A Role for Nitric Oxide-Mediated Peroxynitrite Formation in a Model of Endotoxin-Induced Shock

Salvatore Cuzzocrea, Emanuela Mazzon, Rosanna Di Paola, Emanuela Esposito, Heather Macarthur, George M. Matuschak, and Daniela Salvemini

Department of Clinical and Experimental Medicine and Pharmacology, University of Messina, Italy (S.C., E.M., R.D.P.); IRCCS Centro Neurolesi “Bonino-Pulejo,” Messina, Italy (S.C., E.M., R.D.P.); Department of Experimental Pharmacology, University of Naples “Federico II”, Naples, Italy (E.E.); Department of Pharmacological and Physiological Science, St. Louis University School of Medicine, St. Louis, Missouri (H.M., G.M.M., D.S.); Department of Internal Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, St. Louis University School of Medicine, St. Louis, Missouri (G.M.M., D.S.)

Received May 19, 2006; accepted June 29, 2006

ABSTRACT

The aim of the present study was to assess the relative contributions of peroxynitrite formation following induction of nitric-oxide synthase (iNOS) in the pathophysiology of endotoxin-induced shock in the rat. To this end, we used a selective inhibitor of iNOS, N-(3-(aminomethyl)benzyl)acetamidine (1400W), and a peroxynitrite decomposition catalyst, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron III chloride (FeTTPs). Intravenous (i.v.) administration of Escherichia coli lipopolysaccharide (LPS; 4 mg/kg) elicited a time-dependent fall in mean arterial pressure as well as liver, renal, and pancreatic tissue damage. 1400W (3–10 mg/kg i.v.) administered 30 min before LPS delayed the development of hypotension but did not improve survival. On the other hand, FeTTPs administered (10–100 mg/kg i.v.) inhibited in a dose-dependent manner LPS-induced hypotension, tissue injury, and improved mortality rate. In separate experiments, rats were treated with LPS (4 mg/kg) or saline for control, and their aortas were isolated and placed in organ baths 2 h later. Tissues from LPS-treated rats had significant inhibition of contractile activity to phenylephrine as well as a significantly impaired relaxation response to acetylcholine. FeTTPs, when administered (100 mg/kg i.v.) 1 h before LPS, prevented the LPS-induced aortic contractile and endothelial dysfunction. These results demonstrate that nitric oxide-derived peroxynitrite formation plays an important role in this model of endotoxemia. Our results also suggest that use of an iNOS inhibitor in this setting has little beneficial effect in part because, in the presence of a failing eNOS system, some NO is needed to maintain adequate organ function.

Severe hypotension, development of hyporeactivity to vasopressors, and ultimately, progressive multiple organ dysfunction characterize the pathogenesis of Gram-negative bacterial endotoxic shock (Siegel et al., 1967). The cause of the systemic vasodilation associated with shock, in particular, is still unclear, although overproduction of the potent vasodilator nitric oxide (NO) from the inducible form of nitric-oxide synthase (iNOS) has been implicated (Nathan, 1992; Thiemermann, 1994; Rixen et al., 1997). Even so, results from iNOS knockout mice have been controversial, with some studies reporting reduced endotoxin-related hypotension and others indicating no effects or even detrimental outcomes (Nicholson et al., 1999). Furthermore, the extensive evaluation of selective inhibitors for iNOS, such as amino guanidine and N-iminoethyl-L-lysine in models of shock, have likewise been disappointing and controversial (Thiemermann et al., 1995; Parratt, 1997).

In septic shock, inhibition of the activity of the constitutive endothelial cell isoform of nitric-oxide synthase (eNOS) precedes the induction of iNOS (Lu et al., 1996). Moreover, down-regulation of eNOS occurs at time points similar to those where iNOS induction is seen. Collectively, these results indicate that the beneficial effects of NO from eNOS are probably lost in septic shock associated with Gram-negative endotoxemia. In this context, nitric oxide from eNOS plays an important homeostatic role in maintaining appropriate blood flow to vital organs, such as the kidney, liver, and lungs and exhibits cytoprotective effects, in part, by preventing

