A role for nitric oxide-mediated peroxynitrite formation in a model of endotoxin induced shock

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Abbreviations:
(FeTTPs): 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron III chloride
(LPS): lipopolysaccharide
(INOS): nitric oxide synthase
(NO) nitric oxide
(ONOO-) peroxynitrite
(MAP) mean arterial pressure
(NOx) nitrite/nitrate
(AG) aminoguanidine
(L-NIL) N-iminoethyl-L-lysine
(ecNOS) constitutive endothelial cell isoform of NOS
(AST) aspartate aminotransferase
(ALT) Alanine aminotranferease
(PBS) phosphate buffered saline
(NADPH) nicotinamide adenine dinucleotide phosphate
(PARP) poly(ADP-ribose) polymerase
ABSTRACT

The aim of the present study was to assess the relative contributions of peroxynitrite (ONOO⁻) 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, 1400W and a peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron III chloride (FeTTPs). Intravenous administration of E. coli lipopolysaccharide (LPS; 4 mg/kg) elicited a time-dependent fall in mean arterial pressure (MAP) as well as liver, renal and pancreatic tissue damage. 1400W (3-10 mg/kg i.v.) administered 30 minutes prior to 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. FeTPPs, when administered (100 mg/kg i.v.) 1 h prior to 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.
INTRODUCTION

Severe hypotension, development of hyporeactivity to vasopressors and ultimately, progressive multiple organ dysfunction characterize the pathogenesis of gram-negative bacterial endotoxic shock (Siegel, 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 aminoguanidine (AG) and N-iminoethyl-L-lysine (L-NIL) in models of shock have likewise been disappointing and controversial (Parratt, 1997; Thiemermann et al., 1995).

In septic shock, inhibition of the activity of the constitutive endothelial cell isoform of NOS (ecNOS) precedes the induction of iNOS (Lu et al., 1996). Moreover, down regulation of ecNOS occurs at time points similar to those where iNOS induction is seen. Collectively, these results indicate that the beneficial effects of NO from ecNOS are likely lost in septic shock associated with gram-negative endotoxemia. In this context, nitric oxide from ecNOS plays an important homoeostatic role in maintaining appropriate blood flow to vital organs such as the kidney, liver and lungs and exhibits cytoprotective effects, in part by preventing platelet/neutrophil adhesion to the blood vessel wall (Forsterrmann et al., 1998). It is therefore conceivable that a critical residual fraction of
the 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 pro-apoptotic 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 by-product of the reaction between NO and \(O_2^-\)) has been increasingly implicated in the pathogenesis of endotoxin-induced hemodynamic instability and organ dysfunction (Zingarelli et al., 1998; Beckman et al., 1990; Beckman, 1996).

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 akin to that of a hydroxyl radical (Tsai et al., 1994, Crow et al. 1994). We recently have reported that certain water-soluble iron (III) porphyrins are highly active ONOO\(^-\) decomposition catalysts and that 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 \(NO_2^-\) regenerates 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 (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-tetrakis(4-sulfonatophenyl)porphyrinato iron III chloride (FeTTPs). 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 do demonstrate minimal superoxide dismutase activity and do 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, FeTTPs and other derivatives of similar (for example, FeTMPS) and different (for example F15) chemical class exert potent anti-inflammatory properties across several disease states (Obrosova et al., 2005; Salvemini et al., 1998b; Cuzzocrea et al., 2000). The results of our studies indicate that the function of ecNOS 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 ecNOS is compromised, and that alternative strategies such as removal of peroxynitrite may yield better outcomes overall.
METHODS

Surgical Procedure

Male Sprague Dawley rats (250-300 g) were anesthetized with thiopentone sodium (Intraval 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, sacrificed and their aortas 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 EEC 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 Saint 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, cat.#L4130, lot 024K4077) was purchased from Sigma. 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 EU (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. Then, all animals 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-(Aminomethyl)benzyl)acetamidine (1400W, in 4 mL/kg/h of saline). These infusion of 1400W was preceded by bolus injections of 3 or 10 mg/kg i.v. In an others experimental groups the animals received FeTPPs (10-100 mg/kg i.v.) 1h 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 (1610 x g 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 assessed by measuring the rises 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 non-specific 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, PBS) at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Sections (thickness 7 µm) were de-paraffinized with xylene, stained with hematoxylin and eosin and studied using light microscopy (Dialux 22 Leitz).

