The apolipoprotein A-I mimetic peptide 4F prevents defects in vascular function in endotoxemic rats

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Abstract **High density lipoprotein (HDL) and apolipoprotein A-I (apoA-I) reduce inflammatory responses to lipopolysaccharide (LPS). We tested the hypothesis that the apoA-I mimetic peptide 4F prevents LPS-induced defects in blood pressure and vascular reactivity. Systolic blood pressure (SBP) was measured in rats at baseline and 6 h after injection of LPS (10 mg/kg) or saline vehicle. Subgroups of LPS-treated rats also received 4F (10 mg/kg) or scrambled 4F (Sc-4F). LPS administration reduced SBP by 35% compared with baseline. 4F attenuated the reduction in SBP in LPS-treated rats (17% reduction), while Sc-4F was without effect. Ex vivo studies showed a reduced contractile response to phenylephrine (PE) in aortae of LPS-treated rats (ED_{50} = 459 ± 83 nM) compared with controls (ED_{50} = 57 ± 6 nM). This was associated with nitric oxide synthase 2 (NOS2) upregulation. 4F administration improved vascular contractility (ED_{50} = 60 ± 9 nM), reduced aortic NOS2 protein, normalized plasma levels of NO metabolites, and reduced mortality in LPS-treated rats. These changes were associated with a reduction in plasma endotoxin activity. In vivo administration of ^{14}C-4F and Bodipy-LPS resulted in their colocalization and retention in the HDL fraction. It is proposed that 4F promotes the localization of LPS to the HDL fraction, resulting in endotoxin neutralization. 4F may thus prevent LPS-induced hemodynamic changes associated with NOS2 induction.**—Dai, L., G. Datta, Z. Zhang, H. Gupta, R. Patel, J. Honavar, S. Modi, J. M. Wyss, M. Palgunachari, G. M. Anantharamaiah, and C. R. White. **The apolipoprotein A-I mimetic peptide 4F prevents defects in vascular function in endotoxemic rats.** ***J. Lipid Res.*** **2010.** 51: **2695–2705.**

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Sepsis is a major cause of death in hospitalized patients. Approximately 50% of patients in intensive care units develop sepsis, and the overall mortality rate is 29% (1). Mortality is due, in large part, to the cytotoxic actions of lipopolysaccharide (LPS), an endotoxic component of the outer membrane of Gram-negative bacteria. LPS is released from bacterial membranes and activates Toll-like receptors (TLR) on monocytes, neutrophils, and other target cells (2–5). TLRs transduce LPS action by activating nuclear factor (NF)-κB-dependent signaling (4–6). By this mechanism, LPS stimulates the synthesis/release of inflammatory cytokines, which play an important role in the innate immune response (7, 8). Dysregulation of this inflammatory response can lead to intravascular coagulation and multiple organ failure. LPS-induced cytokine production also increases expression of nitric oxide synthase 2 (NOS2) and cyclooxygenase 2 (COX-2), thus enhancing the synthesis of nitric oxide (NO) and arachidonic acid-derived vasoactive prostanoids. These products have been implicated in hemodynamic disturbances in sepsis by reducing peripheral vascular resistance (9–14). Cardiovascular (CV) failure, characterized by severe hypotension and cardiac dysfunction, is linked to increased mortality in patients with severe sepsis (15–17).

Lipoproteins are thought to play a role in the neutralization/detoxification of endotoxin (18–20). Low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), and high-density lipoprotein (HDL) bind to LPS and direct it to the liver for metabolism and excretion (18–20). HDL is most effective in clearing endotoxin, a property attributed to its relatively high phospholipid content compared with LDL and VLDL (18, 21). With increasing

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Abbreviations: apoA-I, apolipoprotein A-I; COX-2, cyclooxygenase 2 LPS, lipopolysaccharide; NO, nitric oxide; NOS2, nitric oxide synthase 2; PE, phenylephrine; SBP, systolic blood pressure; Sc-4F, scrambled 4F.

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severity of sepsis, HDL levels are reduced, and clinical data suggest that low HDL is associated with an increase in mortality in septic patients (6, 22, 23). It follows that increasing plasma HDL concentration reduces sepsis-dependent complications in humans and animal models (22–24). While raising plasma HDL represents an important goal in the treatment of sepsis, obtaining therapeutic quantities of the lipoprotein is impractical.