ABBREVIATIONS: NO, nitric oxide; FeTTPs, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron III chloride; LPS, lipopolysaccharide; iNOS, inducible nitric-oxide synthase; ONOO−, peroxynitrite; MAP, mean arterial pressure; NOx, nitrite/nitrate; eNOS, endothelial nitric-oxide synthase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NADPH, nicotinamide adenine dinucleotide phosphate; 1400W, N-(3-(aminomethyl)benzyl)acetamidine; PARP, poly(ADP-ribose) polymerase; i.v., intravenous.
platelet/neutrophil adhesion to the blood vessel wall (Forstermann et al., 1998). Therefore, it is conceivable that a critical residual fraction of NO derived from iNOS is required for vital organ perfusion and blood vessel potency. Thus, its inhibition will be overall detrimental. This may explain, at least in part, the controversial reports on iNOS inhibitors. We reasoned that blocking peroxynitrite, a powerful and potent proapoptotic and proinflammatory mediator (Salvemini et al., 1998a), would yield better outcomes. This hypothesis stems from the fact that removal or antagonism of ONOO− in endotoxic shock will not deplete homeostatic levels of NO produced from iNOS but rather only attenuate the harmful effects of NO overproduced from iNOS (which are mediated by ONOO−). Peroxynitrite (the byproduct of the reaction between NO and O2−) has been increasingly implicated in the pathogenesis of endotoxin-induced hemodynamic instability and organ dysfunction (Beckman et al., 1996; Beckman, 1996; Zingarelli et al., 1998).

Peroxynitrite is known to undergo acid-catalyzed decomposition by two distinct pathways (Salvemini et al., 1998b; Groves, 1999). Isomerization to nitrate is the major decay route, but a significant portion of the decomposition produces a species with reactivity related to that of a hydroxyl radical (Crow et al., 1994). Certain water-soluble iron (III) porphyrins are highly active ONOO− decomposition catalysts, and they function by catalyzing the isomerization of ONOO− almost exclusively to nitrate (Stern et al., 1996). Catalysis is proposed to proceed via an oxo-Fe(IV) intermediate generated from the metal-promoted cleavage of the O–O bond. Subsequent recombination with NO2 generates the Fe(III) state and produces nitrate. These catalysts thus dramatically increase the rate of ONOO− isomerization, preempting the formation of oxidizing radical species and generating the harmless nitrate anion. This mode of catalysis manifests itself by dramatic shifts in the resulting nitrite-to-nitrate ratio when compared with the proton-catalyzed decomposition (Stern et al., 1996; Salvemini et al., 1998b; Shimanovich et al., 2001).

In the present study, we have examined the role of ONOO− by using the peroxynitrite decomposition catalysts 5,10,15,20-tetakis(4-sulfonatophenyl)porphyrinato iron III chloride (FeTPPs). This compound is a water-soluble Fe (III) porphyrin complex that catalyzes rapid isomerization of ONOO− to nitrate (NO3−) under physiologically relevant conditions (pH 7.4, 37°C) (Jensen and Riley 2002). The cytoprotective actions of FeTPPs have been characterized (Stern et al., 1996; Misko et al., 1998). In addition to peroxynitrite-decomposing effect, FeTPPs demonstrates minimal superoxide dismutase activity and does not complex with NO (Lee et al., 1998; Misko et al., 1998; Imam et al., 2000). In fact, the peroxynitrite-decomposing effect increases considerably if superoxide and peroxynitrite are present in the same milieu as a result of the formation of catalytic cycle (Lee et al., 1998).

As shown in previous studies, FeTPPs and other derivatives of similar (e.g., FeTMPS) and different (e.g., F15) chemical class exert potent anti-inflammatory properties across several disease states (Salvemini et al., 1998b; Cuzzocrea et al., 2000; Obrosova et al., 2005). The results of our studies indicate that the function of eNOS was compromised during endotoxic shock and that, although iNOS inhibitors attenuated arterial hypotension, they had no effect on overall survival. In contrast, FeTPPs prevented hypotension, improved mortality, preserved multiple organ function, and mitigated endothelial cell dysfunction. These results strongly suggest that use of iNOS inhibitors has little benefit early after endotoxemia where eNOS is compromised and that alternative strategies, such as removal of peroxynitrite, may yield better outcomes overall.

