Peroxynitrite Decomposition Catalysts Prevent Myocardial Dysfunction and Inflammation in Endotoxemic Rats

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OBJECTIVES

The aim of this study was to test whether peroxynitrite neutralizers would reduce peroxynitrite accumulation and improve myocardial contractile dysfunction and inflammation in endotoxin-treated rats.

BACKGROUND

Release of endogenous proinflammatory cytokines such as tumor necrosis factor (TNF)-alpha in response to endotoxin is responsible for the production of large amounts of nitric oxide (NO), which may exert detrimental effects on the myocardium in animal models, isolated hearts, and isolated cardiac myocytes. Recent studies have indicated that many of the deleterious effects of NO are mediated by peroxynitrite, a powerful oxidant generated from a fast diffusion-limited reaction of NO and superoxide anion.

RESULTS

Mercaptoethylguanidine sodium succinate and FeTPPS largely prevented the accumulation of peroxynitrite as measured by plasma rhodamine fluorescence and heart nitrotyrosine staining. Interestingly, MEG sodium succinate and FeTPPS improved endotoxin-induced myocardial contractile dysfunction, which was associated with reduced degradation of nuclear factor kappa B inhibitory protein I-kappa-B, plasma TNF-alpha levels, and microvascular endothelial cell-leukocyte activation.

CONCLUSIONS

These observations suggest that the beneficial effects of MEG and FeTPPS on endotoxin-induced myocardial contractile dysfunction could be related to the unique effects of these compounds on cardiovascular inflammation processes. (J Am Coll Cardiol 2004;43:2348–58) © 2004 by the American College of Cardiology Foundation

Impaired myocardial contractile function is a well-documented feature in both experimental and human septic shock (1–4) that greatly contributes to the mortality associated with this pathologic condition (5,6). Evidence from our laboratory and from others suggests that exposure of animal to bacterial endotoxin (lipopolysaccharides) exerts deleterious effects on myocardial function (3,7,8). Several hypotheses have been proposed to explain endotoxin-induced cardiovascular failure, including microvascular dysfunction, the presence of activated leukocytes, and the effects of various circulating and/or locally produced proinflammatory cytokines, such as tumor necrosis factor (TNF)-alpha and interleukin (IL)-1beta (9). Most of the deleterious effects of proinflammatory cytokine release have been attributed to the production of large amounts of nitric oxide (NO), which may exert detrimental effects on the myocardium in animal models, isolated hearts, and isolated cardiac myocytes (7,10,11). Deleterious effects of NO have been mainly reported in biological conditions in which reactive oxygen species generation is also enhanced (12–16). The relevance of these biological events in mediating myocardial dysfunction is further illustrated by the finding that antioxidant therapy (17) and inhibition of NO synthase activity can improve endotoxin and cytokine-induced contractile dysfunction (10,17–20). However, this contention has been challenged by the results showing that selective inducible nitric oxide synthase (iNOS) inhibitors may worsen myocardial function in sepsis (21,22) and that genetic deficiency of iNOS was associated with no myocardial protection or reduction in survival in endotoxin-treated mice (23,24).

As a different concept, NO may interact with oxidants (e.g., O$_2$) to form peroxynitrite, a potent oxidant that can exert deleterious effects on biological materials (12,13,25). Cellular targets of peroxynitrite include lipid peroxidation, nitration of tyrosine residues, oxidation of sulfhydryl groups, deoxyribonucleic acid-strand breakage, and inhibition of mitochondrial respiration, leading to tissue injury (26–29). For example, endogenous formation of peroxynitrite contributes to myocardial stunning in ischemia reperfusion injury (26), cytokine and endotoxin-induced contractile dysfunction (25,27), and to spontaneous loss of cardiac...
function in the isolated working heart (28). In these pathophysiological models, one very important defect resulting from peroxynitrite generation is endothelial injury, which is manifested by enhanced expression of adhesion molecules and P-selectin in human endothelial cells (30), and IL-8 expression in human leukocytes (31). In human neutrophils, peroxynitrite triggers the downregulation of L-selectin expression, and upregulation of CD11/CD18 expression (31). These effects are likely to be mediated by the ability of peroxynitrite to trigger nuclear factor (NF)-κB activation (32).

