mg/kg EUK-8 given intraperitoneally at t = 1 h	4
	Lipopolysaccharide given intraperitoneally at t = 0 h, and saline given intraperitoneally at t = 1 h	8
	Lipopolysaccharide given intraperitoneally at t = 0 h, and 500 mg/kg *N*-acetylcysteine given intraperitoneally at t = 1 h	7
	Lipopolysaccharide given intraperitoneally at t = 0 h, and 30 mg/kg EUK-8 given intraperitoneally at t = 1 h	6
2	Measurements of HPV 22 h after lipopolysaccharide or saline challenge and 21 h after intratracheal administration of scavengers	
	Saline given intraperitoneally at t = 0 h, and saline given intratracheally at t = 1 h	6
	Saline given intraperitoneally at t = 0 h, and 100 mg/kg *N*-acetylcysteine given intratracheally at t = 1 h	4
	Saline given intraperitoneally at t = 0 h, and 4 mg/kg EUK-8 given intratracheally at t = 1 h	4
	Lipopolysaccharide given intraperitoneally at t = 0 h, and saline given intratracheally at t = 1 h	6
	Lipopolysaccharide given intraperitoneally at t = 0 h, and 100 mg/kg *N*-acetylcysteine given intratracheally at t = 1 h	6
	Lipopolysaccharide given intraperitoneally at t = 0 h, and 4 mg/kg EUK-8 given intratracheally at t = 1 h	6
3	Measurements of HPV 22 h after lipopolysaccharide or saline challenge and immediately after intravenous administration of EUK-8	
	Saline given intraperitoneally at t = 0 h, and 30 mg/kg EUK-8 given intravenously at t = 22 h	3
	Saline given intraperitoneally at t = 0 h, and vehicle given intravenously at t = 22 h	3
	Lipopolysaccharide given intraperitoneally at t = 0 h, and 30 mg/kg EUK-8 given intravenously at t = 22 h	4
	Lipopolysaccharide given intraperitoneally at t = 0 h, and vehicle given intravenously at t = 22 h	3

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.[23] 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.[24] 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-

Table 2. Hemodynamic Measurements: Intraperitoneal Scavenger Group

Challenge (intraperitoneal; t = 0 h) / Treatment (intraperitoneal; t = 1 h)		Saline + Saline (n = 8)	Saline + *N*-acetylcysteine (n = 4)	Saline + EUK-8 (n = 4)	Lipopolysaccharide + Saline (n = 7)	Lipopolysaccharide + *N*-acetylcysteine (n = 7)	Lipopolysaccharide + EUK-8 (n = 6)
HR (beats/min)	Baseline	544 ± 57	610 ± 61	624 ± 31	541 ± 39	475 ± 58†	558 ± 48
	LMBO	548 ± 76	570 ± 66	610 ± 40	542 ± 47	489 ± 59	562 ± 50
SAP (mmHg)	Baseline	86 ± 14	72 ± 5	80 ± 8	70 ± 19	97 ± 20	75 ± 12
	LMBO	79 ± 17	72 ± 7	83 ± 4	67 ± 19	94 ± 20	74 ± 4
PAP (mmHg)	Baseline	14 ± 2	14 ± 3	13 ± 3	15 ± 2	14 ± 2	13 ± 1§
	LMBO	15 ± 3	15 ± 3	14 ± 4	16 ± 3	14 ± 3	14 ± 1#
QLPA ($\mu l \cdot min^{-1} \cdot g^{-1}$ bw)	Baseline	84 ± 18	85 ± 9	87 ± 10	81 ± 14	95 ± 15	102 ± 15
	LMBO	45 ± 8††	44 ± 5**	45 ± 4**	62 ± 9†**	62 ± 12††	65 ± 14††
ΔQLPA (%)		45 ± 5	49 ± 6	48 ± 5	23 ± 8‡	35 ± 3*§	37 ± 6*‖
LPVR ($mmHg \cdot ml^{-1} \cdot min^{-1} \cdot g^{-1}$ bw)	Baseline	82 ± 21	92 ± 27	88 ± 13	131 ± 21†	114 ± 20	97 ± 14‖
	LMBO	165 ± 26††	178 ± 43**	185 ± 22††	169 ± 41	187 ± 36††	162 ± 26††
ΔLPVR (%)		106 ± 24	95 ± 26	111 ± 10	23 ± 12‡	58 ± 6*§	68 ± 10*‖

