Nitro-oleic acid protects against endotoxin-induced endotoxemia and multiorgan injury in mice

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Wang H, Liu H, Jia Z, Olsen C, Litwin S, Guan G, Yang T. Nitro-oleic acid protects against endotoxin-induced endotoxemia and multiorgan injury in mice. Am J Physiol Renal Physiol 298: F754–F762, 2010. First published December 23, 2009; doi:10.1152/ajprenal.00439.2009.—Nitroalkene derivatives of nitro-oleic acid (OA-NO2) are endogenous lipid products with potent anti-inflammatory properties in vitro. The present study was undertaken to evaluate the in vivo anti-inflammatory effect of OA-NO2 in mice given LPS. Two days before LPS administration, C57BL/6j mice were chronically infused with vehicle (LPS vehicle) or OA-NO2 (LPS OA-NO2) at 200 μg·kg−1·day−1 via osmotic minipumps; LPS was administered via a single intraperitoneal (ip) injection (10 mg/kg in saline). A third group received an ip injection of saline without LPS or OA-NO2 and served as controls. At 18 h of LPS administration, LPS vehicle mice displayed multiorgan dysfunction as evidenced by elevated plasma urea and creatinine (kidney), aspartate aminotransferase (AST) and alanine aminotransferase (ALT; liver), and lactate dehydrogenase (LDH) and reduced ejection fraction (heart). In contrast, the severity of multiorgan dysfunction was less in LPS OA-NO2 animals. The levels of circulating cGMP and PGE2 contents, were greater in LPS vehicle vs. control mice, but were attenuated in LPS OA-NO2 animals. Similar patterns of changes in the expression of inflammatory mediators were observed in the liver. Together, pretreatment with OA-NO2 ameliorated the inflammatory response and multiorgan injury in endotoxin-induced endotoxemia in mice.

lipopolysaccharide; TNF-α; sepsis; acute kidney injury; COX-2

SEPSIS IS A SYSTEMIC INFLAMMATORY RESPONSE to a blood-borne infection that is associated with an extremely high rate of morbidity and mortality. The incidence of septic shock is increasing, affecting 750,000 patients each year, with an overall mortality rate of 20–50% (28). It is the second leading cause of death among patients in noncoronary intensive care units (ICUs) (32) and the tenth leading cause of death overall in the United States (19). The high mortality often results from the failure of multiple organs, including the kidney and liver. In particular, acute kidney injury (AKI) secondary to sepsis is a highly prevalent diagnosis in the ICU setting in which the mortality rate can reach as high as 70% (24, 40); the mortality rate for septic patients with acute renal failure (ARF) is approximately double compared with patients with sepsis alone (29). ARF is considered a critical prognostic factor in sepsis (37), while the management of sepsis and sepsis-induced ARF is largely supportive. Therefore, novel therapies to prevent or treat this devastating disease are urgently required.

Recently, nitrated fatty acids (NO2-FAs), notably nitroalkene derivatives of linoleic acid (nitrolinoleic acid; LNO2) and nitro-oleic acid (OA-NO2), are identified as endogenous molecules with several attractive signaling properties. These derivatives are formed via nitric oxide (NO)-dependent oxidative reactions (30, 36). Plasma concentrations are ~0.5 μM for LNO2 (3) and ~0.6 μM for OA-NO2 (3) in healthy human blood, and combined blood levels of these two fatty acid derivatives exceed 1 μM, indicating their capability to act in physiological concentration ranges. To date, there are three major mechanisms by which nitroalkenes appear to mediate cell signaling. First, LNO2 and OA-NO2, at physiological concentrations, serve as potent ligands for proxisome proliferator-activated receptor subtype-γ (PPARγ) (39). The second is as a NO-storage form; thus nitroalkenes can mediate vessel relaxation through a cGMP-dependent pathway (38). Aqueous decay and release of NO by nitroalkenes occur via a modified Nef reaction and leads to the regulation of NO-sensitive cell-signalizing pathways (38). Last, nitroalkenes are electrophiles and thus can nitroalkylate proteins and small peptides such as glutathione through reaction with cysteine thiols and histidine (4).