Numerous therapeutic approaches have been used in the treatment of sepsis, including administration of antibiotics, sympathomimetic amines, corticosteroids, nonsteroidal anti-inflammatory drugs (NSAID), and tumor necrosis factor-α (TNF-α) antibodies (15–17, 25, 26). These treatments, however, have yielded variable effects on the resolution of sepsis complications and survival. Drotrecogin alfa (recombinant human-activated protein C) is currently the only treatment for sepsis approved by the Food and Drug Administration (27). The PROWESS study showed that this treatment improved survival in high-risk patients (28). In contrast, the RESOLVE study, which was designed to test effects of drotrecogin alfa on prevention of organ failure and mortality, revealed negative results (29). The search for an ideal drug to treat sepsis and its complications continues.

Apolipoprotein (apo) mimetic peptides have been shown to attenuate several lipid-mediated inflammatory diseases in a manner similar to apoA-I and/or HDL (30). We previously reported that the apoA-I mimetic peptide 4F antagonizes the inflammatory effects of LPS in cell culture models (31). The goal of the current study was to determine whether 4F administration improves vascular function in vivo and ex vivo. To this end, we report that 4F administration blunts LPS-induced hypotension and restores vasoconstrictor sensitivity of arterial segments assayed under ex vivo conditions. Our data suggest that the principal action of the peptide in improving vascular function is to reduce circulating endotoxin activity, thus preventing the induction of NOS2 by LPS.

METHODS

Materials

LPS (*Escherichia coli*, serotype 026:B6) was obtained from Sigma. Antibodies to NOS-2 (Transduction Laboratories) and β-actin (Santa Cruz, Inc.) were obtained commercially. Bodipy-LPS was obtained from Molecular Probes, Inc. Superose 6 columns were from Amersham Biosciences (NJ). Plasma endotoxin levels were measured using a QCL 1000 Limulus amebocyte lysate (LAL) kit (Lonza, Inc., Walkersville, MD). TNF-α levels were measured by ELISA (BD OptEIA; BD Biosciences, San Diego, CA). HDL cholesterol was measured using a commercially available kit (Wako, Inc.).

ApoA-I mimetic peptide synthesis

4F, whose amino acid sequence is Ac-DWFKAFYDKVAEKFKEAF-NH_2, was synthesized using *L*-amino acids by the solid phase peptide synthesis method as previously described (32). The scrambled peptide Sc-4F (Ac-DWFAKDYFKKAFVEEFAK-NH_2) was designed by rearranging the amino acid sequence of 4F so that it was unable to form an amphipathic α-helix, and it was used as a control for 4F (33). Peptide purity was ascertained by mass spectral analysis and analytical HPLC. Peptide concentration was determined using molar extinction coefficients of tryptophan and tyrosine (34). ^{14}C-4F was synthesized by the same procedure as 4F except that acetylation of the N-terminal was carried out with ^{14}C-labeled acetic acid (American Radiolabeled Chemicals, St Louis, MO) instead of normal acetic acid, as previously described (35).

Animals

Ten-week-old, male Sprague-Dawley rats were purchased from Charles Rivers Breeding Laboratories (Wilmington, MA) and allowed a 1 week recovery period prior to initiating experimental protocols. All rats received standard laboratory chow (Teklad Diets, Inc.) and water ad libitum. Rats were maintained at constant humidity (60 ± 5%), temperature (24 ± 1°C), and light cycle (6 AM to 6 PM). All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 96-01, revised 2002).

Measurement of systolic blood pressure

SBP was measured noninvasively in untreated rats by tail cuff plethysmography using a model 300 sphygmomanometer (Narco Biosystems, Inc.). Conscious rats were conditioned to the tail pressure cuff for time periods up to 50 min. Baseline SBP was then determined and is reported as the average of five successive measurements. Rats were randomized to receive intravenous injection with LPS (10 mg/kg) or an equivalent volume of saline vehicle. Immediately afterward, subgroups of LPS-treated rats additionally received intraperitoneal injection with 4F (10 mg/kg) or Sc-4F (10 mg/kg). As an additional control, 4F was administered to rats in the absence of LPS treatment. SBP measurements were repeated at the 6 h time point. The dosage of 4F used in these studies is consistent with a recent clinical study showing that 4F administration at 6 mg/kg significantly improves anti-inflammatory properties of HDL in humans (36).

Measurement of plasma endotoxin, inflammatory mediators, and HDL cholesterol

Plasma samples were collected from the tail vein 0, 2, and 6 h after receiving vehicle control, LPS, or LPS+4F. Endotoxin levels in these samples were measured as previously described (37). Briefly, plasma aliquots were diluted 1:10 in pyrogen-free water and heated at 75°C for 10 min to degrade endogenous inhibitors of endotoxin. LPS (*E. coli*, serotype 026:B6) standards were treated in the same manner. Endotoxin levels were then determined using a Limulus amebocyte lysate (LAL) kit. Tumor necrosis factor-α (TNF-α) levels in plasma were measured by ELISA. HDL cholesterol was measured using a commercially available kit after precipitation of apoB-containing lipoproteins per the manufacturer's instructions (Wako, Inc.).