### Materials and Methods

#### Surgical Procedure

Male Sprague-Dawley rats (250–300 g) were anesthetized with thiopentone sodium (intravital sodium; 120 mg/kg intraperitoneally). The trachea was cannulated to facilitate respiration, and body temperature was maintained at 37°C by means of a heating pad. The left femoral vein was cannulated for administration of drugs. The left femoral artery was cannulated and connected to a pressure transducer to allow for the monitoring of blood pressure and when required for blood collections. In some experiments, rats were treated with Escherichia coli lipopolysaccharide (LPS) for 2 h and sacrificed, and their aortas were isolated and removed for organ bath experiments as described below. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the European Economic Committee regulations (O.J. of E.C. L 358/1 12/18/1986). Animals were also cared for in accordance with the guidelines of the institutional Animal Care and Use Committee of St. Louis University Health Science Center and in accordance with the National Institute of Health Guidelines on Laboratory Animal Welfare.

#### Drug Administration

LPS (serotype 0111:B4) was purchased from Sigma (St. Louis, MO). Lipopolysaccharides contain endotoxin levels of not less than 500,000 EU (endotoxin units)/mg unless otherwise noted. One nanogram of endotoxin is equivalent to 5 (Limulus lysate assay) and 10 EU (chromogenic assay). LPS (4 mg/kg) was administered by bolus intravenous (i.v.) injection in a volume of 0.3 ml as a slow injection over 15 min. All animals then received a continuous infusion of saline (4 mg/kg/h i.v.). A further group of animals received 30 min before the administration of endotoxin an infusion of either a low dose (3 mg/kg/h) or a high dose (10 mg/kg/h) of the selective iNOS inhibitor N-(3-aminomethylbenzyl)acetamide (1400 W, in 4 ml/kg/h saline). Infusion of 1400 W was preceded by bolus injections of 3 or 10 mg/kg i.v. In other experimental groups, the animals received FeTPPs (10–100 mg/kg i.v.) 1 h prior to LPS. Surviving animals were sacrificed at 9 h after the administration of LPS or saline as dictated by the animal experimental protocol. Ten rats per group were used.

#### Quantization of Organ Function and Injury

Six hours after LPS or saline injection, blood samples were collected from all animals (n = 10 for each group). The blood sample was centrifuged (1610g for 3 min at room temperature) to separate plasma. All plasma samples were analyzed within 24 h by a veterinary clinical laboratory using standard laboratory techniques. The following marker enzymes were measured in the plasma as biochemical indicators of organ injury/dysfunction of the kidneys, pancreas, and liver. 1) Renal dysfunction was determined and assessed by measuring the increases in plasma levels of creatinine (an indicator of reduced glomerular filtration rate). 2) Pancreatic injury was inferred from increases in serum levels of lipase and amylase, and 3) liver injury was assessed by measuring the rise in plasma levels of bilirubin, alkaline phosphatase, aspartate aminotransferase (AST, a nonspecific marker for hepatic injury), and alanine aminotransferase (ALT, a specific marker for hepatic parenchymal injury).

#### Light Microscopy

Standardized liver biopsies were taken at the end of experiments, fixed for 1 week in buffered formaldehyde solution (10% in phosphate-buffered saline) at room temperature, dehydrated by graded ethanol, and embedded in Paraplast (Sherwood...
Medical, Mahwah, NJ). Sections (thickness 7 μm) were deparaffinized with xylene, stained with hematoxylin and eosin, and studied using light microscopy (Dialux 22 Leitz).

**Immunohistochemical Localization of Nitrotyrosine.** Nitrotyrosine nitration, an index of the nitration of proteins by peroxynitrite and/or oxygen-derived free radicals, was determined by immunohistochemistry as described previously (Misko et al., 1993). At 6 h after LPS or saline injection, the relevant organs were fixed in 10% buffered formaldehyde, and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H2O2 in 60% methanol for 30 min, after which sections were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 20 min. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in phosphate-buffered saline for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin. The sections were then incubated overnight with 1:1000 dilution of primary antinitrotyrosine antibody or with control solutions. Controls included buffer alone or non-specific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex. To confirm that the immunoreactions for the nitrotyrosine were specific, some sections were also incubated with the primary antibody (antinitrotyrosine) in the presence of excess nitrotyrosine (10 nM) to verify the binding specificity.