Increasing in vitro evidence demonstrates that nitroalkenes exert potent anti-inflammatory actions. For example, nitroalkenes reduce human neutrophil superoxide generation, degranulation, and integrin expression and thrombin-induced Ca2+ elevations and platelet aggregation (9, 10, 26). Moreover, in cultured RAW264.7 cells, a murine macrophage cell line, nitroalkenes attenuate the LPS-elicited inflammatory response via diverse mechanisms involving activation of mitogen-activated protein kinase phosphatase 1 (21) and nitroalkylation of NF-κB p65 (11). Compared with the detailed in vitro analysis of signaling properties of nitroalkenes, there are few studies that investigate their biological function in vivo. We previously demonstrated that OA-NO2 had protective effects on renal injury in the ischemia-reperfusion mouse model (27). The present study seeks to examine the potential therapeutic effects of OA-NO2 in LPS-induced inflammation and renal injury.

MATERIALS AND METHODS

Materials. 9-Nitro-oleic acid and 10-nitro-oleic acid are two regioisomers of OA-NO2, which are formed by nitration of oleic acid (OA) in approximately equal proportions in vivo (3). The two compounds were purchased from Cayman Chemical (Ann Arbor, MI) (9-nitro-oleic acid: catalog no. 10008042; 10-nitro-oleic acid: catalog no. 10008043), dissolved in DMSO, and used as a 1:1 mixture of the
isomers. OA was purchased from the same company (catalog no. 90260) and dissolved in the same solvent. All other reagents were purchased from Sigma-Aldrich unless otherwise specified. All protocols employing mice were conducted in accordance with the principles and guidance of the University of Utah Institutional Animal Care and Committee.

Animal protocol. Male C57BL/6 mice (8–10 wk old) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained on a standard rodent chow and had free access to water. OA-NO₂ was dissolved in 100% DMSO at 1 mg·mL⁻¹. Mice were pre-treated for 48 h with DMSO (LPS vehicle) or OA-NO₂ (LPS OA-NO₂) at 0.2 mg·kg⁻¹·day⁻¹ via a micro-osmotic pump (DURECT, Cupertino, CA), and then both groups were treated with a single intraperitoneal (ip) injection of LPS at 10 mg/kg (Escherichia coli 0127:B8, Sigma, St. Louis, MO; dissolved in saline). The infusion rate of the pump was 6 μL/day. A third group received an ip injection of saline only and served as controls. Functional studies were done at 18 h after LPS injection. A separate experiment was performed to compare the anti-inflammatory effects of OA-NO₂ vs. OA; OA was delivered at the same dose (0.2 mg·kg⁻¹·day⁻¹) via the same route as OA-NO₂.

Measurement of body temperature and hematocrit. Rectal temperature was measured before and 18 h after LPS injection using a digital thermometer. At 18 h after LPS injection, hematocrit was determined as previously described (49). Briefly, 5–10 μL of blood was collected from a tail cut using a 10-μL capillary glass (Idaho Technology). One side of the tube was sealed with Hemato-Seal and then centrifuged for 4 min in a Thermo IEC microcentrifuge. The total height of the sample and height of the red blood cell column were measured. The hematocrit reflects the ratio between the red blood cell column and total height.

Echocardiography. In vivo cardiac function was assessed using echocardiography. The animals were lightly anesthetized with isoflurane and were imaged in the left lateral decubitus position with a linear 13-MHz probe (Vivid V echocardiograph, General Electric). Digital images were obtained at a frame rate of 180 images/s. Two-dimensional images were recorded in parasternal long- and short-axis projections with guided M-mode recordings at the midventricular level in both views.

Blood pressure measurements. Mean arterial pressure (MAP) was determined by telemetry. The radiotelemetric device was implanted in mice through catheterization of the carotid artery (model TA11PA-
Measurement of circulating TNF-α. Circulating TNF-α was measured by using a commercially available enzyme immunoassay kit (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions.