Vessel reactivity studies

In subsequent studies, LPS-treated rats were randomized to receive intraperitoneal injection with the COX-2 inhibitor NS-398 (10 mg/kg), the NOS2 inhibitor 1400W (10 mg/kg), or vehicle. These doses of 1400W and NS-398 have been shown to maximally inhibit NOS2 and COX-2 activities in septic rats (38, 39). Separate control groups received vehicle injection, 4F, or 1400W, but no LPS. Animals were euthanized after 6 h. The aorta was then excised and cleansed of fat and adhering tissue in preparation for measurement of isometric tension. The vessel was cut

into individual ring segments (3 mm in width) and suspended from a force-displacement transducer in an isolated tissue bath (Radnoti, Inc.). Ring segments were bathed in Krebs-Henseleit (K-H) buffer of the following composition (mM): NaCl 118; KCl 4.6; $NaHCO_3$ 27.2; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 1.75; Na_2EDTA 0.03; and glucose 11.1. Buffer was maintained at 37°C and aerated with 95% O_2/5% CO_2. A passive load of 2 g was applied to all ring segments, corresponding to the optimal range of the length-tension relationship for this arterial preparation. Vasoconstrictor responsiveness was tested in arterial ring segments by cumulative addition of phenylephrine (PE; 10^{-9}-3×10^{-6} M). In some experiments, PE dose responses were performed in arterial ring segments from LPS-treated rats in the presence of the nonspecific COX inhibitor indomethacin (5 μM). Standardized dose-response profiles for PE were constructed and analyzed for differences in developed tension. ED_{50} values for PE, the concentration of PE eliciting 50% of the maximum contractile response, were calculated and used as an index of vessel sensitivity. In other experiments, endothelium-dependent relaxation was tested in preconstricted ring segments by cumulative addition of acetylcholine (Ach; $10^{-9} - 3 \times 10^{-6}$ M).

Western blot analyses

Western blot analyses were performed on aortic tissue homogenates to assess the effects of 4F on NOS2 expression in arteries of LPS-treated rats. Tissue was homogenized and proteins extracted as previously described (31). Protein lysates (50 μg) were subjected to 7.5% SDS-PAGE under denaturing conditions and transferred to nitrocellulose membranes (Hybond, Amersham). Nitrocellulose was blocked for 60 min with 1% BSA, 0.01% Tween 20 in Tris-buffered saline. The blots were incubated with primary antibody overnight (rabbit polyclonal NOS2) followed by incubation for 1–2 h with secondary antibody (horseradish peroxidase conjugated anti-rabbit IgG). Immunoreactive bands were visualized using electrochemiluminescence (ECL; Amersham). Membranes were reprobed with primary mouse β-actin antibody (Santa Cruz, Inc.) to control for protein loading. Effects of 4F on NOS2 expression are presented as a percentage decrease compared with LPS treatment alone.

Chemiluminescent detection of nitric oxide metabolites in rat plasma

Plasma samples of vehicle- and LPS-treated rats were collected for the measurement of total NO metabolites (NOx), including nitrate (NO_3^-), nitrite (NO_2^-), S-nitrosothiols, and C- and N-nitroso compounds. NOx was reduced to NO using a vanadium chloride (VnCl) solution (0.4g Vn/50 ml 2 M HCl) boiled (90°C under a weak vacuum) and bubbled with helium gas. Plasma samples were injected into a reaction chamber containing VnCl, and NO gas that evolved from the reaction mixture was passed to a chemiluminometer (Sievers) by a stream of helium. Total NOx levels are reported.

Chromatographic colocalization of 4F and LPS in HDL

Our previous studies suggested that 4F physically associates with LPS in conditioned cell-culture medium (31). To test whether a similar binding interaction occurs in vivo, the following localization studies were performed. 4F was radiolabeled (^{14}C) as previously described (35). A solution of LPS was prepared containing 400 μg Bodipy-LPS. ^{14}C-4F (10 mg/kg) and Bodipy-LPS were then administered to control rats (n = 3) by separate intravenous injections. In some experiments, Bodipy-LPS was administered to rats (n = 3) in the absence of LPS treatment. Blood samples were collected after 10 min. Plasma was then fractionated by size-exclusion chromatography using a Bio-Logic fast-protein liquid chromatography (FPLC) system (Biorad, CA). Two Superose 6 columns (Pharmacia) in tandem were equilibrated with PBS (pH 7.4). Fractions (0.5 ml) were collected, and the presence of LPS was detected by measuring fluorescence emission at 530 nm (λ_{ex} 485 nm) using a BioTek plate reader (BioTek Instruments, VT). ^{14}C-4F counts in each fraction were measured using a scintillation counter (Beckman Instruments). The cholesterol content of collected fractions was determined enzymatically using a commercially available kit (Wako). Profiles were decomposed into component peaks and analyzed for relative area using PeakFit (SPSS Science, Chicago, IL).

