

Radical Scavengers Protect Murine Lungs from Endotoxin-induced Hyporesponsiveness to Inhaled Nitric Oxide

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Background: Sepsis is associated with an impaired pulmonary vasodilator response to inhaled nitric oxide (NO). A combination of NO and other inflammatory mediators appears to be responsible for endotoxin-induced pulmonary vascular hyporesponsiveness to inhaled NO. The authors investigated whether scavengers of reactive oxygen species could preserve inhaled NO responsiveness in endotoxin-challenged mice.

Methods: The vasorelaxation to inhaled NO was studied in isolated, perfused, and ventilated lungs obtained from mice 16 h after an intraperitoneal challenge with saline or 50 mg/kg *Escherichia coli* lipopolysaccharide. In some mice, challenge with saline or lipopolysaccharide was followed by intraperitoneal administration of *N*-acetylcysteine, dimethylthiourea, EUK-8, or polyethylene glycol-conjugated catalase.

Results: The pulmonary vasodilator response of U46619-pre-constricted isolated lungs to ventilation with 0.4, 4, and 40 ppm inhaled NO in lipopolysaccharide-challenged mice was reduced to 32, 43, and 60%, respectively, of that observed in saline-challenged mice ($P < 0.0001$). Responsiveness to inhaled NO was partially preserved in lipopolysaccharide-challenged mice treated with a single dose of *N*-acetylcysteine (150 or 500 mg/kg) or 20 U/g polyethylene glycol-conjugated catalase (all $P < 0.05$ vs. lipopolysaccharide alone). Responsiveness to inhaled NO was fully preserved by treatment with either dimethylthiourea, EUK-8, two doses of *N*-acetylcysteine (150 mg/kg administered 3.5 h apart), or 100 U/g polyethylene glycol-conjugated catalase (all $P < 0.01$ vs. lipopolysaccharide alone).

Conclusions: When administered to mice concurrently with lipopolysaccharide challenge, reactive oxygen species scavengers prevent impairment of pulmonary vasodilation to inhaled NO. Therapy with scavengers of reactive oxygen species may provide a means to preserve pulmonary vasodilation to inhaled NO in sepsis-associated acute lung injury.

LOW concentrations of inhaled nitric oxide (NO) gas is used to treat infants¹ and adults^{2,3} with hypoxemia or pulmonary hypertension caused by a variety of acute and chronic pulmonary diseases. However, 30–45% of patients with acute respiratory distress syndrome fail to

respond with selective pulmonary vasodilation to NO inhalation. Nonresponders are more frequent (approximately 60%) in patients with sepsis-associated acute respiratory distress syndrome.^{2,3} Impaired pulmonary vasorelaxation to both endothelium-dependent^{4,5} and -independent⁵ vasodilators was demonstrated in isolated pulmonary vessels obtained from acutely injured lungs. We previously reported that pulmonary vasorelaxation to inhaled NO was impaired in isolated, perfused, and ventilated lungs obtained from endotoxin-challenged rats^{6,7} and mice.⁸ Taken together, these clinical and laboratory observations suggest that endotoxemia and sepsis impair pulmonary vasodilation to inhaled NO.

Sepsis induces the abundant production of NO *via* increased expression of the inducible isoform of nitric oxide synthase (NOS2).⁹ Mice congenitally lacking the NOS2 gene demonstrate resistance to endotoxin-induced impairment of pulmonary responsiveness to inhaled NO.⁸ When supplied with exogenous sources of NO *via* breathing ambient NO after endotoxin challenge, NOS2-deficient mice demonstrate impaired pulmonary vasorelaxation to inhaled NO, comparable to that observed in wild-type mice.⁸ NO breathing alone in unchallenged wild-type or NOS2-deficient mice, however, did not alter the pulmonary responsiveness to inhaled NO.⁸ These findings suggest that high concentrations of NO, either endogenously produced or exogenously supplied, are necessary but are not sufficient to impair pulmonary vasorelaxation to inhaled NO after endotoxin challenge.

Sepsis and endotoxemia induce the cellular production of an excessive amount of reactive oxygen species (ROS).^{9,10} ROS have been implicated in the expression of many proinflammatory cytokines and mediators important in inducing acute inflammatory responses associated with sepsis.¹¹ The concurrent overproduction of ROS and NO is implicated in the pathophysiology of acute lung injury (ALI), possibly by producing the toxic reactive nitrogen species peroxynitrite.^{9,10} We therefore examined the role that ROS play in the endotoxin-induced impairment of pulmonary vasodilation to inhaled NO. *N*-acetylcysteine scavenges $\cdot\text{HO}$, H_2O_2 , hypochlorous acid,^{12,13} and peroxynitrite¹⁴ and decreases nitrotyrosine formation.¹⁵ Dimethylthiourea has been shown to react with $\cdot\text{HO}$, H_2O_2 , hypochlorous acid,¹⁶ and O_2^{-17} and prevents protein nitration by peroxynitrite.¹⁸ EUK-8, a novel nonselective manganese-containing ROS scavenger, has been shown to neutralize O_2^{-} and H_2O_2 .¹⁹ Related manganese-containing compounds have been shown to scavenge peroxynitrite²⁰ and prevent protein

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nitrotyrosylation.^{20,21} Superoxide dismutase (SOD) and catalase are selective O_2^- and H_2O_2 scavengers, respectively.¹⁰ We hypothesized that treatment with ROS scavengers could prevent or attenuate the endotoxin-induced impaired pulmonary vasodilation to inhaled NO.

We report that administration of scavengers of ROS and reactive nitrogen species to endotoxin-challenged mice prevents the impairment of pulmonary vascular responsiveness to inhaled NO.