The development of peroxynitrite neutralizers has provided a more direct approach to assess the role of peroxynitrite in organ injury in a variety of inflammation states (33,34). For example, mercaptoethylguanidine (MEG), a peroxynitrite scavenger and iNOS inhibitor (33) and 5,10,15,20-tetrakis(4-sulfonatophenyl)-porphyrinato iron (III) (FeTPPS), which catalyzes the isomerization of peroxynitrite to nitrate anion (34), may decrease the generation of highly reactive intermediates such as nitrogen dioxide and hydroxyl radicals. While peroxynitrite neutralizers may attenuate inflammatory processes associated with ischemia reperfusion (35), colitis (36), and carrageenan-paw edema and pleurisy (37), their effects have not yet been evaluated in endotoxin-induced cardiovascular inflammation. In the present study, the implication of peroxynitrite generation in the septic myocardial dysfunction was tested in rats infused with Escherichia coli endotoxin. First, we tested whether MEG and FeTPPS would reduce peroxynitrite accumulation and improve myocardial contractile dysfunction in endotoxin-treated rats. Second, we tested whether MEG and FeTPPS would attenuate inflammatory response in terms of degradation of nuclear factor (NF)-κB inhibitory protein I-κB-α, plasma TNF-alpha, heart iNOS expression, and endothelial cell-leukocyte activation.

**METHODS**

**Animal preparation.** Sprague Dawley rats (Dépré, Saint Doulchard, France) (weight 300 to 350 g) were housed for six days in groups of six in standard cages and supplied with laboratory chow and tap water. Endotoxemia was carried out by intravenous injection of 10 mg/kg body weight endotoxin, *E. coli* 055:B5 (Sigma, Saint Quentin Fallavier, France), in 1 ml under brief ether anesthesia. As a control, other animals received injection of an equal volume of sterile saline. In vivo treatment with N-nitro-L-arginine methyl ester (L-NAME) (25 and 50 mm/kg, intraperitoneally) (Sigma), MEG sodium succinate (10 mg/kg, intravenously) (Inotek Pharmaceuticals Corp., Beverly, Massachusetts), and FeTPPS (30 mg/kg, intravenously) (Calbiochem, VWR International SAS, Strasbourg, France) were administered with either saline or endotoxin. After treatment, rat hearts were prepared for either physiological measurements or for in vitro assays. In parallel, blood samples were collected via abdominal aorta puncture. The study was performed in accordance with National Institutes of Health guidelines for the use of experimental animals and with approval from our institution’s animal research committee.

**Isolated and perfused heart preparation.** Myocardial contractile function was studied using a modified Langendorff isolated heart preparation as we have previously described (8). Briefly, after heparinization and ether anesthesia, the heart was rapidly excised and placed into ice-cold Krebs-Henseleit (KH) buffer solution. Then the heart was mounted onto a Langendorff heart perfusion apparatus and perfused in a retrograde fashion via the aorta at a constant flow rate of 10 ml/min with aerated (95% O2 to 5% CO2) KH buffer at 37°C. Cardiac contractile function was assessed using a water-filled latex balloon inserted into the left ventricular cavity and connected to a pressure transducer. This balloon was then adjusted to a left ventricular end-diastolic pressure of 5 mm Hg. The heart was paced at 300 beats/min and allowed to equilibrate for 30 min. Left ventricular developed pressure, its first derivatives (dP/dtmax and dP/dt(min)) and coronary perfusion pressure were monitored and recorded using a Biopac Data Acquisition System (Biopac Systems Inc., Goleta, California).

**Peroxynitrite determination in plasma.** The formation of peroxynitrite was estimated by means of peroxynitrite-dependent oxidation of dihydrorhodamine 123 (Molecular Probes, Eugene, Oregon) to rhodamine as previously described (37,38). Briefly, plasma samples were taken for rhodamine fluorescence evaluation using a fluorometer at an excitation wavelength of 500 nm and an emission wavelength of 530 nm.

**TNF-alpha and antiadhesive molecule endocan determination in plasma.** Plasma levels of TNF-alpha were determined 4 h after treatment by use of commercial immunoassay kits (ELISA) specific for rat cytokines (Quantikine Murine rat TNF, R&D Systems, Abingdon Oxford, United Kingdom). Reading was realized in a microplate readerDigiscan (Spectracount Packard, Packard Instrument Company, Meriden, Connecticut).