^{14}C-4F and Bodipy-LPS plasma clearance studies

Time course studies were performed to determine the clearance of 4F and LPS from plasma of rats. Under isoflurane anesthesiology, polyethylene catheters (PE10) were inserted into the femoral vein and artery. At time zero, a 250 μl arterial blood sample was collected. Next, LPS (10 mg/kg) or an equivalent volume of saline vehicle was injected intravenously. This was immediately followed by intravenous injection with ^{14}C-4F (10 mg/kg). Arterial blood samples were collected after 1, 5, 10, 20, 30, 45, 60, 120, 180, and 240 min. The plasma concentration of ^{14}C-4F radioactive counts for each time point was measured and expressed as a percentage of counts present in the 1 min sample, as previously described (40). A similar procedure was used to measure the clearance of LPS from the circulation. After collecting a baseline blood sample, rats received intravenous injection with 4F (10 mg/kg) or saline vehicle, followed by injection with Bodipy-LPS (10 mg/kg). Blood samples were collected for time points up to 240 min, followed by measurement of Bodipy-dependent fluorescence in plasma. Similar to measurement of ^{14}C-4F clearance, Bodipy-LPS fluorescence was normalized to the signal obtained in the 1 min sample.

Survival studies in LPS-treated rats

Because hemodynamic changes may contribute to dysfunction of multiple organs and mortality in the context of sepsis, we assessed whether 4F administration improved survival in LPS-treated rats. As the dosage of LPS used in previous studies is sublethal, we administered LPS at a dosage of 30 mg/kg by intraperitoneal injection, as previously described (41). LPS-treated animals were randomized to receive either vehicle or 4F (10 mg/kg) by intravenous injection. A control group was composed of vehicle-treated rats.

Statistical methods

All results are reported as the mean ± SEM. Statistical analysis was performed using SigmaStat 3.5 software (Systat Software, Inc.). Differences between the groups were assessed by one-way ANOVA (ANOVA) with post hoc testing (Student-Neuman-Keuls test). Survival data was assessed by log-rank analysis (Kaplan-Meier survival method). A difference in survival between individual groups was determined using the Holm-Sidak method. $P < 0.05$ was considered statistically significant.

RESULTS

In initial experiments, we assessed the effect of 4F administration on LPS-induced changes in rodent blood pressure in vivo. SBP was measured in 10-week-old, male Sprague-Dawley rats using tail cuff plethysmography. As shown in **Fig. 1**, SBP was similar at time zero in all animals. After obtaining this baseline recording, rats were randomized to receive intravenous injection with LPS (10 mg/kg; n = 9) or an equivalent volume of saline vehicle (n = 6).

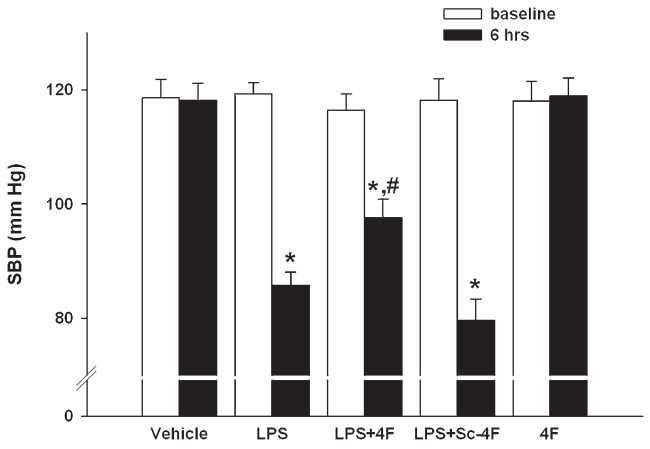

Fig. 1. 4F attenuates the hypotensive response to LPS. At baseline, systolic blood pressure was measured in rats using tail-cuff plethysmography. Rats were then randomized to receive in vivo treatment with saline vehicle (n = 6), 4F (10 mg/kg; n = 6), LPS (10 mg/kg; n = 9), LPS+4F (10 mg/kg each; n = 7) or LPS plus Sc-4F (10 mg/kg each; n = 7). Blood pressure measurements were repeated at 6 h. Data are means ± SEM. * denotes a significant difference compared with baseline blood pressure ($P < 0.05$). # denotes a significant difference compared with LPS treatment ($P < 0.05$). LPS, lipopolysaccharide; SBP, systolic blood pressure; Sc-4F, scrambled 4F.