Methods

Materials

N-nitro-L-arginine-methyl-ester, indomethacin, bovine serum albumin, dextran, dimethylthiourea, polyethylene glycol (PEG), PEG-conjugated SOD (PEG-SOD), and PEG-conjugated catalase (PEG-catalase) were obtained from Sigma Chemical Co. (St. Louis, MO), Hanks' balanced salt solution was obtained from GibcoBRL (Grand Island, NY), and U46619 was obtained from Cayman Chemicals (Ann Arbor, MI). EUK-8¹⁹ was generously provided by Eukarion Inc. (Bedford, MA). *N*-acetylcysteine was obtained from American Regent Laboratories Inc. (Shirley, NY), peroxyxynitrite was obtained from Upstate Biotechnology (Lake Placid, NY), *E. Coli* 0111:B4 lipopolysaccharide was obtained from Difco Laboratories (Detroit, MI), and NO gas was obtained from INO Therapeutics (Port Allen, LA).

Lung Isolation and Perfusion

The isolated mouse lung perfusion system was previously described.⁸ Briefly, after the mice were killed with pentobarbital sodium (200 mg/kg), they were ventilated *via* an endotracheal tube, the pulmonary artery and left atrium were cannulated, and the pulmonary artery pressure (PAP) and left atrial pressure were measured and recorded continuously. Vascular perfusion was initiated at a constant flow of $50 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. The perfusate consisted of Hanks balanced salt solution with 5% bovine serum albumin and 5% dextran. Indomethacin (30 μM final concentration) and *N*-nitro-L-arginine-methyl-ester (1 mM final concentration) were added to the perfusate to inhibit endogenous prostaglandin and NO synthesis, respectively.

Pulmonary Vasorelaxation to Inhaled Nitric Oxide

After stable pulmonary vasoconstriction was achieved with U46619, a thromboxane analog, a vasodilator dose-response curve to inhaled NO was obtained by sequentially ventilating the lungs with 0.4, 4, and 40 ppm NO.⁸ The vasorelaxation to inhaled NO (ΔPAP) was calculated as the reduction in PAP produced by inhaled NO (PAP pre-NO minus PAP after 5 min of NO inhalation) and expressed as a percentage of the increase in baseline PAP induced by infusing U46619.

RNA Blot Hybridization

RNA was extracted from lungs using the guanidine isothiocyanate-cesium chloride method,²² and 10 μg was fractionated in formaldehyde-agarose gels and transferred to nylon membranes. Membranes were hybridized with a ^{32}P -labeled 0.3-kb mouse *NOS2* cDNA probe.²³ Equal loading of RNA on gels was confirmed by staining 28S and 18S ribosomal RNA with ethidium bromide.

Pulmonary Nitrotyrosine Concentrations

Nitrotyrosine concentrations in mouse lungs were estimated using semiquantitative immunoblotting. Lungs were homogenized in Laemmli buffer,²⁴ boiled for 5 min, and centrifuged at 15,000g for 30 min. Soluble proteins (80 mg per lane) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters. Each gel was loaded with 0.5 μg of a nitrated bovine serum albumin standard (Upstate Biotechnology, Lake Placid, NY) to produce a positive control, with an additional positive control generated by pretreating the supernatant protein solution (1 mg/ml) with peroxyxynitrite (100 μl). Membranes were blocked at room temperature with 5% dry milk in phosphate-buffered saline for 30 min and incubated overnight at 4°C with rabbit polyclonal antinitrotyrosine antiserum (Biomol Research Laboratories, Plymouth Meeting, PA; diluted 1:500 in 5% milk-phosphate buffered saline with Tween 20). Bound antibody was detected using horseradish-peroxidase protein A (Boehringer Mannheim Biochemicals, Indianapolis, IN) and visualized using chemiluminescence (Renaissance Western Blot Chemiluminescence Reagent Plus; NEN Life Science Products, Boston, MA).

Animals and Experimental Groups

The investigations were approved by the Subcommittee for Research Animal Care of the Massachusetts General Hospital (Boston, MA). We studied wild-type SV129B6/F1 mice (F1-generation progeny of SV129 and C57BL/6 mice) of both sexes, weighing 16–30 g and aged 2–4 months (table 1). All drugs were administered intraperitoneally unless specified otherwise. Drugs were dissolved in saline so that the volume of each intraperitoneal injection was 0.01 ml/g body weight (except EUK-8, which was administered in 0.02 ml distilled water per gram). Endotoxin-challenged mice received 50 mg/kg body weight *E. coli* 0111:B4 lipopolysaccharide 16 h before lungs were isolated and perfused.⁸

Animals in group 6 were studied after prolonged exposure to inhaled NO. Mice were housed in chambers and breathed 20 ppm NO⁸ in air during the 16 h after intraperitoneal administration of saline, lipopolysaccharide, or lipopolysaccharide and 150 mg/kg *N*-acetylcysteine. Mice were studied 1 h after discontinuation of NO inhalation, allowing ample time for any vasodilator action of inhaled NO to dissipate.

Table 1. Mice Treatment Groups and Studies

| Group | Challenge and Time | Number of Mice |
|--|---|----------------|
| Pulmonary vasorelaxation to inhaled NO after LPS | | |
| 1. | Saline | 10 |
| | LPS | 10 |
| | } Given at t = 0 h | |
| Pulmonary vasorelaxation to inhaled NO after LPS and a non-selective ROS scavenger | | |
| 2. | LPS and 30 mg/kg NAC | 5 |
| | LPS and 150 mg/kg NAC | 6 |
| | LPS and 500 mg/kg NAC | 8 |
| | LPS given at t = 0, with 150 mg NAC given at t = 0 h and at t = 3.5 h | 8 |
| | Saline and 150 mg/kg NAC given concomitantly at t = 0 h | 6 |
| 3. | LPS and 333 mg/kg DMTU | 10 |
| | Saline and 330 mg/kg DMTU | 5 |
| | LPS and 30 mg/kg EUK-8 | 7 |
| | Saline and 30 mg/kg EUK-8 | 6 |
| 4. | LPS was given at t = 0 h and 150 mg/kg NAC at t = 15¼ h | 5 |
| Pulmonary vasorelaxation to inhaled NO after LPS and a selective ROS scavenger | | |
| 5. | LPS and 20 U/g PEG-catalase | 8 |
| | LPS and 100 U/g PEG-catalase | 8 |
| | LPS and 50 U/g PEG-SOD | 6 |
| | LPS and 15.4 mg/kg PEG | 5 |
| | Saline and 100 U/g PEG-catalase | 8 |
| | Saline and 50 U/g PEG-SOD | 5 |
| | } Given concomitantly at t = 0 h | |
| Effect of prolonged NO exposure on subsequent vasorelaxation to inhaled NO in LPS- and NAC-treated lungs | | |
| 6. | Saline | 5 |
| | LPS | 5 |
| | LPS and 150 mg/kg NAC | 5 |
| | } Given at t = 0 h and followed by 16 h of NO exposure (20 ppm) | |

Mice were injected intraperitoneally with 50 mg/kg lipopolysaccharide with or without a ROS scavenger, 16 h before lung perfusion. Groups of mice treated with saline, with or without a ROS scavenger served as controls. In some experiments, mice were subjected to 16 h of NO breathing.