Hemodynamic parameters before (baseline) and 5 min after left mainstem bronchus occlusion (LMBO). Mice were challenged with intraperitoneal saline or endotoxin (lipopolysaccharide) 22 h before hemodynamic measurements. Control mice received saline, *N*-acetylcysteine, or EUK-8 intraperitoneally 1 h after saline challenge. Mice in the endotoxin group received intraperitoneal saline (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 control groups and endotoxin-challenged groups: * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ *versus* corresponding control; § $P < 0.05$, ‖ $P < 0.01$ *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.

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*-acetylcysteine on lung MPO activity because *N*-acetylcysteine 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 guanidine 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 ^{32}P-labeled 0.3-kb mouse-inducible NO synthase-2 (NOS2) cDNA probe and subsequently with a 15-fold excess of a ^{32}P-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*-acetylcysteine 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*-acetylcysteine or EUK-8. In contrast, LMBO increased

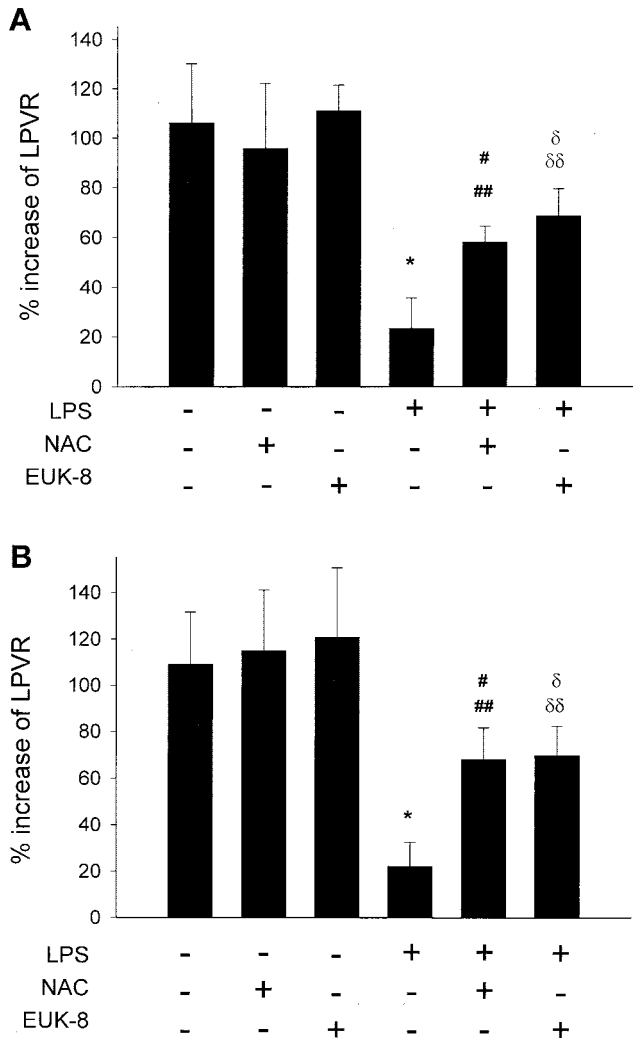

Fig. 1. (*A*) Percent increase of left lung pulmonary vascular resistance (LPVR) in response to left mainstem bronchus occlusion (LMBO) in mice challenged with intraperitoneal saline solution (LPS−) or endotoxin (LPS+) 22 h earlier. Some mice in both groups further received intraperitoneal saline, *N*-acetylcysteine (NAC), or EUK-8 1 h after challenge (see table 1 for experimental groups). *$P < 0.001$ *versus* saline-challenged mice; #$P < 0.05$ *versus* endotoxin-challenged mice; ##$P < 0.05$ *versus* saline-challenged mice treated with *N*-acetylcysteine; δ$P < 0.01$ *versus* endotoxin-challenged mice; δδ$P < 0.05$ *versus* saline-challenged mice treated with EUK-8. (*B*) Percent increase of LPVR in response to LMBO in mice challenged with intraperitoneal saline (LPS−) or endotoxin (LPS+) 22 h earlier. Some mice in both groups further received intratracheal aerosolized saline, *N*-acetylcysteine, or EUK-8 1 h after challenge (see table 1 for experimental groups). *$P < 0.001$ *versus* saline-challenged mice; #$P < 0.05$ *versus* endotoxin-challenged mice; ##$P < 0.05$ *versus* saline-challenged mice treated with *N*-acetylcysteine; δ$P < 0.05$ *versus* endotoxin-challenged mice; δδ$P < 0.05$ *versus* saline-challenged mice treated with EUK-8.