Measurement of biochemical parameters. Blood samples from anesthetized mice were collected by puncturing the vena cava using a 1-ml insulin syringe containing 50 μl of 1 mM EDTA in the absence of protease inhibitors. Plasma levels of both urea and creatinine were measured by using a commercially available enzyme immunoassay kit (Cayman Chemical) and normalized by protein concentrations. Protein concentrations were determined using Coomassie reagent. The tissue PGE2 content was determined using an EIA kit (Cayman Chemical) and normalized by protein concentrations.

Statistical analysis. Values shown represent means ± SE. Data were analyzed using unpaired t-test or ANOVA followed by a Bonferroni posttest. A P value <0.05 was considered significant.
RESULTS

Body temperature and hematocrit. The body temperature decreased significantly at 18 h after LPS injection (29.0 ± 0.55 vs. 36.9 ± 0.6°C, P < 0.01) (Fig. 1A). Pretreatment of OA-NO2 for 2 days significantly improved LPS-induced hypothermia (31.76 ± 1.14 vs. 29.0 ± 0.55°C, P < 0.05) (Fig. 1A). LPS is reported to induce erythrocytopenia and leukocytopenia, resulting in a reduction of hematocrit, particularly at the later phase of septic shock (20, 23). We found that at 18 h post-LPS, hematocrit was significantly decreased that was attenuated by pretreatment with OA-NO2 (control: 53.1 ± 2.6%; LPS vehicle: 43.7 ± 1.4%; LPS OA-NO2: 49.5 ± 0.5%).

Renal function. Renal function was reflected by plasma blood urea nitrogen (BUN) and creatinine (Fig. 2). LPS injection elevated plasma BUN from 27.3 ± 3.9 to 108.8 ± 3.4 mg/dl (P < 0.05) and plasma creatinine from 0.22 ± 0.03 to 0.34 ± 0.04 mg/dl (P < 0.05). Pretreatment with OA-NO2 attenuated the rise of plasma BUN (95 ± 1.4 vs. 108.8 ± 3.4 mg/dl, P < 0.05) and almost completely normalized plasma creatinine levels (0.23 ± 0.03 vs. 0.34 ± 0.04 mg/dl, P < 0.05). Renal histological changes in response to LPS were not evident as previously reported (13) and thus were not used as a parameter for evaluating the effect of OA-NO2 (data not shown).

Proinflammatory cytokines, chemokines, and adhesion molecules. TNF-α is a well-established pathogenic factor in the inflammatory responses in sepsis. Therefore, emphasis was placed on the analysis of renal TNF-α mRNA expression and circulating TNF-α. By real-time RT-PCR, renal TNF-α mRNA exhibited a 14-fold increase in LPS vehicle vs. control mice. Compared with LPS vehicle mice, this increase was reduced by 50% in the LPS OA-NO2 animals (Fig. 3A). Similar results were obtained concerning circulating TNF-α as assessed by enzyme immunoassay (Fig. 3B). Additionally, we examined renal mRNA expression of chemokines and adhesion molecules, including monocyte chemotactrant protein-1 (MCP-1), ICAM-1, and VCAM-1. The fold-inductions between LPS vehicle and LPS OA-NO2 groups were 27.1- vs. 9.7-fold for MCP-1, 12.0- vs. 4.8-fold for ICAM-1, and 4.2- vs. 1.8-fold for VCAM-1 (Fig. 4). Taken together, OA-NO2 pretreatment ameliorated the expression of proinflammatory cytokines, chemokines, and adhesion molecules in endotoxic shock.

Renal iNOS expression. iNOS is highly inducible by proinflammatory stimuli and is a major source of enhanced NO generation in sepsis. Furthermore, iNOS-derived NO has been implicated in the pathogenesis of LPS-induced AKI (45). In the present study, the expression of iNOS was examined at both mRNA and protein levels. At 18 h post-LPS, renal iNOS mRNA exhibited a >1,000-fold increase that was reduced by...
80% in LPS OA-NO2 mice (Fig. 5A). Similarly, LPS injection induced a 70-fold increase in renal iNOS protein expression that was reduced by 90% with OA-NO2 (Fig. 5B). Renal cGMP content exhibited a similar pattern of changes as renal iNOS expression (Fig. 5C).