Endocan, previously called endothelial-cell-specific molecule-1, a newly described endothelial proteoglycan molecule that binds directly to the integrin CD11a/CD18 (LFA-1) and blocks the binding to intercellular adhesion molecule-1, was determined by the use of specific mouse/rat endocan ELISA developed by P. Lassalle (INSERM U416) (39).
Nitrite/nitrate concentration in plasma. Nitrite/nitrate levels, an indicator of NO synthesis, were measured in plasma samples as previously described. First, nitrate in the plasma was reduced to nitrite by adding nitrate reductase (25 mU/ml; Sigma) and NADPH (200 μM; Calbiochem) at room temperature. After 3 h, samples were deproteinized by adding a solution of ZnSO4 30%; 15 min later, samples were centrifuged at 2,000 g for 10 min. Nitrite concentration in the samples was measured by the Griess reaction: 100 μl of Griess reagent (0.1% naphthalethylenediamine dihydrochloride in H2O and 1% sulfanilamide in 5% concentrated H3PO4; vol 1:1; Molecular Probes) were added to 100 μl of supernatants. The optical density at 550 nm (OD550) was measured using a microplate reader. Nitrate concentrations were calculated by comparison with OD550 of standard solutions of sodium nitrite (35).

Measurements of heart myeloperoxidase activity. Myeloperoxidase activity in the left ventricle was used as an index of leukocyte infiltration (35). Briefly, left ventricles were placed in a 20-mM phosphate buffer (pH 7.4) at 10% w/vol and homogenized; 1 ml of the homogenate was then adjusted to a total volume of 10 ml with 20 mM phosphate buffer (pH 7.4) and centrifuged at 6,000 g for 20 min at 4°C. The pellet was re-homogenized and sonicated for 10 s in 1 ml of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl-ammonium bromide detergent (Sigma). After a centrifugation at 12,500 g for 3 min, 20 μl of the prepared samples were used in reactions for myeloperoxidase activity determined spectrophotometrically (650 nm) by measuring hydrogen peroxide-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine (Sigma).

Heart morphologic analysis for leukocyte infiltration. Left ventricular tissue sections (5-mm thick) from hearts fixed in 10% formaldehyde solution were dehydrated and embedded in paraffin. Serial sections (4-μm thick) were stained with hematoxylin and eosin for myocardial leukocyte content. The number of random fields needed to count 60 leukocytes in each group at ×400 magnification was recorded. The average number of leukocytes per ×400 field was then determined as a semiquantitative assessment of myocardial leukocyte content (Mocha Software, Jandel, San Rafael, California) (40).

Leukocyte adhesion in the mesenteric venules. Rats were anesthetized with 50 mg/kg ketamine xylazine intramuscularly, and the carotid artery was cannulated with a polyethylene 50 tube connected to a pressure transducer (Kontron, Basel, Switzerland) to monitor mean arterial pressure. The abdomen was then opened via a midline laparotomy, and a segment of the distal ileum was gently exposed and mounted in an optical chamber. Thanks to this design, the exposed bowel wall within the chamber was superfused with a thermostat-controlled saline solution maintained at 37°C. After a 30-min equilibration period, the mesenteric microcirculation was observed with the use of an intravital microscope. An Eclipse E800 Nikon microscope (Nikon, Tokyo, Japan) fitted with a Xenon light
Table 1. Effects of L-NAME, MEG Sodium Succinate, and FeTPPS on Endotoxin-Induced Myocardial Dysfunction at 16 h After Treatment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Endotoxin</th>
<th>Endotoxin L-NAME</th>
<th>Endotoxin MEG</th>
<th>Endotoxin FeTPPS</th>
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<tr>
<td>LVDP</td>
<td>98 ± 8</td>
<td>70 ± 4*</td>
<td>65 ± 7*</td>
<td>92 ± 6†</td>
<td>93 ± 8†</td>
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<tr>
<td>dP/dt&lt;sub&gt;max&lt;/sub&gt;</td>
<td>2,200 ± 220</td>
<td>1,700 ± 135*</td>
<td>1,500 ± 150*</td>
<td>1,950 ± 345†</td>
<td>2,000 ± 140†</td>
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<tr>
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<td>1,300 ± 100*</td>
<td>1,100 ± 250*</td>
<td>1,700 ± 140†</td>
<td>1,840 ± 105†</td>
</tr>
<tr>
<td>CPP</td>
<td>48 ± 10</td>
<td>43 ± 2</td>
<td>68 ± 3†</td>
<td>46 ± 1</td>
<td>45 ± 2</td>
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Myocardial function was evaluated at 16 h in rats treated with saline, endotoxin (10 mg/kg), endotoxin and N-nitro-L-arginine methyl ester (L-NAME) (50 mg/kg), endotoxin and mercaptoethylguanidine (MEG) sodium succinate (10 mg/kg), and endotoxin and 5,10,15,20-tetrakis(4-sulfonatophenyl)-porphyrinato iron (III) (FeTPPS) (30 mg/kg). See Methods section for treatment group design. Results are expressed as mean ± SEM (n = 6 in each group). *p < 0.01 compared with control; †p < 0.01 compared with endotoxin-treated rats.