**Isolated Tissue Experiments.** Four aortic rings were prepared from each of 10 rats. In brief, thoracic aortas were removed from male Sprague-Dawley rats and treated with LPS (4 mg/kg i.v.), LPS plus FeTPPs (100 mg/kg i.v.), or an equal volume of saline. Thoracic aortas were removed at 2 h post-treatment, and connective tissue was carefully trimmed to avoid damage to the endothelium. Rings were cut into 3 mm lengths and placed into a 10-ml tissue bath. In some experiments, the endothelium was removed by gentle rubbing of the ring preparation. Successful removal of the endothelium was confirmed by the lack of a relaxation response to acetylcholine (10–6 M). Aortic rings were maintained at 37°C in Krebs’ bicarbonate buffer, pH 7.4, bubbled with 5% CO2/95% O2. Rings were preloaded with 1 g of tension and equilibrated for 30 min with two to three buffer changes. After stabilization of the baseline, the rings were contracted with phenylephrine. Doses of phenylephrine that produced 90 to 100% contraction were chosen for subsequent experiments. NO-mediated relaxation of the precontracted endothelium-intact rings was produced by cumulative increases in the concentration of exogenously added acetylcholine. Endothelium-independent relaxation was evoked by administration of the NO donor, sodium nitroprusside, using endothelium-denuded rings. Isometric tension was recorded, and relaxation was determined as the percentage of maximal tone developed to phenylephrine.

**Nitrite/Nitrate Assay.** NO reacts with oxygen to yield nitrate and nitrite. Therefore, we measured these breakdown products of NO by means of the fluorometric assay for the measurement of nitrite as described in detail by Misko et al. (1993). Plasma samples were first passed through an Ultra-MC filter (10,000 molecular weight cut-off; Millipore, Bedford, MA). Nitrate in plasma aliquots (5 μl) was converted to nitrite by the addition of nitrate reductase (14 μl) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH, 1 nmol) with incubation for 10 min at room temperature. The reaction was terminated by dilution with water and the addition of the dianonaphthene reagent (Misko, 1993). Nitrite concentrations in these samples were determined fluorimetrically (excitation at 365 nm and emission at 450 nm) with sodium nitrite used as a standard. All determinations were performed in duplicate, and results were expressed as plasma NOx (levels in micromolars). Plasma NOx were measured up to 6 h post-LPS.

**Materials.** The composition of the Krebs’ buffer was as follows: 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO4, 2.5 mM CaCl2, 1.0 mM NaH2PO4, 26.2 mM NaHCO3, and 10 mM HEPES, pH 7.4. FeTPPs was obtained from Calbiochem (Inalco, Milan, Italy). LPS, phenylephrine, acetylcholine, sodium nitroprusside, and aminoguanidine were all obtained from Sigma. 1400W was purchased from Alexis Biochemicals (San Diego, CA).

Fig. 1. A, administration of *E. coli* LPS serotype 0111:B4 (4 mg/kg) results in the development of irreversible hypotension in the anesthetized rat. Treatment with 1400W (3–10 mg/kg) 30 min prior to LPS delays but does not prevent this fall in MAP and mortality (A). When given alone at the highest dose tested, 1400W (10 mg/kg) had no effect on MAP (A). B, in addition, the increase in plasma levels of NOx seen at 2, 3, and 6 h but not at 1 h post-LPS is attenuated in a dose-dependent manner by 1400W. Each point represents the mean ± S.E.M. of 10 rats for each group. *p < 0.01 versus Saline; #, p < 0.001 versus LPS.
Statistical Analysis. All values in the figures and text are expressed as mean ± S.E.M. of \( n \) observations, where \( n \) represents the number of vascular rings or rats studied (10 rats or rings from two to three independent experiments). For the in vivo studies, \( n \) represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. The results were analyzed by one-way analysis of variance followed by a Bonferroni’s post hoc test for multiple comparisons. A \( p \) value of less than 0.05 was considered significant.