**Renal COX-2 expression.** Like iNOS, COX-2 is highly inducible by proinflammatory stimuli and is another important marker of inflammatory responses in sepsis. In the present study, COX-2 expression was evaluated at mRNA and protein levels as well as at the level of the enzyme activity. Renal COX-2 mRNA and protein were 8.3- and 17.1-fold greater, respectively, in LPS vehicle vs. control mice; they were 2.1- and 8.9-fold greater in LPS OA-NO2 vs. control mice (Fig. 6, A and B). At 18 h post-LPS, renal content of PGE2, a major product of COX-2, exhibited a 1.5-fold increase that was almost normalized by pretreatment with OA-NO2 (Fig. 6C).

**Hepatic injury.** LPS injection elevated plasma AST from 176.7 ± 43.3 to 264.8 ± 12.8 U/l (P < 0.05) (Fig. 7A) and plasma ALT from 81.1 ± 2.2 to 99.5 ± 3.1 U/l (P < 0.05) (Fig. 7B). Pretreatment with OA-NO2 lowered plasma AST to 207.8 ± 15.9 U/l (P < 0.05) (Fig. 7A) and plasma ALT to 85.6 ± 5.6 U/l (P = 0.051) (Fig. 7B). ELISA and quantitative (q)RT-PCR were subsequently performed to evaluate markers of inflammatory response in the liver. By ELISA, the increase in hepatic TNF-α content was less in LPS OA-NO2 vs. LPS vehicle mice (Fig. 7C). LPS injection induced parallel increases in mRNA expression of hepatic TNF-α, IL-1, ICAM-1, MCP-1, iNOS, and COX-2; these increases were all attenuated by OA-NO2 (Fig. 8).

**Cardiac injury and hypotension.** Echocardiography revealed that LPS injection significantly reduced the ejection fraction, an index of left ventricular dysfunction, which was almost completely corrected by OA-NO2 (Fig. 9A). Plasma LDH was elevated by LPS injection (P < 0.05) that was attenuated by OA-NO2 (P < 0.05) (Fig. 9B). TNF-α content in the heart tended to show a similar pattern of changes as in the kidney and liver, but no statistical significance was detected between any groups (Fig. 9C). Hemodynamic parameters in LPS vehicle and LPS OA-NO2 mice were monitored using radiotelemetry. LPS-induced hypotension was evident in both groups. MAP in LPS OA-NO2 mice tended to be consistently ~10 mmHg higher than in LPS vehicle animals (Fig. 10A). The statistical significance between the two groups can be detected by an unpaired t-test but not ANOVA, reflecting a small effect of OA-NO2. Similar results were obtained with heart rate (Fig. 10B).

**Effectiveness of OA.** Separate experiments determined whether the protective effect of OA-NO2 in endotoxin-induced endotoxemia was specific to the nitrated (i.e., OA-NO2) or native form (i.e., OA). The indices of systemic inflammation (body temperature and hematocrit) and renal dysfunction (plasma BUN and creatinine) in the endotoxemic mice were significantly attenuated by OA-NO2 but were unaffected by OA (Fig. 11), documenting the lack of anti-inflammatory effect.
NO2, are naturally occurring products (11, 12). Abundant cytokines (14). In patients with ARF, plasma cytokine Toll-like receptor 4 (TLR4) initiates the release of inflammatory bacterial constituents, such as LPS. The binding of LPS to cellular function and inflammation, caused by the release of mediators of responses and improved multiorgan dysfunction in the kidney, and heart in septic C57/BL6 mice.

**DISCUSSION**

OA-NO2 in endotoxic shock is attributable to the nitration of fatty acid. This finding suggests that the protective effect of OA-NO2 in endotoxic shock is attributable to the nitration of fatty acid.

**Discussion**

NO2-FA, notably nitroalkene derivatives of LNO2 and OA-NO2, are naturally occurring products (11, 12). Abundant evidence demonstrates that nitroalkenes exert potent anti-inflammatory actions in vitro. The present study explored the in vivo anti-inflammatory effect of OA-NO2 in a mouse model of LPS administration. LPS is an endotoxin derived from the outer membrane of Gram-negative bacteria, and it is strongly associated with septic shock. Many of the pathophysiological abnormalities of human endotoxic shock can be reproduced in experimental animals by administration of LPS (18). In particular, murine models of LPS administration are frequently used to investigate the pathophysiology and therapies of endotoxin-induced ARF. Here, we report that preventative administration of OA-NO2 attenuated the systemic and local inflammatory responses and improved multiorgan dysfunction in the kidney, liver, and heart in septic C57/BL6 mice.