LPVR in endotoxin-challenged mice by only 23 ± 12% ($P < 0.001$ *vs.* saline-challenged mice). In mice treated with 500 mg/kg intraperitoneal *N*-acetylcysteine 1 h after endotoxin challenge, the LMBO-induced increase in LPVR was partially preserved (58 ± 6%; $P < 0.05$ *vs.* endotoxin-challenged mice, $P < 0.05$ *vs.* saline-challenged mice). Similarly, in mice treated with 30 mg/kg intraperitoneal EUK-8 1 h after endotoxin challenge, the LMBO-induced increase in LPVR was also 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).

Table 3. Hemodynamic Measurements: Intratracheal Scavenger Group

Challenge (intraperitoneal; t = 0 h) Treatment (intratracheal; t = 1 h)		Saline (n = 6)	Saline + N-acetylcysteine (n = 4)	Saline + EUK-8 (n = 4)	Lipopolysaccharide + Saline (n = 8)	Lipopolysaccharide + N-acetylcysteine (n = 7)	Lipopolysaccharide + EUK-8 (n = 6)
HR (beats/min)	Baseline	568 ± 67	620 ± 107	540 ± 29	549 ± 38	513 ± 43	569 ± 59
	LMBO	555 ± 64	602 ± 85	535 ± 23	556 ± 40	508 ± 69	581 ± 68
SAP (mmHg)	Baseline	72 ± 15	66 ± 11	67 ± 5	79 ± 11	93 ± 24	91 ± 20
	LMBO	73 ± 14	64 ± 12	66 ± 6	75 ± 11	87 ± 22	83 ± 23‖
PAP (mmHg)	Baseline	13 ± 2	13 ± 1	12 ± 1	15 ± 3	12 ± 1	13 ± 2
	LMBO	13 ± 2	13 ± 1	13 ± 1‖	15 ± 3	13 ± 1	14 ± 2‖
QLPA ($\mu l \cdot min^{-1} \cdot g^{-1}$ bw)	Baseline	93 ± 9	103 ± 5	102 ± 4	95 ± 11	91 ± 25	92 ± 19
	LMBO	42 ± 3**	45 ± 3**	43 ± 3**	76 ± 10‡#	56 ± 19	60 ± 15**
ΔQLPA (%)		54 ± 4	55 ± 3	55 ± 4	21 ± 4‡	38 ± 9†§	35 ± 4†§
LPVR ($mmHg \cdot ml^{-1} \cdot min^{-1} \cdot g^{-1}$ bw)	Baseline	90 ± 7	91 ± 12	97 ± 11	113 ± 42	91 ± 13	100 ± 23
	LMBO	180 ± 36#	194 ± 23#	212±52‖	136 ± 41	154 ± 29**	168 ± 31**
ΔLPVR (%)		109 ± 22	114 ± 26	120 ± 29	22 ± 10‡	67 ± 13*§	69 ± 12*§

Hemodynamic parameters before (baseline) and 5 min after left mainstem bronchus occlusion (LMBO). Mice were challenged with intraperitoneal saline or endotoxin (lipopolysaccharide) 22 h before hemodynamic measurements. Control mice received saline, *N*-acetylcysteine, or EUK-8 intratracheally 1 h after saline challenge. Mice in the endotoxin group received intratracheal saline (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.