CPP = coronary perfusion pressure; dP/dt<sub>max</sub> = maximum rate of left ventricular pressure rise; dP/dt<sub>min</sub> = maximum rate of left ventricular pressure fall; LVDP = left ventricular developed pressure.

source and epi-fluorescence assembly was used with filter sets for acridine orange (excitation: 470 nm FWHM 40; emission 540 nm FWHM 40). A video-camera HAMAMATSU C2400-08 (Hamamatsu City, Japan) mounted on the microscope projected the image onto a monitor, and the images (720 × 576 pixels) were recorded for playback analysis with digital video cassette recorder SONY DVR 30. Magnification ×40 was used. Consequently, the final resolution was 0.5 μm per pixel. As previously described, single unbranched mesenteric venules (25 to 40 μM in diameter) were selected for study of each animal. Red blood cell velocity (VRBC) was measured off-line as the distance through which packed red blood cells or plasma break traveled within two subsequent video frame time intervals of 40 ms. Mean red blood cell velocity was determined as VRBC/1.6, and wall shear rate was calculated based on the Newtonian definition: shear rate = (VRBC/Dv) × (8s<sup>–1</sup>), where Dv is the venular diameter. The leukocyte behavior in the venules was observed for a 1-min period after the injection of 1 ml of 1% w/v acridine orange into the carotid artery. Leukocyte rolling flux was determined off-line during play-back of videotaped images by counting the number of leukocytes distinguishable from the blood stream passing a line perpendicular to the vessel axis. Flux of rolling leukocytes was measured as the number of white blood cells that could be seen rolling past a fixed perpendicular line in the venule during a 1-min interval. Quantification of venular endothelium leukocyte adherence was performed off-line during play-back of videotaped images by counting the number of leukocytes that stuck and remained stationary for a period >30 s per 100 μm of venule (40). The number of emigrated leukocytes was determined off-line during play-back of videotaped images by counting the number of leukocytes surrounding the venule (40). Leukocyte emigration was expressed as the number of leukocytes per 100 μm of venule.

**Immunohistochemistry for nitrotyrosine and Western blot analysis for I-kappa-B and iNOS.** Immunolocalization of nitrotyrosine, a “footprint” of peroxynitrite formation, was evaluated 4 h after treatment. Paraffin sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Sections were treated with hydrogen peroxide to block endogenous peroxidase activity, and were rinsed briefly in PBS. To detect nitrotyrosine, routine histochemical procedure was applied as previously described (14). Rabbit polyclonal antinitrotyrosine antibody (Upstate Biotechnology, Lake Placid, New York) was applied in a dilution of 1:500 overnight at 4°C. Immunoreactivity was detected with a biotinylated horse antirabbit secondary antibody and the avidin–bixin-peroxidase complex, both supplied by the Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, California). 3,3’–diaminobenzidine (Vector Laboratories) was used to reveal peroxidase activity. Negative controls were obtained by omitting the primary antibody. Sections were counterstained with hematoxylin and mounted.

For Western blot analysis, hearts were homogenized in RIPA buffer (in mmol/l: Tris 10, NaCl 140, EDTA 5, PMSF 1, with Triton X-100 1%, Deoxycholate 1%, SDS 0.1%, and in μg/ml: aprotinin 10, leupeptin 10, pepstatin 10, at pH 7.4). Proteins (50 μg) were run on a 12% SDS-PAGE. The proteins on the gel were electrophoretically transferred to nitrocellulose membranes. After blocking, membranes were treated with mouse monoclonal anti–I–kappa-B (Santa Cruz Biotechnology Inc.), rabbit polyclonal anti-iNOS (Upstate Biotechnology), and rabbit polyclonal anti-G3PDH (Trevigen) antibodies. Membranes were then incubated with horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit immunoglobulin G (IgG) secondary antibody (Biorad), washed, and bound antibodies were detected using chemiluminescence with an ECL Plus kit (Amersham Biosciences Europe GmbH).