ROS = reactive oxygen species; NO = nitric oxide; LPS = lipopolysaccharide; NAC = N-acetylcysteine; DMTU = 1,3-dimethyl-2-thiourea; PEG = polyethylene glycol; SOD = superoxide dismutase.

Lung Wet/Dry Ratio

After perfusion, both lungs, excluding hilar structures, were excised and weighed. Thereafter, the tissue was dried in a microwave oven for 60 min and reweighed as described previously.⁸ Lung wet/dry ratios were calculated.

Statistical Analysis

All data are expressed as mean \pm SEM. The overall responsiveness to the three doses of inhaled NO was compared between groups by analysis of variance for repeated measurements (Statistica for Windows; StatSoft, Inc., Tulsa, OK). A *P* value < 0.05 indicated significance of the overall responsiveness to inhaled NO. Differences in wet/dry ratios between groups were determined using a two-tailed Student *t* test.

Results

In each murine lung perfusion, U46619 produced a stable increase of PAP that was reversible after discontinuing U46619. The dose of U46619 required to increase PAP by 5 or 6 mmHg did not differ in saline- or lipopolysaccharide-treated mice (data not shown).

Effect of Lipopolysaccharide Challenge on

Pulmonary Vasodilation to Inhaled Nitric Oxide

Pulmonary vasorelaxation to 0.4, 4, and 40 ppm inhaled NO in saline-treated mice (table 1, group 1) was $34 \pm 4\%$, $60 \pm 5\%$, and $75 \pm 4\%$, respectively (fig. 1). After endotoxin challenge, pulmonary vasorelaxation to 0.4, 4, and 40 ppm inhaled NO was $11 \pm 2\%$, $26 \pm 4\%$, and $45 \pm 4\%$, respectively (*P* < 0.0001 vs. saline; fig. 1).

Early Administration of Nonselective Reactive Oxygen Species Scavengers after Lipopolysaccharide Challenge

To study the role of ROS in endotoxin-induced impairment of pulmonary vasodilation to inhaled NO, we administered *in vivo* concurrent with the lipopolysaccharide challenge one of three chemically unrelated ROS scavengers, each of which possesses nonselective antioxidant activity. N-acetylcysteine is a virtually nontoxic scavenger possessing beneficial effects in ALI in humans.²⁵ Mice were given a single intraperitoneal dose of 30, 150, or 500 mg/kg N-acetylcysteine immediately after lipopolysaccharide challenge (table 1, group 2), and lungs were isolated and perfused 16 h later. Administration of 30 mg/kg N-acetylcysteine did not alter the en-

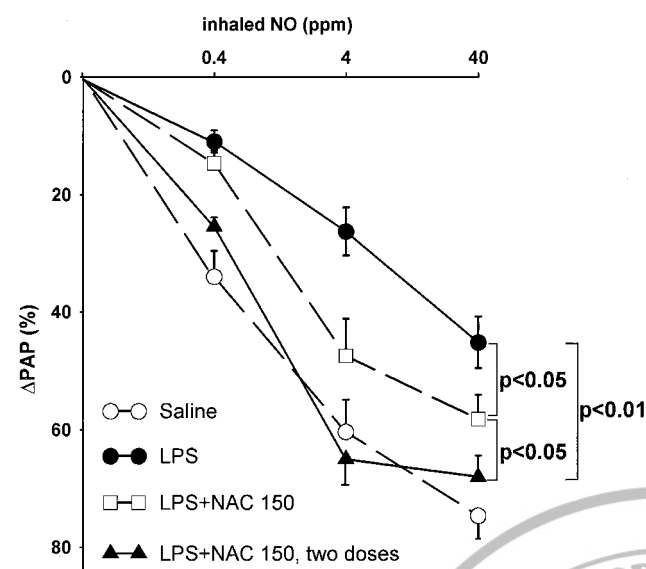


Fig. 1. The pulmonary vasorelaxation to 0.4, 4, and 40 ppm inhaled nitric oxide (NO) in mice, 16 h after intraperitoneal challenge with saline (open circles) or lipopolysaccharide (LPS; closed circles). Endotoxin attenuated the pulmonary vasorelaxation to inhaled NO ($P < 0.001$). Responsiveness to inhaled NO was partially preserved in mice treated with lipopolysaccharide and given 150 mg/kg intraperitoneal *N*-acetylcysteine (NAC; open squares) immediately thereafter. Responsiveness to inhaled NO was fully preserved after lipopolysaccharide and two doses of 150 mg/kg *N*-acetylcysteine given 3.5 h apart (closed triangles; $P =$ nonsignificant *vs.* saline). Δ PAP = decrease in pulmonary artery pressure as percentage of the U46619-induced increase.

dotoxin-induced impairment of vasorelaxation to inhaled NO (data not shown). The pulmonary vasorelaxation produced by 0.4, 4, and 40 ppm inhaled NO in mice challenged with endotoxin and treated with 150 mg/kg *N*-acetylcysteine was $15 \pm 2\%$, $47 \pm 6\%$, and $58 \pm 4\%$, respectively ($P < 0.05$ *vs.* lipopolysaccharide alone). A single intraperitoneal dose of 500 mg/kg *N*-acetylcysteine (data not shown) did not confer greater preservation of the vasodilator response to inhaled NO than a single dose of 150 mg/kg. Similar preservation of the vasodilator response to inhaled NO was observed when 150 mg/kg *N*-acetylcysteine was administered subcutaneously (*vs.* intraperitoneally) immediately after lipopolysaccharide challenge (data not shown).