Endotoxic shock represents a systemic inflammatory response arising from a complicated interplay of mediators of cellular function and inflammation, caused by the release of bacterial constituents, such as LPS. The binding of LPS to Toll-like receptor 4 (TLR4) initiates the release of inflammatory cytokines (14). In patients with ARF, plasma cytokine levels are positively correlated with mortality (42). Among various proinflammatory cytokines induced by LPS, TNF-α is of major importance (31). The pathogenic role of TNF-α in endotoxic renal failure is demonstrated by the observations that administration of TNF-α to intact animals or isolated, perfused kidneys reduced the glomerular filtration rate (5, 44). Along this line, TNF-α neutralization with a TNF-α antibody or a TNF-α-soluble receptor (TNFsRp55) protects against septic shock in baboons (43) and mice (6, 25). More importantly, mice deficient in TNFRI, the major receptor subtype for the inflammatory action of TNF-α, are resistant to LPS-induced ARF (12) despite the lack of improvement of mortality (33, 35); the cross-kidney transplantation between TNFR +/+ and −/− mice further demonstrates that LPS-induced ARF is caused by the direct renal action of TNF-α (12). In the present study, we found that pretreatment with OA-NO2 effectively reduced renal and hepatic TNF-α mRNA expression as well as circulating TNF-α level in septic mice. We suspect that suppression of TNF-α expression may be a primary mechanism responsible for the protective action of OA-NO2 in endotoxic shock. The inhibition of TNF-α appears to be direct since exposure of cultured RAW264.7 cells to OA-NO2 as well as LNO2 significantly reduced the release of TNF-α in response to LPS (11).

LPS administration is well known to trigger an influx of inflammatory cells, namely, neutrophils and macrophages, to various organs, including the liver, lung, and kidney (1, 22, 47). This process is directed by local expression of adhesion molecules and chemokines. For example, the adhesion molecule ICAM-1 is mainly expressed on the endothelial cell surfaces, interacting with ligands present on circulating leukocytes, thereby allowing attachment and adhesion of the leukocytes to the endothelium, a key event of the inflammatory response. The importance of ICAM-1 in sepsis is demonstrated by the observation that mice deficient in ICAM-1 are resistant to LPS-induced ARF (47), with improved mortality (48). We found that pretreatment with OA-NO2 attenuated LPS-induced expression of ICAM-1 as well as VCAM-1 in the kidney or/and liver. We suspect that OA-NO2 may directly target vascular endothelial cells, where it attenuates attachment of circulating leukocytes via suppressing local expression of adhesion molecules. Since the expression of MCP-1 is similarly suppressed by OA-NO2, it is possible that the nitrated lipid may also attenuate macrophage recruitment via suppressing chemokine expression.

Other inflammatory mediators including iNOS and COX-2 are well known to be responsive to proinflammatory stimuli such as LPS. NO is generated by three NOS isoenzymes: endothelial (eNOS), neuronal (nNOS), and iNOS. The involvement of NO in endotoxic organ failure has been extensively studied, revealing distinct actions of NO derived from different enzymatic sources. eNOS-derived NO protects the cardiovascular system (41), while iNOS-derived NO mediates kidney injury in endotoxic shock (25). In particular, the iNOS inhibitor 1-NAME-(1-iminoethyl)-lysine and iNOS deficiency protect against endotoxic renal failure (25, 46). COX exists in two isoforms: constitutive (COX-1) and inducible (COX-2). Like iNOS, COX-2 is highly inducible by proinflammatory stimuli. COX-2 −/− mice are resistant to LPS-induced inflammation and mortality, accompanied by blunted induction of renal iNOS expression (15, 16). The finding suggests that LPS-induced iNOS expression may be mediated by COX-2-derived products. Along this line, we observed a robust induction
of iNOS and COX-2 in response to LPS administration in the kidney, which was suppressed in parallel by pretreatment with OA-NO₂. To date, there are no reports as to whether nitraolkenes are capable of directly suppressing COX-2 expression in the context of inflammation. Of note, the changes in the cGMP concentration in the kidney correspond to the expression level of iNOS in control, LPS, and LPS + OA-NO₂ mice, suggesting that the increased renal cGMP production after LPS administration may be primarily due to iNOS-derived NO and therefore may be detrimental. However, cGMP is renoprotective in other experimental disease settings such as renal ischemia-reperfusion injury and nephrectomy (7, 34). Likely, cGMP can exert distinct roles in renal pathophysiology depending on the coupling to a specific NOS isoform.