Subgroups of LPS-treated rats additionally received 4F (10 mg/kg; n = 7) or the scrambled control peptide Sc-4F (10 mg/kg; n = 7) by intraperitoneal injection. As an additional control, 4F was administered by intravenous injection to rats (n = 6) in the absence of LPS treatment. Blood pressure measurements were repeated 6 h after treatment. Fig. 1 shows that SBP was unaltered in saline- or 4F-treated control rats over the 6 h time period. In contrast, LPS administration significantly reduced SBP at 6 h post treatment by 28% compared with the baseline. Administration of the control peptide Sc-4F, which does not form an amphipathic α-helix, did not influence SBP in LPS-treated rats. In contrast, 4F administration significantly blunted the hypotensive response to LPS (17% reduction compared with the baseline) (Fig. 1).

Because administration of 4F significantly attenuated LPS-induced hypotension at 6 h, subsequent experiments were designed to study underlying mechanisms of apoA-I mimetic peptide action. The contractile properties of blood vessels isolated from LPS-treated rats were assessed, and functional effects of 4F administration were tested. LPS significantly reduced the sensitivity of isolated aortic ring segments to the α-adrenergic agonist PE compared with vehicle-treated controls (**Fig. 2**). The ED_{50} for PE-induced contraction in ring segments of LPS-treated rats was 459 ± 83 nM compared with 57 ± 6 nM for vehicle-treated controls ($P < 0.001$). 4F administration in the absence of LPS treatment did not alter the response to PE (ED_{50} = 69 ± 8 nM). Administration of 4F to LPS-treated rats resulted in a contractile response that was similar to that of controls (ED_{50} = 60 ± 9 nM), thus preventing the inhibitory effect of LPS on vascular function (Fig. 2). Endothelium-dependent relaxation was also tested in ring segments by cumulative addition of Ach and was similar in all treatment groups (**Fig. 3**). These results suggest that the altered sensitivity to PE in LPS-treated rats is not related to changes in endothelial nitric oxide synthase (NOS3) activity.

As vasoactive metabolites of COX-2 and NOS2 have been implicated in the depression of vasoconstrictor function in sepsis, additional ex vivo studies were performed to determine the relative contribution of these enzyme systems. To test whether this impaired response was associated with increased formation of COX-2-derived vasoactive prostanoids, we administered LPS and the specific COX-2 inhibitor NS-398 (10 mg/kg) concurrently to rats. NS-398 administration did not improve the vasoconstrictor response to PE under ex vivo conditions compared with LPS

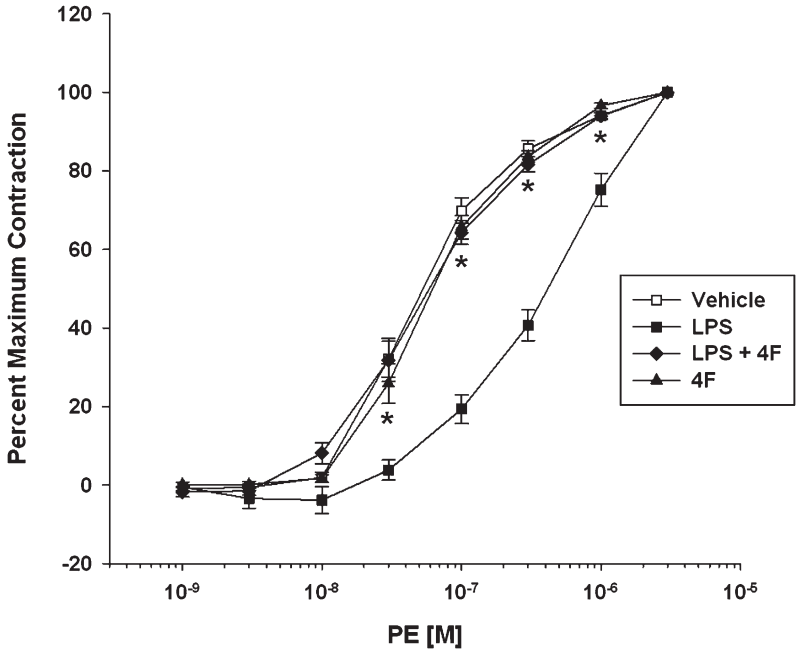

Fig. 2. 4F improves the contractile response to PE in isolated aortic ring segments of LPS-treated rats. Rats were treated in vivo with saline vehicle, LPS, and peptides as described in Fig. 1. Vasoconstrictor sensitivity was tested ex vivo by cumulative addition of PE to ring segments from rats treated with vehicle (n = 14), 4F (n = 16), LPS (10 mg/kg; n = 17) or LPS+4F (10 mg/kg each; n = 14). Data are means ± SEM. * denotes a significant difference compared with LPS treatment ($P < 0.05$). LPS, lipopolysaccharide; PE, phenylephrine.