Because *N*-acetylcysteine has a short plasma half-life as a result of rapid biotransformation and renal clearance,²⁵ we also assessed pulmonary vasorelaxation after two doses of 150 mg/kg *N*-acetylcysteine were given 3.5 h apart (table 1, group 2). After two doses of *N*-acetylcysteine, pulmonary vasorelaxation to 0.4, 4, and 40 ppm inhaled NO was $25 \pm 2\%$, $65 \pm 5\%$, and $68 \pm 4\%$, respectively ($P < 0.01$ *vs.* lipopolysaccharide alone; fig. 1) and did not significantly differ from vasorelaxation in saline-challenged mice. Therefore, two doses of *N*-acetylcysteine resulted in greater protection than a single dose ($P < 0.05$ *vs.* lipopolysaccharide and single *N*-acetylcysteine dose).

In lungs from mice treated with lipopolysaccharide followed by 330 mg/kg dimethylthiourea (table 1, group 3), the pulmonary vasorelaxation produced by 0.4, 4, and 40 ppm inhaled NO was $24 \pm 3\%$, $55 \pm 4\%$, and $71 \pm 3\%$, respectively ($P < 0.01$ *vs.* lipopolysaccharide alone, $P =$ nonsignificant *vs.* saline challenge; fig. 2). In mice treated with lipopolysaccharide followed by 30 mg/kg EUK-8, the pulmonary vasorelaxation produced by 0.4, 4, and 40 ppm inhaled NO was $19 \pm 10\%$, $55 \pm 6\%$, and $72 \pm 3\%$, respectively ($P < 0.01$ *vs.* lipopolysaccharide alone, $P =$ nonsignificant *vs.* saline challenge; fig. 2).

To examine whether fully developed impairment of inhaled NO responsiveness could be acutely reversed or ameliorated by late treatment with a ROS scavenger, mice were treated with 150 mg/kg intraperitoneal *N*-acetylcysteine at 15.25 h after lipopolysaccharide challenge and 45 min before they were killed (table 1, group 4). The impairment of pulmonary vasorelaxation to inhaled NO did not differ in mice challenged with lipopolysaccharide whether or not *N*-acetylcysteine was administered 15.25 h after lipopolysaccharide administration (data not shown).

The vasorelaxation to inhaled NO in lungs 16 h after treatment with saline followed immediately with either 150 mg/kg *N*-acetylcysteine, 330 mg/kg dimethylthiourea, or 30 mg/kg EUK-8 (table 1, groups 2 and 3) did not significantly differ from that of lungs of mice treated with saline alone (data not shown).

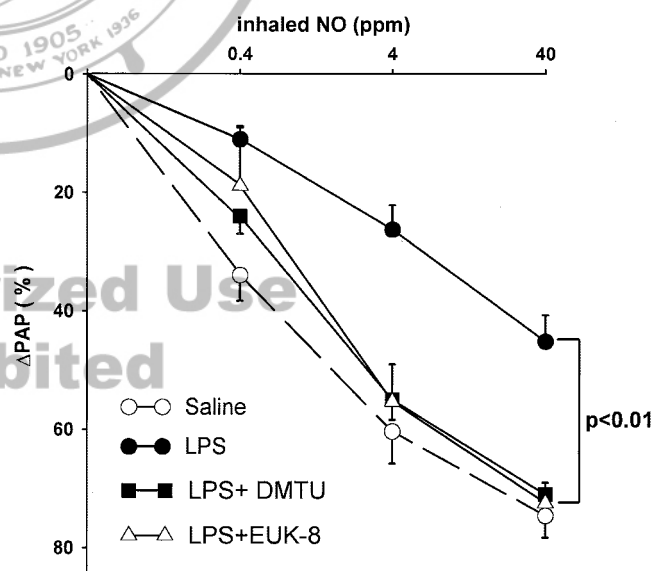


Fig. 2. The pulmonary vasorelaxation to inhaled nitric oxide (NO) in lipopolysaccharide (LPS)- (closed circles) and saline-challenged (open circles) mice and lipopolysaccharide-challenged mice treated concomitantly with dimethylthiourea (DMTU) (closed squares) or EUK-8 (open triangles). Responsiveness to inhaled NO was fully preserved after treatment with lipopolysaccharide combined with either dimethylthiourea or EUK-8 ($P < 0.01$). Δ PAP = decrease in pulmonary artery pressure as percentage of the U46619-induced increase.

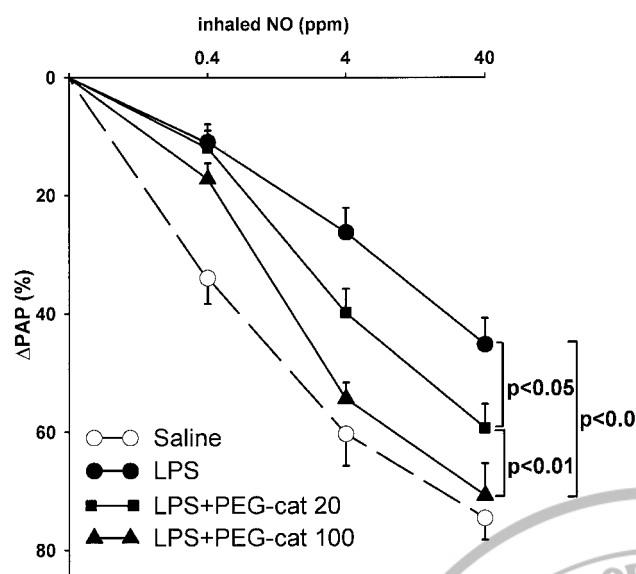


Fig. 3. The pulmonary vasorelaxation to inhaled nitric oxide (NO) in lipopolysaccharide (LPS)- (closed circles) and saline-challenged (open circles) mice and in lipopolysaccharide-challenged mice treated concomitantly with 20 (closed squares) or 100 U/g (closed triangles) polyethylene glycol-catalase (PEG-cat) administered intraperitoneally. Vasorelaxation to inhaled NO was partially preserved in mice treated with lipopolysaccharide and 20 U/g PEG-cat ($P < 0.05$ vs. lipopolysaccharide alone). Responsiveness to inhaled NO was fully preserved after treatment with lipopolysaccharide and 100 U/g PEG-cat ($P < 0.01$ vs. lipopolysaccharide alone and vs. 20 U/g PEG-cat).