Fig. 10. Radiotelemetric determination of mean arterial pressure (MAP; A) and heart rate (B) in LPS vehicle and LPS OA-NO₂ mice. The arrow indicates the time of LPS injection. The immediate rise of MAP and heart rate following LPS injection reflects the influence from stress. LPS: n = 8; LPS + OA-NO₂: n = 10. Values are means ± SE. *P < 0.05 vs. basal values in the same group. #P < 0.05 vs. LPS alone at the corresponding period.

Fig. 11. Comparison of the effects of OA-NO₂ vs. oleic acid (OA) on body temperature (A), hematocrit (B), plasma BUN (C), and creatinine (D) in endotoxemic C57/BL6 mice; n = 5/group. Values are means ± SE.
Overall, our data suggest that the anti-inflammatory action of nitroalkenes may be attributable to their ability to suppress diverse inflammatory pathways mediated by TNF-α, MCP-1, ICAM-1/VCAM-1, iNOS, and COX-2. Although TNF-α is reported to induce expression of most of these inflammatory mediators, evidence exists to suggest dissociation of TNF-α with iNOS in endotoxemic renal failure in mice (25). In particular, this study showed that TNF neutralization produces a similar degree of renoprotection in endotoxemic iNOS +/+ and −/− mice (25). Despite favorable preclinical elevations, clinical trials attempting to inhibit TNF-α by using either anybodyes (8) or fusion proteins composed of the extracellular domain of TNFR1 or TNFR2 have failed to influence sepsis-related mortality (2, 17). The negative clinical trial data may stimulate interest in targeting an inflammatory mediator(s) upstream of TNF-α, such as NF-κB. Indeed, nitroalkenes are reported to inhibit LPS-induced NF-κB activation via nitroalkylation of p65 (11). Moreover, nitroalkenes activate the mitogen-activated protein kinase phosphatase-1, a negative regulator of LPS-induced STAT signaling, resulting in suppression of iNOS and MCP-1 (21). Most likely, the effective anti-inflammatory effect of nitroalkenes may lie in their ability to intervene in the early event of inflammatory signaling.

The present study is associated with a number of limitations. For example, OA-NO2 was administered in a preventative rather than therapeutic way. The latter was not performed due to the consideration that with the slow infusion rate of minipumps, the effective and stable plasma concentrations of OA-NO2 might not be achieved within the short period post-LPS administration. Second, the molecular mechanisms responsible for the anti-inflammatory action of OA-NO2 were not tested. The mechanisms appear to be complex, involving activation of PPARγ (3, 39), release of NO (26, 38), inhibition of NF-κB (11), and regulation of phosphorylation of STAT (21), etc. Dissection of each of the signaling pathways needs to be performed in future studies.

In summary, the present study is the first to evaluate the in vivo anti-inflammatory action of OA-NO2 in a mouse model of LPS administration. Preventative treatment with OA-NO2 attenuated the systemic and local inflammatory responses and improved multiorgan dysfunction in endotoxic mice. The effective anti-inflammatory effect of OA-NO2 appears to be attributable to the suppression of diverse proinflammatory mediators, including cytokines, chemokines, adhesion molecules, iNOS, and COX-2. Together, nitroalkenes may hold promise for the prevention of and possibly therapy for endotoxic shock.

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DISCLOSURES
No conflicts of interest are declared by the authors.

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