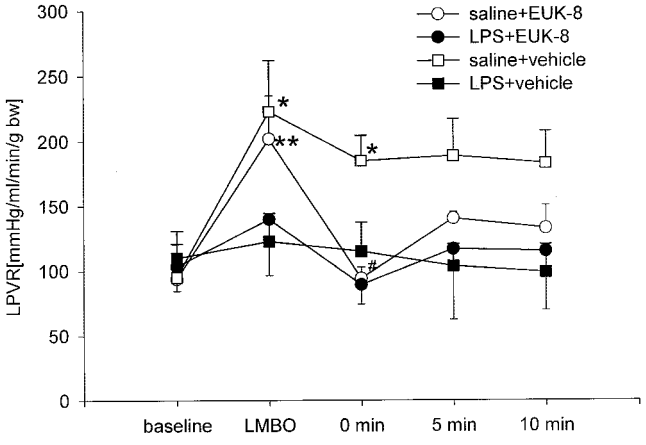

Fig. 2. Left lung pulmonary vascular resistance (LPVR) change in response to bolus intravenous administration of EUK-8 or vehicle in saline-challenged mice (saline + EUK-8 and saline + vehicle, respectively) and endotoxin-challenged mice (LPS + EUK-8 and LPS + vehicle, respectively). Mice were challenged with endotoxin or saline 22 h earlier, and EUK-8 or vehicle was given during left mainstem bronchus occlusion (LMBO); LPVR was measured immediately after the administration of EUK-8 or vehicle (0 min), 5 min later (5 min), and 10 min later (10 min). *$P < 0.05$ *versus* LPVR of saline plus vehicle at baseline, **$P < 0.05$ *versus* LPVR of saline plus EUK-8 at baseline; #$P < 0.01$ *versus* LPVR of saline–vehicle at 0 min; $P < 0.05$ *versus* LPVR of saline plus EUK-8 at LMBO.

Discussion

In the current study, we have demonstrated that early treatment with a single dose of the antioxidants *N*-ace-

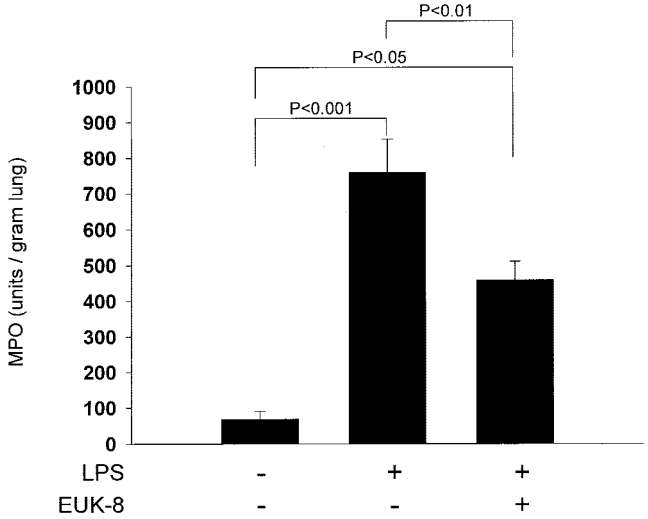

Fig. 3. Myeloperoxide (MPO) levels in lung tissue (units per gram lung) harvested from saline-challenged mice treated with aerosolized saline (LPS−, EUK-8−, n = 3), endotoxin-challenged mice treated with aerosolized saline (LPS+, EUK-8−, n = 4), or endotoxin-challenged mice treated with EUK-8 (LPS+, EUK-8+, n = 4). Mice were challenged with intraperitoneal endotoxin or saline solution 7 h earlier, and EUK-8 or saline solution was administered by aerosol 1 h after endotoxin challenge.