Pulmonary Vasorelaxation to Inhaled Nitric Oxide after Lipopolysaccharide Challenge and Treatment with Reactive Oxygen Species-selective Scavengers

Pulmonary vasorelaxation to 0.4, 4, and 40 ppm inhaled NO in mice challenged with lipopolysaccharide followed by 20 U/g PEG-catalase administered intraperitoneally (table 1, group 5) was $12 \pm 4\%$, $40 \pm 4\%$, and $59 \pm 4\%$, respectively ($P < 0.05$ vs. lipopolysaccharide alone; fig. 3). In mice treated with lipopolysaccharide followed by 100 U/g PEG-catalase, the pulmonary vasorelaxation to 0.4, 4, and 40 ppm inhaled NO was $17 \pm 3\%$, $54 \pm 3\%$, and $71 \pm 5\%$, respectively ($P < 0.01$ vs. lipopolysaccharide alone and vs. lipopolysaccharide plus 20 U/g PEG-catalase, $P =$ nonsignificant vs. saline challenge; fig. 3). This single intraperitoneal dose of PEG-catalase fully preserved the pulmonary vasodilator response to inhaled NO. Because PEG itself has antioxidant properties,²⁶ we examined whether the PEG moiety alone was responsible for the protective effect of PEG-bound catalase observed in endotoxin-challenged lungs. PEG alone (table 1, group 5) did not protect endotoxin-challenged lungs against the impaired responsiveness to inhaled NO (data not shown).

The pulmonary vasorelaxation to 0.4, 4, and 40 ppm inhaled NO in endotoxin-challenged mice treated with 50 U/g PEG-SOD was $10 \pm 3\%$, $35 \pm 5\%$, and $49 \pm 7\%$, respectively ($P =$ nonsignificant vs. lipopolysaccharide alone). PEG-catalase and PEG-SOD had no effect on the

pulmonary vasodilator response to NO in saline-challenged control mice (table 1, group 5; data not shown).

Decreased Pulmonary Nitric Oxide Concentrations Do not Account for the Protective Effect of N-acetylcysteine

N-acetylcysteine²⁷ and other ROS scavengers^{27,28} may attenuate endotoxin-induced induction of NOS2 gene expression. A subsequent decrease of endogenous pulmonary NO concentrations could protect against endotoxin-induced impaired responsiveness to inhaled NO.^{7,8} To examine this possibility, murine pulmonary NOS2 mRNA concentrations were measured 6 h after intraperitoneal administration of saline, lipopolysaccharide, or lipopolysaccharide with 150 mg/kg N-acetylcysteine. This time point was chosen since murine pulmonary NOS2 mRNA concentrations are at their peak approximately 6 h after endotoxin challenge.^{29,30} After saline challenge, murine lungs had undetectable concentrations of NOS2 mRNA. In contrast, NOS2 gene expression was markedly elevated in lungs 6 h after challenge with lipopolysaccharide. Treatment with N-acetylcysteine did not reduce the ability of lipopolysaccharide to induce NOS2 gene expression (data not shown).

To confirm that the beneficial effects of N-acetylcysteine after endotoxin challenge are not attributable to diminution of pulmonary NO concentrations, responsiveness to inhaled NO was studied in mice that breathed 20 ppm NO for 16 h after challenge with saline, lipopolysaccharide, or lipopolysaccharide and N-acetylcysteine (table 1, group 6). As previously reported,⁸ prolonged NO inhalation did not alter the responsiveness to subsequent inhaled NO in either saline- or lipopolysaccharide-challenged lungs. The pulmonary vasorelaxation to 0.4, 4, and 40 ppm inhaled NO in mice that breathed 20 ppm NO for 16 h after receiving lipopolysaccharide and 150 mg/kg N-acetylcysteine was $18 \pm 2\%$, $48 \pm 6\%$, and $62 \pm 8\%$, respectively ($P < 0.05$ vs. lipopolysaccharide alone).

Effect of Lipopolysaccharide on Pulmonary Nitrotyrosine Concentrations

To determine whether endotoxin-induced impaired vasorelaxation to inhaled NO in mice was associated with increased formation of peroxynitrite, lungs concentrations of nitrotyrosine, a product of peroxynitrite,³¹ were assessed 7 h after lipopolysaccharide or saline challenge. We chose this time because previous reports suggested that nitrotyrosine could be detected in lungs within 3–12 h after lipopolysaccharide challenge^{30,32} and that maximum pulmonary nitrotyrosine concentrations coincided with maximal NOS2 expression.^{30,33} Nitrotyrosine was not detected in lungs obtained from either saline- or lipopolysaccharide-treated mice but was detected in a nitrated bovine serum albumin standard and in lung proteins pretreated with peroxynitrite (positive controls).

Lung Wet/Dry Ratio

The lung wet/dry ratios did not differ in mice challenged with saline or lipopolysaccharide with or without treatment with a ROS scavenger. The lung wet/dry ratios were not affected by prolonged breathing of 20 ppm NO.

Discussion

The novel finding of this study is that treatment with scavengers of ROS can protect murine lungs from endotoxin-induced hyporesponsiveness to inhaled NO. In accordance with several previous clinical² and experimental studies,⁶⁻⁸ we found in an isolated mouse lung perfusion model that an lipopolysaccharide challenge attenuates the pulmonary vasodilatory effect of inhaled NO. When given concurrently with lipopolysaccharide, the nonselective ROS and reactive nitrogen species scavengers dimethylthiourea, *N*-acetylcysteine, EUK-8, or a selective H₂O₂ scavenger, PEG-catalase, were able to preserve the pulmonary vasodilator response to inhaled NO when measured 16 h after lipopolysaccharide challenge.