Immunohistochemical localization of nitrotyrosine

Tyrosine nitration, an index of the nitration of proteins by peroxynitrite and/or oxygen-derived free radicals, was determined by immunohistochemistry as previously described (Misko, 1993). At six hours 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 de-paraffinization, 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 PBS 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 anti-nitrotyrosine 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. In order to confirm that the immunoreactions for the nitrotyrosine were specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity.
Isolated Tissue Experiments

Four aortic rings were prepared from each of 10 rats. Briefly, thoracic aortas were removed from male Sprague Dawley rats 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 Kreb’s bicarbonate buffer, pH 7.4, bubbled with 5% CO2/95% O2. Rings were preloaded with 1g of tension and equilibrated for 30 min with 2-3 buffer changes. After stabilization of the baseline, the rings were contracted with phenylephrine. Doses of phenylephrine that produced 90-100% contraction were chosen for subsequent experiments. NO-mediated relaxation of the pre-contracted, 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 maximum tone developed to phenylephrine.

Nitrite/Nitrate (NOx) Assay

NO reacts with oxygen to yield nitrate and nitrite. We therefore 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 MWCO, Millipore, Bedford, MA). Nitrate in plasma aliquots (5 µl) was converted to nitrite by the addition of nitrate reductase (14 λ) 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 addition of the diaminonapthalene 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 expressed as plasma NOx levels (µM). 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 (St. Louis, MO, USA). 1400W was purchased from Alexis Biochemicals (San Diego, CA, USA).

Statistical Analysis

All values in the figures and text are expressed as mean ± standard error of the mean of n observations, where n represents the number of vascular rings or rats studied (10 rats or rings from 2-3 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 ANOVA followed by a Bonferroni 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 (Figure 1A). When 1400W (3-10 mg/kg, Figure 1A) was administered 30 minutes prior to LPS infusion, there was a dose-dependent delay in the development of late phase hypotension when compared to control. However, regardless of 1400W pre-treatment, mean arterial pressure fell sharply after 5 h, such that 100% mortality was observed by 7 h (Figure 1A; n = 6). At the highest dose tested 1400W (10 mg/kg, Figure 1A) had no effect of mean arterial pressure, a result consistent with their selectivity for iNOS. LPS also caused a time dependent increase in circulating nitrite/nitrate (NOx) concentrations (Figure 1B). On the other hand, and consistent with its selectivity for iNOS, 1400W blocked in a dose-dependent manner, NOx release at 2, 3 and 6 hr but not 1 hr (NO derived from eNOS) (Figure 1B). Please note that when either other two iNOS inhibitors aminoguanidine (10-100 mg/ kg) or L-NIL (3-30 mg/ kg) were administered 1 h prior to LPS there was a dose-dependent delay in the development of late phase hypotension when compared to 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 between 125 ± 3-130 ± 5 mm Hg and were not significantly different between groups (Figure 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 approximately 7 h (Figure 3). However, administration of FeTPPs 1 h prior to endotoxemia dose dependently inhibited (10-100 mg/kg; Figure 3) arterial hypotension. Furthermore, at doses of 10 and 30 mg/kg, FeTPPs also significantly prevented mortality up to the 9 h time point (Figure 3).

**Effects of the peroxynitrite decomposition catalyst FeTPPs on endotoxin-induced multiple organ dysfunction**

*Renal dysfunction:* In non-endotoxemic control rats, administration of saline did not significantly alter plasma creatinine levels (Figure 3A). In contrast, at 6 hr 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 (Figure 3A).

*Pancreatic injury:* As for plasma creatinine, no significant alterations in plasma levels of lipase and amylase were observed in saline-infused animals (Figure 3BC). In contrast, at 6 hr post LPS injection, significant increases in plasma levels of lipase and amylase were observed (Figure 3BC) and event blocked by FeTPPs (100 mg/kg) (Figure 3BC).

*Liver injury:* No significant elevations in the plasma levels of ALT, AST, bilirubin, and alkaline phosphatase were noted in saline control rats (Figure 4). In contrast, at 6 hr post LPS injection, significant elevations in plasma levels of ALT, AST, bilirubin-and alkaline phosphatase, compatible with the development of hepatocellular injury and cholestasis.
was observed (Figure 4). As for renal and pancreatic markers of organ dysfunction, treatment with 100 mg/kg of FeTPPs, abolished the liver dysfunction caused by LPS (Figure 4).

**Effects of the peroxynitrite decomposition catalysts FeTPPs on endotoxin-induced liver nitrotyrosine formation and histopathology**

Immunohistochemical analysis, using a specific anti-nitrotyrosine antibody, revealed a positive staining in liver from LPS-treated at 6 hours post injection (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. 6A;A1) compared to 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 hr 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 (Figure 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 (Figure 7).