**Statistical analysis.** For in vitro and cardiac function studies, we tested for differences using analysis of variance procedure (SPSS for Windows 9.0). When a significant difference was found, we identified specific differences between groups using a sequentially rejective Bonferroni procedure (41). After application of a Bonferroni correction, significance was achieved with a value of p < 0.01 for comparisons with control. Data are presented as means ± SEM throughout.
RESULTS

Effects of L-NAME, MEG, and FeTPPS on myocardial dysfunction induced by endotoxin. In the first series of experiments, myocardial dysfunction was evaluated at 4 h after endotoxin injection. As shown in Figure 1, left ventricle developed pressure and its maximal first derivatives (i.e., dP/dt max) were significantly decreased 4 h after administration of endotoxin as compared with control animals; MEG hydrochloride, unstable in solution, was unable to improve myocardial function in septic rats (data not shown). Injection of FeTPPS (30 mg/kg) or MEG sodium succinate (10 mg/kg) with endotoxin largely prevented left ventricular systolic function alterations of endotoxin-treated hearts (n = 8 in each group) (Fig. 1). Injection of L-NAME at 25 mg/kg (data not shown) and 50 mg/kg with endotoxin had no effects on left ventricular systolic function of endotoxin-treated hearts (n = 8 in each group). Injection of L-NAME at 50 mg/kg increased coronary perfusion pressure in both saline and endotoxin-treated rats (Fig. 1). Treatments with MEG sodium succinate and FeTPPS had no effects on left ventricular systolic function in saline-treated control rats (n = 5 in each group) (data not shown).

In the second series of experiments, myocardial dysfunction was evaluated at 16 h after endotoxin injection. Left ventricle developed pressure and its maximal first derivatives

Figure 2. (A) Plasma rhodamine fluorescence intensity (n = 8 in each group) and (B) representative heart section (n = 3 in each group) stained for nitrotyrosine by immunohistochemistry of rats treated with saline, endotoxin (10 mg/kg), endotoxin and L-NAME (50 mg/kg), endotoxin and MEG sodium succinate (10 mg/kg), and endotoxin and FeTPPS (30 mg/kg). See Methods section for treatment group design. Results are expressed as mean ± SEM. *p < 0.01 compared with control; #p < 0.01 compared with endotoxin-treated rats. Abbreviations as in Figure 1.
(i.e., dP/dt\text{max}) were significantly decreased 16 h after administration of endotoxin as compared with control animals. Endotoxin-induced myocardial dysfunction was not prevented by L-NAME treatment, whereas FeTPPS (30 mg/kg) or MEG sodium succinate (10 mg/kg) treatment had beneficial effects in endotoxin-treated rats at 16 h (Table 1).

Because myocardial dysfunction was maximal at 4 h after endotoxin injection, evaluation of biological parameters were performed at this time point.

**Peroxy nitrite neutralizers, MEG and FeTPPS, reduced formation plasma rhodamine fluorescence and nitrotyrosine in endotoxin-treated rats.** Injection of endotoxin caused at 4 h a significant increase in rhodamine fluorescence of plasma, which is indicative of peroxynitrite-induced oxidation of dihydrorhodamine 123 to rhodamine. As opposed to L-NAME at 50 mg/kg, MEG sodium succinate and FeTPPS reduced the oxidation of dihydrorhodamine 123 (Fig. 2A); MEG hydrochloride had no effects on peroxynitrite formation in endotoxin-treated rats (data not shown). At 4 h after endotoxin treatment, heart sections were analyzed for the presence of nitrotyrosine, a footprint of peroxynitrite. Myocardial staining for nitrotyrosine was nearly absent in saline-treated rats. In contrast, there was an abundant myocardial nitrotyrosine immunostaining in endotoxin-treated rats at 4 h, which was prevented by MEG sodium succinate and FeTPPS but not by L-NAME (Fig. 2B).

**Figure 3.** Effects of L-NAME (50 mg/kg), MEG sodium succinate (10 mg/kg), and FeTPPS (30 mg/kg) on endotoxin-induced heart I-kappa-B degradation and plasma tumor necrosis factor (TNF)-alpha levels. See Methods section for treatment group design. (A) Degradation of I-kappa-B (n = 5 in each group). Semiquantitative analysis was performed on the basis of relative I-kappa-B/G3PDH density. (B) Plasma TNF-alpha levels (n = 6 in each group). Results are expressed as mean ± SEM. \(*p < 0.01\) compared with control; \(#p < 0.01\) compared with endotoxin-treated rats. Abbreviations as in Figure 1.
FeTPPS in vivo treatment largely prevented I-kappa-B degradation (Fig. 3A).

The rationale for targeting TNF-alpha in plasma was based on previous studies showing that NF-kappa-B is intimately involved in transactivating the gene encoding TNF-alpha (41). Endotoxin induced increases in plasma TNF-alpha levels, which were largely reduced by injection of FeTPPS (30 mg/kg) and MEG sodium succinate (10 mg/kg) (n = 8 in each group) (Fig. 3B). Injection of L-NAME (50 mg/kg) increased plasma TNF-alpha levels at 4 h endotoxin. Treatments with L-NAME, MEG sodium succinate, and FeTPPS had no effects on plasma TNF-alpha levels in saline-treated control rats (n = 5 in each group) (data not shown).