Nonselective Reactive Oxygen Species Scavengers Preserve Pulmonary Vasorelaxation to Inhaled Nitric Oxide after Lipopolysaccharide Challenge

We found that a single dose of EUK-8 or dimethylthiourea fully preserved murine pulmonary vasorelaxation to inhaled NO after lipopolysaccharide challenge, whereas a single dose of *N*-acetylcysteine was only partially protective (fig. 1). We considered the possibility that the incomplete protection of inhaled NO responsiveness achieved after a single intraperitoneal dose of *N*-acetylcysteine was attributable to the short half-life in mice (as has been observed in humans²⁵). We observed that the administration of two intraperitoneal doses of *N*-acetylcysteine, 3.5 h apart, fully preserved pulmonary vasorelaxation to inhaled NO 16 h after lipopolysaccharide challenge. This observation suggests that the process by which ROS impair pulmonary responsiveness to inhaled NO lasts more than a few hours after lipopolysaccharide challenge and that ROS scavengers are required over an extended time period in the early phase of endotoxemia to prevent the induction of hyporesponsiveness to inhaled NO.

We considered the possibility that ROS scavengers may accentuate the vasodilator response to inhaled NO by enhancing the activity of enzymes in the NO-cyclic guanosine monophosphate signal transduction pathway.³⁴ This is unlikely, however, because no improvement in vasorelaxation was observed 16 h after administration of saline and ROS scavengers. We also considered the possibility that ROS scavengers potentiate the vasodilator response to inhaled NO by prolong-

ing the biologic half-life of NO. *N*-acetylcysteine, for instance, may stabilize NO in the form of *S*-nitrosothiols,³⁵ thereby preventing NO from reacting with O₂⁻. However, no enhancement of pulmonary vasorelaxation was observed when intraperitoneal *N*-acetylcysteine was given 15.25 h after endotoxin-challenge and 45 min before lung isolation, although tissue concentrations of *N*-acetylcysteine were likely to have been elevated during perfusion. Our studies suggest that the protective effect of ROS scavengers originates from the early prevention or mitigation of lipopolysaccharide-induced functional alterations that impair NO vasorelaxation.

The Protective Effect of N-acetylcysteine Is Retained during Prolonged Breathing of Nitric Oxide

We recently reported that increased pulmonary NO concentrations produced by NOS2 are necessary to impair responsiveness to inhaled NO in this murine sepsis model.⁸ We considered the possibility that *N*-acetylcysteine preserved responsiveness to inhaled NO by preventing the induction of NOS2 gene expression by endotoxin.²⁷ However, we observed that doses of *N*-acetylcysteine sufficient to preserve responsiveness to inhaled NO did not decrease NOS2 mRNA concentrations 6 h after lipopolysaccharide challenge. Moreover, breathing 20 ppm NO for 16 h after lipopolysaccharide challenge, which is sufficient to impair responsiveness in NOS2-deficient mice,⁸ did not impair inhaled NO responsiveness in wild-type mice treated with *N*-acetylcysteine. Taken together, these results suggest that the protective effects of *N*-acetylcysteine against endotoxin-induced impairment of pulmonary inhaled NO responsiveness is not attributable to inhibition of lipopolysaccharide-stimulated increased pulmonary NO concentrations.

H₂O₂ Is an Important Mediator of Endotoxin-induced Pulmonary Hyporesponsiveness to Inhaled Nitric Oxide

N-acetylcysteine, dimethylthiourea, and EUK-8 all scavenge a wide spectrum of ROS,^{12,13,17,19} peroxynitrite,^{14,20} and decrease nitrotyrosine formation by peroxynitrite.^{15,18,20} To further characterize the role of H₂O₂ and O₂⁻ in endotoxin-induced impaired responsiveness to inhaled NO, the selective ROS scavengers polyethylene PEG-catalase and PEG-SOD, respectively, were given concomitantly with lipopolysaccharide. PEG-conjugated enzymes were studied because they have superior intracellular penetration over the native enzymes,^{36,37} a long plasma half-life (up to 40 h in rats),³⁷ systemic efficacy after intraperitoneal administration,³⁸⁻⁴⁰ and protective effects in animal models of septic ALI.^{41,42} In our murine model of endotoxemia, PEG-catalase conferred a major protective effect, indicating an important role for H₂O₂ in the impairment of inhaled NO responsiveness. PEG-SOD, in contrast, did not preserve responsiveness to inhaled NO. During the

first few hours after administration, PEG-SOD had slower transmembrane penetration than PEG-catalase.³⁶ The recommended dose of PEG-SOD in human is 1 U/g,⁴³ and 2 U/g PEG-SOD was shown to effectively attenuate septic ALI in guinea pigs.⁴² We empirically chose a dose of 50 U/g PEG-SOD administered intraperitoneally. The dose we used may not have been enough to achieve a sufficiently high intracellular SOD concentration. After cellular uptake, PEG-conjugated enzymes are entrapped within endosomes or lysosomes.³⁶ Unlike H₂O₂, which crosses cellular membranes, O₂⁻ is membrane-impermeable,¹⁰ and endosomal or extracellular SOD cannot neutralize cytosolic or mitochondrial O₂⁻. The overproduction of NO and O₂⁻ after lipopolysaccharide challenge may overwhelm the small increase in lung SOD activity conveyed by PEG-SOD, because the reaction of O₂⁻ with NO is much faster than its reaction with SOD.³¹ Augmented SOD activity may even paradoxically exacerbate tissue damage through generation of H₂O₂ (and H₂O₂-derived oxidants)^{10,44} or by directly catalyzing tyrosine nitration.^{9,31}