Results

Effects of iNOS Inhibitor 1400W on E. coli Endotoxin-Induced Shock and Mortality

The administration of \( E. \ coli \) LPS serotype 0111:B4 (4 mg/kg i.v.) elicited a time-dependent fall in mean arterial pressure, which resulted in death at approximately 7 h (Fig. 1A). When 1400W (3–10 mg/kg; Fig. 1A) was administered 30 min prior to LPS, there was a dose-dependent delay in the development of late-phase hypotension compared with control. However, regardless of 1400W pretreatment, mean arterial pressure fell sharply after 5 h, such that 100% mortality was observed by 7 h (Fig. 1A, \( n = 6 \)). At the highest dose tested, 1400W (10 mg/kg; Fig. 1A) did not increase mean arterial pressure, a result consistent with their selectivity for iNOS. LPS also caused a time-dependent increase in circulating NO\(_x\) concentrations (Fig. 1B). On the other hand, and consistent with its selectivity for iNOS, 1400W blocked, in a dose-dependent manner, NO\(_x\) release at 2, 3, and 6 h but not at 1 h (NO derived from eNOS) (Fig. 1B). Please note that when either of the two iNOS inhibitors, aminoguanidine (10–100 mg/kg) or \( N \)-iminoethyl-L-lysine (3–30 mg/kg), was administered 1 h prior to LPS, there was a dose-dependent delay in the development of late-phase hypotension compared with control. However, mean arterial pressure fell sharply after 6 h, resulting in 100% mortality by 7 h (data not shown).

Effect of the Peroxynitrite Decomposition Catalyst FeTPPs on Shock and Survival

Baseline levels of mean arterial blood pressure in all groups of animals ranged from 125 ± 3 to 130 ± 5 mm Hg and were not significantly different between groups (Fig. 2). In saline control rats (no endotoxin), administration of FeTPPs, at the highest dose tested (30 mg/kg i.v.), had no effect on mean arterial blood pressure (data not shown). In endotoxemic rats, there was a time-dependent fall in mean arterial pressure which uniformly resulted in death at ap-
proximately 7 h (Fig. 3). However, administration of FeTPPs 1 h prior to endotoxemia dose dependently inhibited (10–100 mg/kg; Fig. 3) arterial hypotension. Furthermore, at doses of 10 and 30 mg/kg, FeTPPs also significantly prevented mortality up to the 9 h time point (Fig. 3).

Effects of the Peroxynitrite Decomposition Catalyst FeTPPs on Endotoxin-Induced Multiple Organ Dysfunction

Renal Dysfunction. In nonendotoxemic control rats, administration of saline did not significantly alter plasma creatinine levels (Fig. 3A). In contrast, at 6 h post-LPS injection, significant increases in plasma creatinine levels, consistent with de novo renal dysfunction, were observed. Notably, treatment with FeTPPs at the highest dose tested (100 mg/kg) abolished the renal dysfunction caused by endotoxemia (Fig. 3A).

Pancreatic Injury. No significant alterations in plasma levels of lipase and amylase were observed in saline-infused animals (Fig. 3, B and C). In contrast, at 6 h post-LPS injection, significant increases in plasma levels of lipase and amylase were observed (Fig. 3, B and C) and this event was blocked by FeTPPs (100 mg/kg) (Fig. 3, B and C).

Liver Injury. No significant elevations in the plasma levels of ALT, AST, bilirubin, and alkaline phosphatase were noted in saline control rats (Fig. 4). In contrast, at 6 h post-LPS injection, significant elevations in plasma levels of ALT, AST, bilirubin, and alkaline phosphatase, compatible
with the development of hepatocellular injury and cholestasis, were observed (Fig. 4). Treatment with 100 mg/kg FeTPPs abolished the liver dysfunction caused by LPS (Fig. 4).

**Effects of the Peroxynitrite Decomposition Catalysts FeTPPs on Endotoxin-Induced Liver Nitrotyrosine Formation and Histopathology**

Immunohistochemical analysis, using a specific antinitrotyrosine antibody, revealed a positive staining in liver from LPS-treated at 6 h postinjection (Fig. 5B). Positive nitrotyrosine staining was particularly prominent in the nuclei of hepatocytes from LPS-treated rats (Fig. 5B1, see arrows). A marked reduction in nitrotyrosine staining was found in the livers of endotoxemic rats that had been treated with FeTPPs (100 mg/kg) (Fig. 5C). There was no staining for nitrotyrosine in organs obtained from saline-infused rats (Fig. 5A).

In conjunction with these findings and at the same time points, the livers of *E. coli* endotoxin-challenged rats showed substantial histological alterations consistent with LPS-induced organ injury (Fig. 6, A and A1) compared with organs obtained from saline-infused rats (Fig. 6C). Most notably, hepatic damage was characterized by focal damage of the hepatic parenchyma (Fig. 6A1) along with infiltration of inflammatory cells (Fig. 6A, see arrow). These LPS-induced histopathological changes were attenuated in the organs of rats treated with FeTPPs (100 mg/kg; Fig. 6B).