Effects of peroxynitrite neutralizers, MEG and FeTPPS, on endotoxin-induced myocardial leukocyte infiltration and endothelium-leukocyte activation. Heart morphologic analyses for leukocyte count of the left ventricular myocardium (n = 5 in each group) were performed. Compared with saline-treated rats, the number of leukocytes in the myocardium increased at 4 h of endotoxia (25 ± 2 vs. 4 ± 1 leukocytes per field; p < 0.01). As opposed to L-NAME, FeTPPS and MEG sodium succinate prevented increases in myocardial leukocyte count in endotoxin-treated rats (endotoxin: 25 ± 2 vs. endotoxin–L-NAME: 29 ± 5, endotoxin–MEG: 9 ± 2, endotoxin–FeTPPS: 7 ± 2 leukocytes per field; p < 0.01). Treatments with L-NAME, MEG sodium succinate, and FeTPPS had no effects on myocardial leukocyte count in saline-treated control rats (n = 3 in each group) (data not shown).

Myeloperoxidase activity, an index of leukocyte tissue sequestration, was increased in left ventricle of endotoxin-treated rats (Fig. 4A). As opposed to L-NAME and MEG sodium succinate, FeTPPS prevented increases in myocardial myeloperoxidase activity in endotoxin-treated rats (n = 5 in each group). Treatments with L-NAME, MEG sodium succinate, and FeTPPS had no effects in saline-treated control rats on myocardial myeloperoxidase activity (n = 5 in each group) (data not shown).

Endocan, a proteoglycan molecule secreted by endothelial cells, which reduce both the recruitment of circulating leukocyte and integrin-dependent leukocyte adhesion and activation, was elevated in plasma of endotoxin rats treated with MEG sodium succinate and FeTPPS (Fig. 4B). Compared with endotoxin-treated rats, plasma endocan levels were reduced in L-NAME-treated rats.

Leukocyte endothelium interactions within the microvasculature were evaluated using intravital microscopy of the mesentery venule. Endotoxin dramatically reduced shear rate compared with saline-treated rats (n = 6 in each group: 550 ± 60 vs. 205 ± 15 s⁻¹; p < 0.01). In endotoxin-treated rats, L-NAME, MEG sodium succinate, and FeTPPS did not prevent the reduction of erythrocyte velocity and, thus, venular wall shear rate (n = 6 in each group: 315 ± 75, 250 ± 25, 240 ± 30 s⁻¹, respectively). This finding suggests that these compounds do not interfere with the physical forces that modulate leukocyte behavior within the microvasculature. In this context, L-NAME enhanced leukocyte rolling, adhesion, and emigration in the mesentery venule of endotoxin-treated rats (Fig. 5) (n = 6 in each group); MEG sodium succinate and FeTPPS did not prevent the reduction of erythrocyte velocity and, thus, venular wall shear rate (n = 6 in each group: 315 ± 75, 250 ± 25, 240 ± 30 s⁻¹, respectively). This finding suggests that these compounds do not interfere with the physical forces that modulate leukocyte behavior within the microvasculature. In this context, L-NAME enhanced leukocyte rolling, adhesion, and emigration in the mesentery venule of endotoxin-treated rats (Fig. 5) (n = 6 in each group); MEG sodium succinate and FeTPPS did not prevent the reduction of erythrocyte velocity and, thus, venular wall shear rate (n = 6 in each group: 315 ± 75, 250 ± 25, 240 ± 30 s⁻¹, respectively). This finding suggests that these compounds do not interfere with the physical forces that modulate leukocyte behavior within the microvasculature. In this context, L-NAME enhanced leukocyte rolling, adhesion, and emigration in the mesentery venule of endotoxin-treated rats (Fig. 5) (n = 6 in each group); MEG sodium succinate and FeTPPS did not prevent the reduction of erythrocyte velocity and, thus, venular wall shear rate (n = 6 in each group: 315 ± 75, 250 ± 25, 240 ± 30 s⁻¹, respectively). This finding suggests that these compounds do not interfere with the physical forces that modulate leukocyte behavior within the microvasculature. In this context, L-NAME enhanced leukocyte rolling, adhesion, and emigration in the mesentery venule of endotoxin-treated rats (Fig. 5) (n = 6 in each group); MEG sodium succinate and FeTPPS did not prevent the reduction of erythrocyte velocity and, thus, venular wall shear rate (n = 6 in each group: 315 ± 75, 250 ± 25, 240 ± 30 s⁻¹, respectively). This finding suggests that these compounds do not interfere with the physical forces that modulate leukocyte behavior within the microvasculature. In this context, L-NAME enhanced leukocyte rolling, adhesion, and emigration in the mesentery venule of endotoxin-treated rats (Fig. 5) (n = 6 in each group); MEG sodium succinate and FeTPPS did not prevent the reduction of erythrocyte velocity and, thus, venular wall shear rate (n = 6 in each group: 315 ± 75, 250 ± 25, 240 ± 30 s⁻¹, respectively). This finding suggests that these compounds do not interfere with the physical forces that modulate leukocyte behavior within the microvasculature.
1.3 ± 0.4 leukocytes/min; p < 0.01) leukocytes in saline-treated control rats (n = 6 rats in each group).