H₂O₂ may induce ALI *via* activation of the transcription factor NF- κ B (nuclear factor- κ B), which plays a central role in regulating the transcription of cytokines, adhesion molecules, and other mediators involved in endotoxemia and sepsis.¹¹ H₂O₂ is converted to hypochlorous acid by myeloperoxidase or decomposes through the Fenton reaction to yield hydroxyl radical (\cdot HO).¹⁰ Recent investigations have demonstrated that H₂O₂, hypochlorous acid and \cdot HO can directly activate various heme oxygenases (e.g., myeloperoxidase) to generate peroxynitrite and other nitrating intermediates *via* oxidation of nitrite and nitrate, the end products of NO metabolism.⁹ In the current study, nitrotyrosine was not detected by immunoblotting in either control murine lungs or 7 h after lipopolysaccharide administration. Our finding is in accordance those of with Baechtold *et al.*,⁴⁵ who did not detect any change in nitrotyrosine content by immunoblotting in murine lungs 24 h after induction of polymicrobial sepsis. Kristof *et al.*,³⁰ however, reported that lipopolysaccharide challenge in mice elicited a widespread increase in immunostaining for nitrotyrosine in lung slices (peaking at 12 h after challenge). It is possible that immunoblotting is less sensitive than immunohistochemistry, as immunostaining also detected nitrotyrosine in slices obtained from saline-challenged lungs.³⁰ Alternatively, prolonged exposure to endotoxin may be required before detectable quantities of lung nitrotyrosine accumulates.³⁰ Although nitrotyrosine-related oxidative modification of tyrosine, such as 3,3'-dityrosine or 3-chlorotyrosine, may alter cellular pathways in inflammatory diseases similar to nitrotyrosine,⁹ these molecules are not detectable by the common nitrotyrosine assays.^{9,46} However, our finding does not support a major role for protein tyrosine nitrosylation in impaired pulmonary vasorelaxation in endotoxemia.

Nitric oxide inhalation improves oxygenation in patients with acute respiratory distress syndrome by redistributing blood flow toward better-ventilated areas, thereby decreasing intrapulmonary shunting.⁸ Although hyporesponsiveness to inhaled NO importantly limits the clinical efficacy of inhaled NO in acute respiratory distress syndrome, means to treat it are unknown. Although extrapolation from a murine model to human disease requires many continuing research steps, this study suggests that treatment with scavengers of ROS might be used to preserve responsiveness to inhaled NO in ALI and thereby increase the number of patients who respond favorably to inhaled NO. Responsiveness to inhaled NO was preserved by *N*-acetylcysteine treatment in endotoxin-challenged mice breathing 20 ppm NO for 16 h, suggesting that when NO is inhaled by patients to treat pulmonary hypertension or to augment arterial oxygenation, antioxidants may be given concomitantly to scavenge ROS and preserve pulmonary vasodilator responsiveness to inhaled NO.

In summary, our study suggests that, when given to mice early after lipopolysaccharide challenge, nonselective ROS scavengers can protect the lungs from endotoxin-induced hyporesponsiveness to inhaled NO. The salutary effect of PEG-catalase suggests an important role for hydrogen peroxide in mediating the endotoxin-induced hyporeactivity to inhaled NO. This study suggests that a novel therapeutic approach, treatment with scavengers of ROS, may preserve responsiveness and prevent hyporesponsiveness to inhaled NO in ALI.