**Aortic Contractility and Endothelial Function.** In separate experiments, rats were treated with LPS (4 mg/kg) or saline and sacrificed 2 h later with their aortas isolated and placed in organ baths as described previously. Phenylephrine (Phe, $10^{-9}$–$10^{-4}$ M) caused a dose-dependent contraction of saline control tissues with an EC$_{50}$ of $1.9 \times 10^{-8}$ M (Fig. 7). The dose-response curve to phenylephrine in tissues taken from endotoxemic LPS-treated rats showed a significant shift to the right, indicative of an inhibition of contractile activity. The EC$_{50}$ for phenylephrine was thus $1.1 \times 10^{-6}$ M (Fig. 7).

To evaluate the effects of LPS on endothelium-dependent vasorelaxation, endothelium-intact rings were precontracted with a dose of phenylephrine that gave a submaximal contraction ($10^{-7}$ and $5 \times 10^{-6}$ M for tissues taken from saline control or LPS-treated rats, respectively), and a dose-response to acetylcholine was then generated ($10^{-9}$–$10^{-5}$ M; Fig. 8A).
taken from the different groups of rats. Each point represents the mean (± S.E.M. of six experiments). *p < 0.01 versus Saline; #, p < 0.01 versus LPS.

Fig. 7. Administration of phenylephrine (10^-9–10^-4 M) to preparations of aortic rings harvested from saline treated rats caused a dose-dependent contraction in these tissues. The ability of phenylephrine (10^-6–10^-7 M) to evoke such contractions in tissues harvested from LPS-treated rats was significantly inhibited. FeTPPs (100 mg/kg) significantly prevented the LPS-induced contractility dysfunction. Each point represents the mean ± S.E.M. of six experiments. +, p < 0.01 versus Saline; #, p < 0.01 versus LPS.

Fig. 8. A, administration of acetylcholine (10^-9–10^-8 M) to aortic tissues elicited a dose-dependent relaxation (depicted as percentage relaxation of induced tone), indicating the release of NO from the endothelial layer of these tissues. This response was diminished in rings taken from LPS-treated rats compared with control tissues. FeTPPs (100 mg/kg) significantly prevented the LPS-induced endothelial dysfunction. B, in another series of experiments, endothelium-denuded aortic rings (evidenced by lack of vasorelaxation to acetylcholine) from saline, LPS-treated rats, or FeTPPs-treated rats were precontracted with a submaximal concentration of phenylephrine. Administration of sodium nitroprusside (10^-7–10^-6 M) to these tissues elicited a dose-dependent relaxation (depicted as percentage relaxation of induced tone). There was no difference in the response obtained from tissues taken from the different groups of rats. Each point represents the mean ± S.E.M. of six experiments. *, p < 0.01 versus Saline; #, p < 0.01 versus LPS.

Discussion

Persistent hypotension requiring vasopressor support is a major risk factor for mortality in patients with Gram-negative bacterial septic shock, and therefore, its prevention is an important therapeutic goal. Impaired endothelium-dependent vasodilation has been found within 1 to 2 h after endotoxemia in humans (Bhagat et al., 1996) and in animal models (Macarthur et al., 1999, 2000). The mechanisms implicated in LPS-mediated impairment of endothelium-dependent vasodilation include inhibition of eNOS enzymatic activity and/or down-regulation of eNOS expression (Lu et al., 1996). Alterations in eNOS have thus been found at both early and late time points after cellular exposure to endotoxin. For instance, in cultured endothelial cells, LPS and various cytokines decrease agonist-mediated NO release within 30 min (Graier et al., 1994). These same cytokines, including tumor necrosis factor-α, have been shown to attenuate eNOS expression by reductions in eNOS mRNA stability at the later time points (Yoshizumi et al., 1993). Thus, by the time iNOS is up-regulated, there is a well established corresponding down-regulation in eNOS expression (Yoshizumi et al., 1993).