Effects of L-NAME, MEG, and FeTPPS on plasma nitrite/nitrate levels and heart iNOS expression. Endotoxin treatment induced at 4-h increases in nitrite/nitrate plasma levels compared with control rats (117 ± 14 vs. 17 ± 2 μmol/l). In endotoxin–treated rats, treatment with L-NAME reduced nitrite/nitrate plasma levels (117 ± 14 vs. 77 ± 12 μmol/l; p < 0.01), whereas MEG sodium succinate and FeTPPS had no effects (respectively, 124 ± 11; 113 ± 21 μmol/l; p > 0.05 vs. endotoxin–treated rats) (n = 8 in each group). Treatments with L-NAME, MEG sodium succinate, and FeTPPS had no effects in saline–treated control rats on nitrite/nitrate plasma levels (n = 5 in each group) (data not shown). To document that the inducible isoform of NO synthase was involved in enhanced NO generation, we stained myocardial tissue with anti-iNOS antibody. Western blot analysis (n = 6 in each group) showed that iNOS protein expression was not detectable in left ventricle of control rat heart but was increased in left ventricle of endotoxin–treated rat heart. Treatments with MEG sodium succinate and FeTPPS had no effects on myocardial iNOS protein expression in endotoxin–treated rats, whereas L-NAME treatment was associated with increases in iNOS protein expression (Fig. 6).

DISCUSSION

The important new finding of the present study is that peroxynitrite neutralizers, MEG, a peroxynitrite scavenger and iNOS inhibitor, and FeTPPS, which catalyzes the isomerization of peroxynitrite to nitrate anion, largely prevented the accumulation of peroxynitrite and improved myocardial contractile dysfunction in endotoxin–treated rats. Beneficial functional effects of these peroxynitrite neutralizers were associated with the attenuation of endotoxin–induced inflammatory response in terms of degradation of NF-kappa-B inhibitory protein I-kappa-B, plasma TNF-alpha, and vascular endothelial cell-leukocyte activation.

Experimental studies support a critical role for enhanced production of NO in the development of myocardial and vascular dysfunction in sepsis (7,10,16). These findings led to the hypothesis that pharmacologic inhibition of NOS might be of therapeutic value for the treatment of septic myocardopathy. However, NOS inhibition with nonselective and selective iNOS inhibitors failed to improve myocardial function in different models of sepsis (21,22). In our model of endotoxemia in rats, treatment with L-NAME, a nonselective NOS inhibitor, reduced NO production as measured by nitrite/nitrate plasma levels but failed to improve left ventricular contractility. Inhibition of basal NO production has been previously implicated in deleterious effects induced by L-NAME on vascular blood flow and endothelial cell functions (18,22,42,43). We confirmed that, first, endotoxin–treated rats administered with L-NAME

Figure 5. Leukocyte behavior in the mesentery venules of rats treated with saline, endotoxin (10 mg/kg), endotoxin and LNAME (50 mg/kg), endotoxin and MEG sodium succinate (10 mg/kg), and endotoxin and FeTPPS (30 mg/kg). See Methods section for treatment group design. Results are expressed as mean ± SEM (n = 6 in each group). *p < 0.01 compared with control; #p < 0.01 compared with endotoxin–treated rats. Abbreviations as in Figure 1.
had increased coronary perfusion pressure, which may indicate coronary vasoconstriction and potential myocardial ischemia. Second, leukocyte rolling and adhesion on peripheral venular endothelium were increased in the presence of L-NAME in both control and endotoxin-treated rats (42,43). Beside these hemorheological effects, we observed that L-NAME elicited an amplification of heart I-kappa-B degradation in endotoxin-treated rat hearts. Previous studies have demonstrated that inhibition of NO production increased I-kappa-B degradation and may lead to NF-kappa-B translocation to the nucleus (44,45). Activation of NF-kappa-B may, in turn, induce numerous proinflammatory genes, including iNOS, COX-2, adhesion molecules, and proinflammatory cytokines that are critically involved in the septic myocardopathy (46,47). Hence, these molecular events may explain increases in TNF-alpha levels, iNOS protein expression, and leukocyte-endothelial activation observed in endotoxin-treated administered with L-NAME.