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References

1. Cornfield DN, Maynard RC, deRegnier RA, Guiang SF 3rd, Barbato JE, Milla CE: Randomized, controlled trial of low-dose inhaled nitric oxide in the treatment of term and near-term infants with respiratory failure and pulmonary hypertension. *Pediatrics* 1999; 104:1089-94
2. Kraft P, Fridrich P, Fitzgerald RD, Koc D, Steltzer H: Effectiveness of nitric oxide inhalation in septic ARDS. *Chest* 1996; 109:486-93
3. Manktelow A, Bigatello LM, Hess D, Hurford WE: Physiologic determinants of the response to inhaled nitric oxide in patients with acute respiratory distress syndrome. *ANESTHESIOLOGY* 1997; 87:297-307
4. McIntyre RC Jr, Sheridan B, Agrafojo J, Fullerton DA: Selective inhibition of receptor-mediated pulmonary vasorelaxation in endotoxin-induced acute lung injury. *Shock* 1997; 7:36-41
5. Sheridan BC, McIntyre RC Jr, Agrafojo J, Meldrum DR, Meng X, Fullerton DA: Neutrophil depletion attenuates endotoxin-induced dysfunction of cGMP-mediated pulmonary vasorelaxation. *Am J Physiol* 1996; 271:L820-8
6. Holzmann A, Bloch KD, Sanchez LS, Filippov G, Zapol WM: Hyporesponsiveness to inhaled nitric oxide in isolated, perfused lungs from endotoxin-challenged rats. *Am J Physiol* 1996; 271:L981-6
7. Holzmann A, Manktelow C, Taut FJ, Bloch KD, Zapol WM: Inhibition of nitric oxide synthase prevents hyporesponsiveness to inhaled nitric oxide in lungs from endotoxin-challenged rats. *ANESTHESIOLOGY* 1999; 91:215-21
8. Weimann J, Bloch KD, Takata M, Steudel W, Zapol WM: Congenital NOS2 deficiency protects mice from LPS-induced hyporesponsiveness to inhaled nitric oxide. *ANESTHESIOLOGY* 1999; 91:1744-53
9. van der Vliet A, 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. 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
13. Bernard GR: N-acetylcysteine in experimental and clinical acute lung injury. *Am J Med* 1991; 91:54S-9S
14. 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
15. Cuzzocrea S, Mazzon E, Costantino G, Serrano I, De Sarro A, Caputi AP: Effects of N-acetylcysteine in a rat model of ischemia and reperfusion injury. *Cardiovasc Res* 2000; 47:537-48
16. Wasil M, Halliwell B, Grootveld M, Moorhouse CP, Hutchison DC, Baum H: The specificity of thiourea, dimethylthiourea and dimethyl sulphoxide as scavengers of hydroxyl radicals: Their protection of alpha 1-antiprotease against inactivation by hypochlorous acid. *Biochem J* 1987; 243:867-70
17. Kelner MJ, Bagnell R, Welch KJ: Thioureas react with superoxide radicals to yield a sulfhydryl compound: Explanation for protective effect against paraquat. *J Biol Chem* 1990; 265:1306-11
18. Whiteman M, Halliwell B: Thiourea and dimethylthiourea inhibit peroxynitrite-dependent damage: Nonspecificity as hydroxyl radical scavengers. *Free Radic Biol Med* 1997; 22:1309-12
19. 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
20. Crow JP: Manganese and iron porphyrins catalyze peroxynitrite decomposition and simultaneously increase nitration and oxidant yield: Implications for their use as peroxynitrite scavengers in vivo. *Arc Biochem Biophys* 1999; 371:41-52
21. Rong Y, Doctrow SR, Tocco G, Baudry M: EUK-134: a synthetic superoxide dismutase and catalase mimetic, prevents oxidative stress and attenuates kainate-induced neuropathology. *Proc Natl Acad Sci U S A* 1999; 96:9897-902
22. Bloch KD, Filippov G, Sanchez LS, Nakane M, de la Monte SM: Pulmonary soluble guanylate cyclase, a nitric oxide receptor, is increased during the perinatal period. *Am J Physiol* 1997; 272:L400-6
23. Lyons CR, Orloff GJ, Cunningham JM: Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J Biol Chem* 1992; 267:6370-4
24. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72:248-54
25. Gillissen A, Nowak D: Characterization of N-acetylcysteine and ambroxol in anti-oxidant therapy. *Respir Med* 1998; 92:609-23
26. White CW, Jackson JH, Abuchowski A, Kazo GM, Mimmack RF, Berger EM, Freeman BA, McCord JM, Repine JE: Polyethylene glycol-attached antioxidant enzymes decrease pulmonary oxygen toxicity in rats. *J Appl Physiol* 1989; 66:584-90
27. Pahan K, Sheikh FG, Namboodiri AM, Singh I: N-acetylcysteine inhibits induction of NO production by endotoxin or cytokine stimulated rat peritoneal macrophages, C6 glial cells and astrocytes. *Free Radic Biol Med* 1998; 24:39-48
28. Kim ND, Kang KW, Kim SG, Schini-Kerth VB: Inhibition of inducible nitric oxide synthase expression and stimulation of the endothelial formation of nitric oxide most likely accounts for the protective effect of 2-(allylthio)pyrazine in a murine model of endotoxemia. *Biochem Biophys Res Commun* 1997; 239:310-5
29. Nagasaki A, Gotoh T, Takeya M, Yu Y, Takiguchi M, Matsuzaki H, Takatsuki K, Mori M: Coinduction of nitric oxide synthase, argininosuccinate synthetase, and argininosuccinate lyase in lipopolysaccharide-treated rats: RNA blot, immunoblot, and immunohistochemical analyses. *J Biol Chem* 1996; 271:2658-62
30. Kristof AS, Goldberg P, Laubach V, Hussain SN: Role of inducible nitric oxide synthase in endotoxin-induced acute lung injury. *Am J Respir Crit Care Med* 1998; 158:1883-9
31. Beckman JS, Koppenol WH: Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *J Appl Physiol* 1996; 271:C1424-37
32. Albertini M, Clement MG, Lafortuna CL, Caniatti M, Magder S, Abdulmalek K, Hussain SN: Role of poly(ADP-ribose) synthetase in lipopolysaccharide-induced vascular failure and acute lung injury in pigs. *J Crit Care* 2000; 15:73-83
33. Haddad IY, Pataki G, Hu P, Galliani C, Beckman JS, Matalon S: Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. *J Clin Invest* 1994; 94:2407-13
34. Gupte SA, Rupawalla T, Mohazzab H, Wolin MS: Regulation of NO-elicited pulmonary artery relaxation and guanylate cyclase activation by NADH oxidase and SOD. *J Appl Physiol* 1999; 276:H1535-42
35. Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ, Loscalzo J: S-nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc Natl Acad Sci U S A* 1992; 89:444-8
36. Beckman JS, Minor RL Jr, Freeman BA: Augmentation of antioxidant enzymes in vascular endothelium. *J Free Radic Biol Med* 1986; 2:359-65
37. Beckman JS, Minor RL Jr, White CW, Repine JE, Rosen GM, Freeman BA: Superoxide dismutase and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance. *J Biol Chem* 1988; 263:6884-92
38. Thibeault DW, Rezaiekhaliq M, Mabry S: Lung antioxidant enzymes and cardiopulmonary responses in young rats exposed to hyperoxia and treated intratracheally with PEG catalase and superoxide dismutase. *Exp Lung Res* 1993; 19:137-51
39. Winn LM, Wells PG: Maternal administration of superoxide dismutase and catalase in phenytoin teratogenicity. *Free Radic Biol Med* 1999; 26:266-74
40. Beaman M, Birtwistle R, Howie AJ, Michael J, Adu D: The role of superoxide anion and hydrogen peroxide in glomerular injury induced by puromycin aminonucleoside in rats. *Clin Sci* 1987; 73:329-32
41. Olson NC, Grizzle MK, Anderson DL: Effect of polyethylene glycol-superoxide dismutase and catalase on endotoxemia in pigs. *J Appl Physiol* 1987; 63:1526-32
42. Suzuki Y, Tanigaki T, Heimer D, Wang WZ, Ross WG, Sussman HH, Raffin TA: Polyethylene glycol-conjugated superoxide dismutase attenuates septic lung injury in guinea pigs. *Am Rev Respir Dis* 1992; 145:388-93
43. Viau AT, Abuchowski A, Greenspan S, Davis FF: Safety evaluation of free radical scavengers PEG-catalase and PEG-superoxide dismutase. *J Free Radic Biol Med* 1986; 2:283-8
44. Traber DL, Adams T Jr, Sziebert L, Stein M, Traber L: Potentiation of lung vascular response to endotoxin by superoxide dismutase. *J Appl Physiol* 1985; 58:1005-9
45. Baechtold F, Scott JA, Markert M, Mehta S, McCormack DG, Anglada F, Galaud D, Vaglio M, Waeber B, Feihl F: Does the activation of poly (ADP-ribose) synthetase mediate tissue injury in the sepsis induced by cecal ligation and puncture? *Shock* 2001; 16:137-42
46. Viera L, Ye YZ, Estevez AG, Beckman JS: Immunohistochemical methods to detect nitrotyrosine. *Methods Enzymol* 1999; 301:373-81

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