The findings reported here show that inhibition of iNOS in this model of endotoxic shock elicits short-term hemodynamic stabilization without meaningfully improving survival rate. We believe that such lack of effect is due to the dependence, especially in the later stages of shock, on iNOS as the prime source of NO. Our results in the isolated aortic ring tissue experiments support this contention, as they clearly show a significant loss in eNOS activity (as assessed by monitoring the responses to acetylcholine, an endothelium-dependent vasodilator) at a time point where iNOS was already induced (e.g., 2 h post-LPS). It is also clear from our experiments that responses to the endothelium-independent vasodilator sodium nitroprusside were not affected, thereby indicating that the function of soluble guanylate cyclase in smooth muscle cells was preserved. In the present study, we did not determine whether loss of endothelium-dependent vasorelaxation (and thus NO release) was due to inhibitory effects on eNOS activity or down-regulation of the expression. The former possibility is more plausible because down-regulation of eNOS mRNA and protein levels by LPS/cytokines require at least three or more pathways (Yoshizumi et al., 1993). Other reports have also alluded to this concept of dependence on iNOS as the prime source of homeostatic NO during circulatory shock, especially in critical organs such as the lung. Thus, inhibition of iNOS worsened rather than...
improved acute respiratory distress syndrome in animal models (Cobb et al., 1999). Likewise, endotoxin down-regulates eNOS in renal arteries, leading to impaired endothelium-dependent vasodilation (Lu et al., 1996), and in rodent models of shock, resulting in NO derived from iNOS becoming the major source of this vasodilator (Lu et al., 1996). Finally, septic shock studies involving iNOS knockout mice have demonstrated that such animals have a much greater mortality rate than their wild-type counterparts, again underscoring the importance of iNOS in septic shock (Cobb et al., 1999).

Our data reported herein extend these findings, while raising questions about the efficacy of global iNOS inhibition as a therapy for endotoxic shock and, conceivably, related disease states, such as ischemia/reperfusion injury, in which the activity and/or expression of eNOS are impaired (Kubes, 1993). In contrast, better outcomes have been more consistently observed following inhibition of peroxynitrite formation. Szabó et al. (1995) demonstrated that ONOO⁻ is formed in the earliest stage of endotoxemia (when eNOS-derived NO combines with superoxide). This early formation is associated with important deleterious effects of peroxynitrite on vascular contractile and endothelial dysfunction (Zingarelli et al., 1997). The beneficial effect of FeTTPs observed in the present study may be partially dependent on the inhibition of early ONOO⁻ production. 1400W will not block early formation of ONOO⁻.

These findings suggest that ONOO⁻ is responsible, at least in part, for the development of endotoxin-induced hypotension, endothelial injury, multiple organ dysfunction, and subsequent death. In support of these results, recent data demonstrate that ONOO⁻ is capable of mimicking many of the cardiovascular alterations associated with shock (endothelial dysfunction, vascular hyporeactivity, myocardial impairment, and cellular energetic failure) (O’Connor et al., 1997). Moreover, recently it has been demonstrated that FP15, another ONOO⁻ decomposition catalyst, also exerts vascular protective and anti-inflammatory effects in many models (Obrosova et al., 2005). Furthermore, ONOO⁻ has been implicated in tissue damage, including the intestinal damage that accompanies endotoxic shock (Salvemini et al., 1999) and has been detected in humans with sepsis (Fukuyama et al., 1997). Peroxynitrite can also cause DNA single-strand damage triggering poly(ADP-ribose) polymerase (PARP) activation, ultimately leading to cell death (Szabo et al., 1997). The involvement of PARP in the pathophysiology of endotoxin shock and the utility of PARP inhibitors in this context has been described previously (Wray et al., 1998).

Therefore, we contend that development of therapeutic strategies for the treatment of Gram-negative septic shock that distinguish between the overtly harmful effects of NO overproduction from the beneficial effects of residual homeostatic NO availability is likely to be more promising than approaches that fail to acknowledge these separate NO-mediated actions. Strategies aimed at directly removing ONOO⁻ or inhibiting signaling events engaged by ONOO⁻ (i.e., activation of PARP) may yield a better overall outcome (Fig. 9).

Acknowledgments

We thank Giovanni Pergolizzi and Carmelo La Spada for excellent technical assistance during this study, Caterina Cutrona for secretarial assistance, and Valentina Malvagni for editorial assistance with the manuscript.
References

Beckman JS, Beckman TW, Chen J, Marshall PA, and Freman BA (1990) Apparent hydroxyl radical production by peroxynitrite: implication for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87:1620–1624.


Address correspondence to: Dr. Daniela Salvemini, Department of Internal Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, St. Louis University Hospital, 3635 Vista Avenue, St. Louis, MO 63110-0250. E-mail: salvem@slu.edu