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 1h 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 7h 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.[27] However, the precise roles of ROS in HPV signaling are controversial with some investigators reporting that hypoxia reduced ROS production[27–29] and others reporting that hypoxia increased ROS production.[30,31] Similarly, the administration of antioxidants has been shown to either enhance[32,33]or inhibit HPV.[30] 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,[34] it is also 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 22 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.,*[34] 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.[23] Taken together, these observations suggest that ROS impair pulmonary vascular function early in the course of endotoxemia, and antioxidant therapy can prevent this process when given early after endotoxin challenge.

Given the wide spectrum of antioxidant property of EUK-8,[22] it is tempting to hypothesize that protective effects of EUK-8 against endotoxin-induced impairment of HPV is mediated by inhibition of protein tyrosine nitration by peroxynitrite. However, we could not detect any nitrotyrosine by immunoblotting in the lung of mice that received a higher dose of endotoxin (50 mg/kg) in our recent study.[23] Therefore, our data do not support a major role for protein tyrosine nitrosylation in endotoxin-induced impairment of HPV.

Because EUK-8 decreased MPO activity in murine lung tissue 7 h after endotoxin challenge (fig. 3), it is possible that the protective effects of EUK-8 are partially caused by antiinflammatory effects. This observation is consistent with a previous report that showed pigs challenged with endotoxin and treated with EUK-8 had lower PMN levels in the bronchoalveolar lavage fluid than did pigs challenged with endotoxin alone.[16] Exposure to endotoxin stimulates cells to express NOS2.[35] We have previously reported that increased pulmonary NO levels (produced by NOS2 or inhaled at high levels from exogenous NO sources) are necessary to impair HPV in this murine sepsis model.[24] To learn if the protective effects of antioxidants on HPV are mediated by preventing the endotoxin-induced increase in pulmonary NO levels, we examined *NOS2* gene expression in endotoxin-challenged mice with and without EUK-8 treatment. We found that *NOS2* gene expression was markedly increased by endotoxin challenge but not affected by EUK-8 treatment, suggesting that the beneficial effects of EUK-8 were not mediated by inhibiting NOS2 induction. This is reminiscent of our recent findings that the protective effects of cysteinyl leukotriene receptor-1 (cysLT1) inhibition on endotoxin-induced impairment of murine HPV were also not mediated by reducing NO levels in the lung.[25] It has been reported that cysLT receptor activation leads to the generation of ROS.[36] In view of our current results, it is conceivable that cysLT1 inhibition decreased production of ROS and preserved HPV in mice after endotoxin challenge.[25]

Directed inhalational therapy of the lung has several well-established advantages over the oral and intravenous routes, including a lower incidence of systemic side effects, increased local efficacy of a smaller inhaled drug dose, and selective drug delivery to well-ventilated lung regions.[37] We found that the endotoxin-induced impairment of HPV could be partially prevented by intratracheal administration of antioxidants at a lower dose. The efficiency of the intratracheal aerosolizer we used for delivering drug to the lung has been estimated at 90%.[38] If we assume that 90% of the study drugs were delivered to the lungs, then the effective total dose delivered to the animals would have been 90 mg/kg of *N*-acetylcysteine and 3.6 mg/kg of EUK-8. Because in-

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.

The authors thank Eukarion, Bedford, Massachusetts, for donating EUK-8, and Yu Chiao Chang, Ph.D. (Instructor, Harvard Medical School, Boston, Massachusetts), for statistical advice.