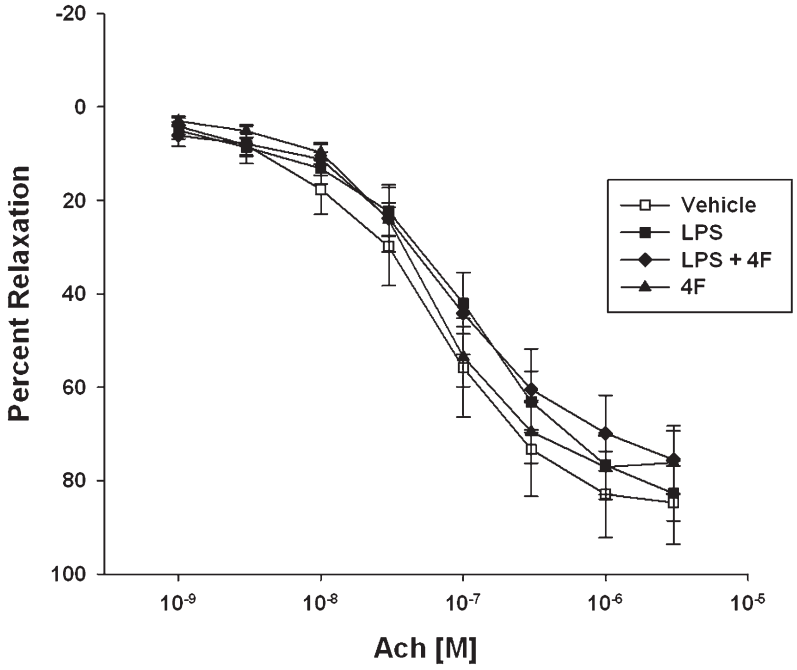

Fig. 3. Endothelium-dependent relaxation is not altered in isolated aortic ring segments of LPS-treated rats. Endothelial function was tested ex vivo by cumulative addition of Ach to ring segments from rats treated with vehicle (n = 8), 4F (n = 8), LPS (10 mg/kg; n = 17) or LPS+4F (10 mg/kg each; n = 11). Data are means ± SEM. No differences in Ach-induced relaxation were noted among groups. Ach, acetylcholine; LPS, lipopolysaccharide.

treatment (**Fig. 4**). In related experiments, we isolated aortic ring segments from LPS-treated rats and acutely exposed them to the nonspecific COX inhibitor indomethacin before administration of PE. Similar to NS-398, indomethacin did not improve the attenuated response to PE in aortic ring segments of LPS-treated rats (Fig. 4).

We next examined the effect of basal NO production on the vasoconstrictor response of isolated blood vessels of LPS-treated rats. In contrast to NS-398 and indomethacin, in vivo treatment of LPS-treated rats with the specific NOS2 inhibitor 1400W (10 mg/kg) significantly increased the sensitivity of ring segments to PE (ED_{50} = 19 ± 3 nM) (Fig. 4). This response suggested that the decreased sensitivity to PE in ring segments of LPS-treated rats was principally mediated by increased NO production and that the protective effect of 4F in vivo may be related to inhibition of NOS2 expression. Administration of 1400W to vehicle-treated rats did not alter the dose-response profile for PE.

The stimulatory effect of endotoxin on NOS2 expression is well known. Therefore, we tested the hypothesis that 4F administration in vivo prevents LPS-induced NOS2 upregulation. Aortae were harvested from control-, LPS-, and LPS+4F-treated rats, homogenized and prepared for SDS-PAGE. Western blot analyses revealed a significant (approximately 3-fold) increase in NOS2 protein in aortae of LPS-treated rats compared with vehicle-treated controls (**Fig. 5A**). 4F completely blocked the induction of NOS2 protein in LPS-treated rats (Fig. 5A). We monitored the plasma concentration of NO metabolites (NOx) as an additional index of NOS2 induction in LPS-treated rats using