In order to evaluate the effects of LPS on endothelium-dependent vasorelaxation, endothelium-intact rings were pre-contracted with a dose of phenylephrine that gave a submaximal contraction ($10^{-7}$ M and $5 \times 10^{-5}$ 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; Figure 8A). In rings taken from control animals, acetylcholine induced relaxation with an EC$_{50}$ of $4.2 \times 10^{-8}$ M. In contrast, tissues from LPS-treated rats had a significantly impaired relaxation response to acetylcholine (EC$_{50}$ for acetylcholine was $3.1 \times 10^{-6}$ M). (Figure 8A). To evaluate the effects of LPS on endothelium-independent vasorelaxation, the endothelium was removed by gentle rubbing of the ring preparation and rings relaxed with the endothelium-independent vasodilator, sodium nitroprusside ($10^{-9}$-$10^{-7}$ M). Successful removal of the endothelium was confirmed by the lack of a relaxation response to acetylcholine ($10^{-5}$ M). There was no difference between the vasorelaxant responses obtained with sodium nitroprusside in tissues derived from saline control or LPS-treated rats, demonstrating that the smooth muscle layer was not impaired by LPS (Figure 8B). Of note, FeTPPs (30 mg/kg, i.v) administered 1 h prior to LPS significantly attenuated the LPS-induced contractile dysfunction (EC$_{50}$ for phenylephrine...
was 2.42 \times 10^{-7} \text{ M} \) and endothelial impairment (EC_{50} for acetylcholine was 5.03 \times 10^{-7} \text{ M} \) (Figures 7, 8A).
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 1-2 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 ecNOS enzymatic activity and/or down regulation of ecNOS expression (Lu et al., 1996). Alterations in ecNOS have thus been found at both early and late time points following 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 also been shown to attenuate ecNOS expression by reductions in ecNOS 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 ecNOS expression (Yoshizumi et al., 1993).

The findings we report 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 dependency, 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 ecNOS activity (as assessed by monitoring the responses to acetylcholine, an endothelium-dependent vasodilator) at a time point where iNOS was already induced.
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 ecNOS activity or down regulation of the expression. The former possibility is more plausible since down regulation of ecNOS mRNA and protein levels by LPS/cytokines require at least 3 or more (Yoshizumi et al., 1993). Other reports have also alluded to this concept of dependency 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 ARDS in animal models (Cobb et al., 1999). Similarly, endotoxin down regulates ecNOS 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 ecNOS are impaired (Kubes, 1993). In contrast, better outcomes have been more consistently observed following inhibition of peroxynitrite formation. Szabò and colleagues (Szabò et al., 1995) have 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 by the peroxynitrite decomposition catalyst. 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 (Wray et al., 1998).

We contend, therefore, 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 are likely to be more promising than approaches which 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 (Figure 9).

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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.


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FIGURE LEGENDS

Figure 1. Administration of *E. coli* LPS serotype 0111:B4 (4 mg/kg) results in the development of irreversible hypotension in the anaesthetized rat (A). Treatment with 1400W (3-10 mg/kg) 30 minutes 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). In addition, the increase in plasma levels of nitrite/nitrate (NOx) seen at 2, 3 and 6 h but not 1 h post LPS is attenuated in a dose-dependent manner by 1400W (B). Each point represents the mean ± SEM of 10 rats for each group. *P < 0.01 versus Saline. #P < 0.01 versus LPS

Figure 2. Administration of LPS (4 mg/kg) results in the development of irreversible hypotension in the anaesthetized rat. Treatment with the active peroxynitrite decomposition catalyst FeTPPs (10-100 mg/kg) prevented in a dose-dependent manner hypotension and mortality when given alone at the highest dose tested, FeTPPs (100 mg/kg) had no effect on MAP. Each point represents the mean ± SEM of 10 rats for each group. *P < 0.01 versus Saline. #P < 0.01 versus LPS

Figure 3. Effect of FeTPPs on pancreatic and renal injury. Creatinine (A), amylase (B) and lipase (C) plasma levels resulted significantly increased in LPS-treated rats. FeTPPs (30 mg/kg) treatment significantly decreases all these parameters. Data are means of mean ± SEM of 10 rats for each group. *P < 0.01 versus Saline. #P < 0.01 versus LPS
**Figure 4.** Effect of FeTPPs on liver injury. Plasma levels of AST (A), ALT (B), bilirubin (C) and alkaline phosphatase (D) resulted significantly increased in LPS-treated rats. FeTPPs (100 mg/kg) treatment significantly reduced all these parameters. Data are means of mean ± SEM of 10 rats for each group. *P < 0.01 versus Saline. #P < 0.01 versus LPS.

**Figure 5.** Immunohistochemical localization of nitrotyrosine in the rat liver. No positive staining for nitrotyrosine was observed in the liver obtained from saline-infuse rats (A). In contrast, nitrotyrosine staining was found in the liver from LPS-treated rats (B). In particular, the positive stainings are mainly localized in the nuclei of hepatocytes (B1 see arrows). There was no detectable immunostaining in the liver (C) of LPS-treated rats treated with FeTPPs. Figure is representative of at least 3 experiments performed on different experimental days.