References

1. Ward JP, Aaronson PI: Mechanisms of hypoxic pulmonary vasoconstriction: Can anyone be right? Respir Physiol 1999; 115:261–71
2. Dantzker DR, Brook CJ, Dehart P, Lynch JP, Weg JG: Ventilation-perfusion distributions in the adult respiratory distress syndrome. Am Rev Respir Dis 1979; 120:1039–52
3. Marshall BE, Hanson CW, Frasch F, Marshall C: Role of hypoxic pulmonary vasoconstriction in pulmonary gas exchange and blood flow distribution. 2. Pathophysiology. Intensive Care Med 1994; 20:379–89
4. Chang SW, Feddersen CO, Henson PM, Voelkel NF: Platelet-activating factor mediates hemodynamic changes and lung injury in endotoxin-treated rats. J Clin Invest 1987; 79:1498–509
5. Theissen JL, Loick HM, Curry BB, Traber LD, Herndon DN, Traber DL: Time course of hypoxic pulmonary vasoconstriction after endotoxin infusion in unanesthetized sheep. J Appl Physiol 1991; 70:2120–5
6. Weir EK, Mlczoch J, Reeves JT, Grover RF: Endotoxin and prevention of hypoxic pulmonary vasoconstriction. J Lab Clin Med 1976; 88:975–83
7. Hales CA, Sonne L, Peterson M, Kong D, Miller M, Watkins WD: Role of thromboxane and prostacyclin in pulmonary vasomotor changes after endotoxin in dogs. J Clin Invest 1981; 68:497–505
8. Stevens T, Morris K, McMurtry IF, Zamora M, Tucker A: Pulmonary and systemic vascular responsiveness to TNF-alpha in conscious rats. J Appl Physiol 1993; 74:1905–10
9. Van D, V, Eiserich JP, Shigenaga MK, Cross CE: Reactive nitrogen species and tyrosine nitration in the respiratory tract: Epiphenomena or a pathobiologic mechanism of disease? Am J Respir Crit Care Med 1999; 160:1–9
10. Chabot F, Mitchell JA, Gutteridge JM, Evans TW: Reactive oxygen species in acute lung injury. Eur Respir J 1998; 11:745–57
11. Blackwell TS, Blackwell TR, Holden EP, Christman BW, Christman JW: In vivo antioxidant treatment suppresses nuclear factor-kappa B activation and neutrophilic lung inflammation. J Immunol 1996; 157:1630–7
12. Spapen H, Zhang H, Demanet C, Vleminckx W, Vincent JL, Huyghens L: Does N-acetyl-L-cysteine influence cytokine response during early human septic shock? Chest 1998; 113: 1616–24
13. Bernard GR, Lucht WD, Niedermeyer ME, Snapper JR, Ogletree ML, Brigham KL: Effect of *N*-acetylcysteine on the pulmonary response to endotoxin in the awake sheep and upon in vitro granulocyte function. J Clin Invest 1984; 73:1772–84
14. Zhang H, Spapen H, Nguyen DN, Benlabed M, Buurman WA, Vincent JL: Protective effects of N-acetyl-L-cysteine in endotoxemia. Am J Physiol 1994; 266:H1746–54
15. Modig J, Sandin R: Haematological, physiological and survival data in a porcine model of adult respiratory distress syndrome induced by endotoxaemia. Effects of treatment with *N*-acetylcysteine. Acta Chir Scand 1988; 154:169–77
16. Gonzalez PK, Zhuang J, Doctrow SR, Malfroy B, Benson PF, Menconi MJ, Fink MP: EUK-8, a synthetic superoxide dismutase and catalase mimetic, ameliorates acute lung injury in endotoxemic swine. J Pharmacol Exp Ther 1995; 275:798–806
17. Suter PM, Domenighetti G, Schaller MD, Laverriere MC, Ritz R, Perret C: *N*-acetylcysteine enhances recovery from acute lung injury in man. A randomized, double-blind, placebo-controlled clinical study. Chest 1994; 105:190–4
18. Galley HF, Howdle PD, Walker BE, Webster NR: The effects of intravenous antioxidants in patients with septic shock. Free Radic Biol Med 1997; 23:768–74