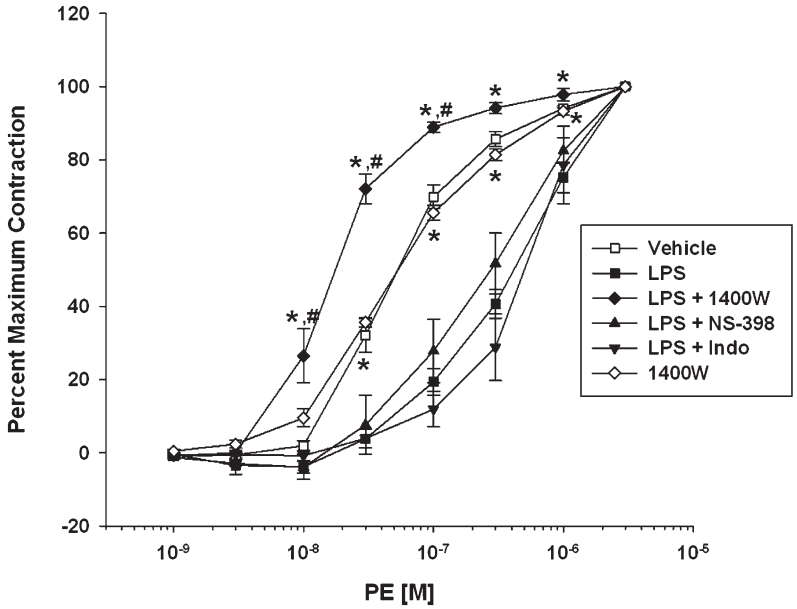

Fig. 4. In vivo administration of 1400W, but not NS-398, improves vasoconstrictor responsiveness to PE in isolated aortic ring segments of LPS-treated rats. Vasoconstrictor sensitivity was tested ex vivo by cumulative addition of PE to ring segments from rats treated with vehicle (n = 14), LPS (10 mg/kg; n = 17), the NOS2 inhibitor 1400W (10 mg/kg each; n = 9), LPS plus 1400W (10 mg/kg each; n = 15), or LPS plus the COX-2 inhibitor NS-398 (10 mg/kg each; n = 8). In some experiments, ring segments from LPS-treated rats were preincubated (30 min) in vitro with the nonselective COX inhibitor indomethacin (5 μM; n = 5). 1400W treatment significantly improved the sensitivity of blood vessels from LPS-treated rats to PE, while NS-398 and indomethacin were without effect. Data are means ± SEM. * denotes a significant difference compared with LPS treatment ($P < 0.05$). # denotes a significant difference compared with the vehicle-treated group ($P < 0.05$). COX-2, cyclooxygenase 2; LPS, lipopolysaccharide; NOS2, nitric oxide synthase 2; PE, phenylephrine.