**Figure 6.** Liver histopathology

No histological alteration was observed in the liver collected from saline control (C). Representative liver sections from LPS-treated rats demonstrate hepatic injury characterized by focal damage of hepatic parenchyma (A1) and inflammatory cells infiltration (A see arrow). Liver sections from LPS-treated rats treated with FeTPPs (B) demonstrate a significantly less hepatic damage and no inflammatory cell infiltration. Figure is representative of at least 3 experiments performed on different experimental days.
Figure 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^{-9}-10^{-4} M) to evoke such contractions in tissues harvested from LPS treated rats was significantly inhibited. FeTPPs (100 mg/kg) significantly prevent the LPS-induced contractility dysfunction. Each point represents the mean ± SEM of six experiments. *P < 0.01 versus Saline. #P < 0.01 versus LPS

Figure 8. Administration of acetylcholine (10^{-9}-10^{-5} M) to aortic tissues elicited a dose-dependent relaxation (depicted as % relaxation of induced tone) indicating the release of NO from the endothelial layer of these tissues (A). This response was diminished in rings taken from LPS-treated rats when compared to control tissues. FeTPPs (100 mg/kg) significantly prevent the LPS-induced endothelial dysfunction. In another series of experiments (B), endothelium denuded aortic rings (evidenced by lack of vasorelaxation to acethylcholine) from saline, LPS treated rats or FeTPPs-treated rats were pre-contracted with a submaximal concentration of phenylephrine. Administration of sodium nitroprusside (10^{-9}-10^{-7} M) to these tissues elicited a dose-dependent relaxation (depicted as % 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 ± SEM of six experiments. *P < 0.01 versus Saline. #P < 0.01 versus LPS

Figure 9. When a pathological event occurs (e.g. immune response or inflammation), lipopolysaccharide (LPS), cytokines, or both rapidly shift the equilibrium
towards phosphorylated nNOS or eNOS, resulting in the abrupt fall in intracellular NO levels (i.e. very low NO levels). This situation can favor the activation of inducible NOS (iNOS) and the production of high levels of NO. The NO so generated combines with superoxide, forming peroxynitrite. Peroxynitrite exerts direct cellular toxicity through oxidative and nitrosative damage and PARP activation. NO, and perhaps peroxynitrite, activate inflammatory cascades through the activation of NF-κB, enhancing inflammation manifested by augmented cytokine expression, STAT3 activation, and tissue neutrophil accumulation. Collectively, these actions result in organ damage and dysfunction. In this schema, PN decomposition catalyst, SOD mimic, or PARP inhibitor each may prevent organ dysfunction and improve outcome.
Figure 1

A

- Saline
- Saline + 1400W 10 mg/kg
- LPS
- LPS + 1400W 3 mg/kg
- LPS + 1400W 10 mg/kg

B

- Control
- 1400W 3 mg/kg
- 1400W 10 mg/kg

Mean arterial blood pressure (mmHg)

Plasma Nitrate/Nitrite (µM)

Time (h)

0 1 2 3 4 5 6 7 8 9

0 20 40 60 80 100 120 140

0 1 2 3 6

Time points post LPS (h)

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Figure 2

![Graph showing mean arterial blood pressure over time for different conditions: Saline, LPS, LPS+FeTPPS 100 mg/kg, LPS+FeTPPS 10 mg/kg, LPS+FeTPPS 30 mg/kg. The graph includes error bars and asterisks indicating statistical significance.]
Figure 3

A

CREATININE (μM)

Vehicle
FeTTPS

SALINE
LPS

B

AMYLASE (U/L)

SALINE
LPS

C

LIPASE (U/L)

SALINE
LPS

* indicates significant difference from saline group.
# indicates significant difference from FeTTPS group.
Figure 6
Figure 7

[Graph showing the effect of phenylephrine on different conditions: Saline, LPS, and FeTTPs. The x-axis represents phenylephrine concentration (M), and the y-axis represents % contraction.]
Figure 8

A

[Graph showing the relaxation of induced tone in response to varying concentrations of acetylcholine.]

B

[Graph showing the relaxation of induced tone in response to varying concentrations of sodium nitroprusside.]
Figure 9

LPS or immune cytokines

nNOS or eNOS

nNOS-Y-(P) or eNOS -Y-(P)

iNOS

O2-

SODm

PN decomposition Catalyst

Very low NO Levels

High NO Levels, ONOO-

NFκB/ IκB

Inflammation
• cytokine expression
• STAT activation
• PMN accumulation

Direct Toxicity
• Oxidative stress

DNA strand breaks

Organ Injury

PARP inhibitors

PARP activation