19. Aruoma OI, Halliwell B, Hoey BM, Butler J: The antioxidant action of *N*-acetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med 1989; 6:593–7
20. Bernard GR: *N*-acetylcysteine in experimental and clinical acute lung injury. Am J Med 1991; 91:54S–9S
21. Foresti R, Sarathchandra P, Clark JE, Green CJ, Motterlini R: Peroxynitrite induces haem oxygenase-1 in vascular endothelial cells: A link to apoptosis. Biochem J 1999; 339(Pt 3):729–36
22. Jung C, Rong Y, Doctrow S, Baudry M, Malfroy B, Xu Z: Synthetic superoxide dismutase/catalase mimetics reduce oxidative stress and prolong survival in a mouse amyotrophic lateral sclerosis model. Neurosci Lett 2001; 304:157–60
23. Raveh Y, Ichinose F, Orbach P, Bloch KD, Zapol WM: Radical scavengers protect murine lungs from endotoxin-induced hyporesponsiveness to inhaled nitric oxide. ANESTHESIOLOGY 2002; 96:926–33
24. Ullrich R, Bloch KD, Ichinose F, Steudel W, Zapol WM: Hypoxic pulmonary blood flow redistribution and arterial oxygenation in endotoxin-challenged NOS2-deficient mice. J Clin Invest 1999; 104:1421–9
25. Ichinose F, Zapol WM, Sapirstein A, Ullrich R, Tager AM, Coggins K, Jones R, Bloch KD: Attenuation of hypoxic pulmonary vasoconstriction by endotoxemia requires 5-lipoxygenase in mice. Circ Res 2001; 88:832–8
26. Bergeron Y, Ouellet N, Deslauriers AM, Simard M, Olivier M, Bergeron MG: Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice. Infect Immun 1998; 66:912–22
27. Archer SL, Huang J, Henry T, Peterson D, Weir EK: A redox-based O2 sensor in rat pulmonary vasculature. Circ Res 1993; 73:1100–12
28. Mohazzab KM, Fayngersh RP, Kaminski PM, Wolin MS: Potential role of NADH oxidoreductase-derived reactive O2 species in calf pulmonary arterial PO2-elicited responses. Am J Physiol 1995; 269:L637–44
29. Mohazzab KM, Wolin MS: Sites of superoxide anion production detected by lucigenin in calf pulmonary artery smooth muscle. Am J Physiol 1994; 267:L815–22
30. Waypa GB, Chandel NS, Schumacker PT: Model for hypoxic pulmonary vasoconstriction involving mitochondrial oxygen sensing. Circ Res 2001; 88:1259–66
31. Marshall C, Mamary AJ, Verhoeven AJ, Marshall BE: Pulmonary artery NADPH-oxidase is activated in hypoxic pulmonary vasoconstriction. Am J Respir Cell Mol Biol 1996; 15:633–44
32. Reeve HL, Weir EK, Nelson DP, Peterson DA, Archer SL: Opposing effects of oxidants and antioxidants on K+ channel activity and tone in rat vascular tissue. Exp Physiol 1995; 80:825–34
33. Abdalla S, Will JA: Potentiation of the hypoxic contraction of guinea-pig isolated pulmonary arteries by two inhibitors of superoxide dismutase. Gen Pharmacol 1995; 26:785–92
34. Archer SL, Peterson D, Nelson DP, DeMaster EG, Kelly B, Eaton JW, Weir EK: Oxygen radicals and antioxidant enzymes alter pulmonary vascular reactivity in the rat lung. J Appl Physiol 1989; 66:102–11
35. Gillissen A, Nowak D: Characterization of *N*-acetylcysteine and ambroxol in anti-oxidant therapy. Respir Med 1998; 92:609–23
36. Woo CH, Lee ZW, Kim BC, Ha KS, Kim JH: Involvement of cytosolic phospholipase A2, and the subsequent release of arachidonic acid, in signalling by rac for the generation of intracellular reactive oxygen species in rat-2 fibroblasts. Biochem J 2000; 348(Pt 3):525–30
37. Newman SP: Aerosol deposition considerations in inhalation therapy. Chest 1985; 88:152S–60S
38. Beck SE, Laube BL, Adams R, Chesnut K, Flotte TR, Guggino WB: Deposition and distribution of aerosolized AAV vectors in the lungs of macaques. Ped Pulm 1999; 228(suppl 19):229