Peroxynitrite neutralizers, (i.e., MEG sodium succinate and FeTPPS) reduced endotoxin-induced peroxynitrite accumulation as measured by plasma rhodamine fluorescence intensity and heart nitrotyrosine staining. Interestingly, septic rats treated with MEG and FeTPPS had improved myocardial function at 4 h and even at 16 h after endotoxin challenge. These observations suggest that peroxynitrite generation may be relevant in mediating endotoxin-induced

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**Figure 6.** Effects of L-NAME (50 mg/kg), MEG sodium succinate (10 mg/kg), and FeTPPS (30 mg/kg) on endotoxin-induced heart inducible nitric oxide synthase (iNOS) protein expression and plasma nitrite/nitrate levels. See Methods section for treatment group design. (A) Heart iNOS protein expression (n = 6 in each group). Semiquantitative analysis was performed on the basis of relative I-kappa-B/G3PDH density. (B) Plasma nitrite/nitrate levels (n = 6 in each group). Results are expressed as mean ± SEM. *p < 0.01 compared with control; #p < 0.01 compared with endotoxin-treated rats. Abbreviations as in Figure 1.
myocardial depression. Beneficial effects of peroxynitrite neutralizers have been attributed to their antioxidant and leukocyte antiadhesive properties in various models of inflammation (34–37). Indeed, we observed that MEG and FeTPPS largely prevented myocardial leukocyte sequestration and peripheral venular endothelium-leukocyte rolling and adhesion during endotoxemia. We speculated that attenuation of cardiovascular inflammatory processes by MEG and FeTPPS could have positively affected septic myocardial depression. This contention is based on previous findings showing that prevention of endothelial and leukocyte activation is critical for heart function in models of endotoxemia (40,48,49).

Proposed molecular mechanisms involved in anti-inflammatory effects of peroxynitrite neutralizers, such as FeTPPS and FP-15, include prevention of NF-kappa-B translocation into the nucleus and related proinflammatory gene transcription (30,31,50–52). In endotoxin-treated rats, we observed that peroxynitrite neutralizers, MEG and FeTPPS, prevented heart I-kappa-B degradation suggesting reduced NF-kappa-B activation. Decreased plasma TNF-alpha levels in endotoxin-treated rats in the presence of FeTPPS and MEG may be attributed to the effects of these compounds on NF-kappa-B activation. The reason why, in the presence of peroxynitrite neutralizers, reduced NF-kappa-B activation was not associated with prevention of iNOS protein expression is not readily available. However, in vivo uncoupling of a nuclear event (NF-kappa-B activation) from a functional event (iNOS protein expression) has been previously demonstrated (32). One possibility is that other mediators generated within the in vivo cell environment could interfere with mechanisms that allow translation into specific functional events in response to NF-kappa-B activation (32,50).

In our study, changes in heart adhesion molecule expression associated with endotoxemia and peroxynitrite neutralizer treatments were not explored. However, the fact that, in septic rats, MEG and FeTPPS increased plasma endothelial levels and reduce leukocyte rolling and adhesion on the venular endothelium is consistent with previous observations demonstrating that reducing peroxynitrite accumulation prevents increases in adhesion molecule expression in models of inflammation (30–37). Indeed, endocan (previously called endothelial-cell-specific molecule-1), is a newly described proteoglycan molecule that binds directly to CD11a/CD18 integrin (LFA-1) and blocks the binding to intercellular adhesion molecule-1 (39). Endocan may, thus, reduce recruitment of circulating leukocytes to inflammatory sites and LFA-1-dependent leukocyte adhesion and activation.

In conclusion, these observations strongly suggest that in this in vivo model of endotoxemia, cardiovascular inflammation causes myocardial contractility depression at 4 h and 16 h, which was prevented by MEG, a peroxynitrite scavenger, and FeTPPS, a peroxynitrite decomposition catalyst. The further use of these peroxynitrite neutralizers as pharmacologic tools may lead to a better understanding of when and where peroxynitrite plays a key role(s) in the development of inflammatory processes. This, in turn, should provide more effective treatment strategies in the clinic for disease such as sepsis.

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