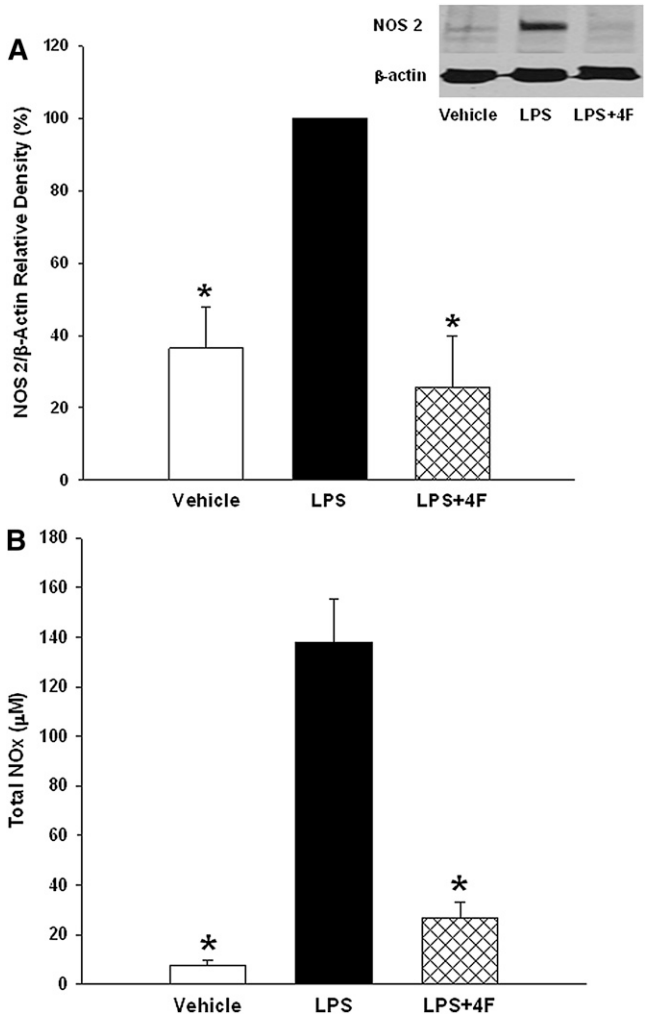

Fig. 5. 4F prevents NOS2 induction and NO formation in LPS-treated rats. A: Protein was isolated from aortae of vehicle control, LPS-treated, and LPS+4F-treated rats (6 h post treatment) and subjected to SDS-PAGE. NOS2 levels were determined using a polyclonal antibody and were normalized to the β-actin content of aortic tissue homogenates. LPS induced approximately a 3-fold increase in NOS2 protein expression compared with saline-treated controls. In LPS+4F-treated rats, the NOS2/β-actin ratio was significantly reduced. N = 5–6 for each treatment group. B: Plasma samples were collected by cardiac puncture from rats receiving saline vehicle (n = 6), LPS (n = 7), or LPS+4F (n = 9). The plasma concentration of total NO metabolites (NOx) was measured using NO chemiluminescence. Data are means ± SEM with n = 6–9 for each treatment group. * denotes a significant difference compared with LPS treatment alone ($P < 0.05$). LPS, lipopolysaccharide; NO, nitric oxide; NOS2, nitric oxide synthase 2.

NO-dependent chemiluminescence. There was a significant increase in plasma NOx in LPS-treated rats that was reversed almost to the level observed in the control group by treatment with 4F (Fig. 5B). The observed changes in plasma NOx levels mirrored the pattern of NOS2 protein expression in aortae from each treatment group, thus indicating that the principal effect of the peptide in the restoration of contractile function is due to inhibition of LPS-induced NOS2 upregulation. NOS2 induction is associated with vasomotor dysfunction and end-organ injury in humans and animal models of sepsis (42). As these responses are associated with an increase in mortality, we tested whether 4F treatment improved survival in LPS-treated rats. Over a 24 h observation period, there was no mortality in vehicle-treated control rats (**Fig. 6**). In contrast, administration of 30 mg/kg LPS increased mortality between the 12 and 21 h time points (60% mortality). Over the same time period, there was only 10% mortality in LPS-treated rats that received 4F (Fig. 6).

To test whether protective effects of 4F were related to a reduction in circulating endotoxin, we measured time-dependent changes in plasma LPS activity using the LAL assay. At baseline, LAL activity was low in all rats. Vehicle treatment did not alter plasma endotoxin activity at 2 or 6 h (**Fig. 7A**). In contrast, LPS administration significantly increased endotoxin activity after 2 h, reflecting a significant increase in circulating LPS. Endotoxin activity in LPS-treated rats returned to control levels by 6 h. Administration of 4F to LPS-treated rats significantly reduced plasma LAL activity at 2 h (Fig. 7A). Since an increase in TNF-α secretion is an early response to LPS, we measured circulating levels of the cytokine (43). Patterns of TNF-α release into the circulation mirrored plasma endotoxin activity, with a significant increase in TNF-α levels in plasma of LPS-treated rats at 2 h and a reduction by 6 h (Fig. 7B). 4F treatment significantly attenuated the increase in plasma TNF-α in LPS-treated rats at the 2 h time point (Fig. 7B).

Previous studies suggest that class A amphipathic peptides, such as 4F, may form HDL-like particles in plasma (44). The plasma concentration of HDL in 4F-treated rats is of potential importance as previous studies show that HDL, which is rich in phospholipid, can effectively neutralize LPS (45). HDL cholesterol was measured in rats at baseline and was 38 ± 3 mg/dl (n = 24). Animals were then randomized to receive either LPS+vehicle or LPS+4F. Plasma cholesterol measurements were repeated after 6 h. LPS administration was associated with a modest reduction

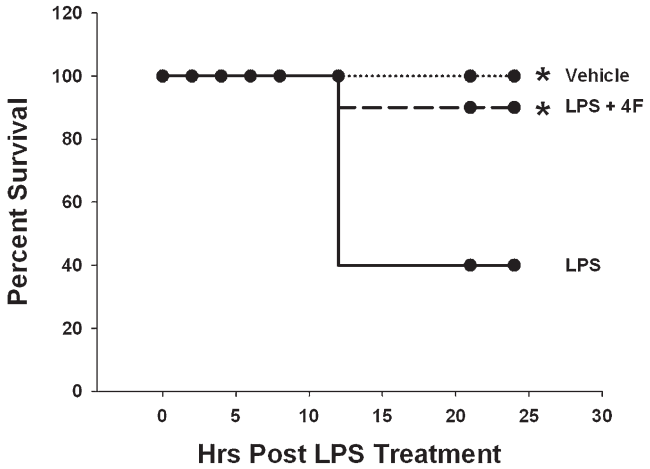

Fig. 6. 4F improves survival in LPS-treated rats. Rats were randomized to receive either saline vehicle (n = 10) or LPS (30 mg/kg) by intraperitoneal injection. Subgroups of LPS-treated rats were further randomized to receive intravenous injection with 4F (10 mg/kg; n = 20) or saline (n = 20). Animals were monitored over a 24 h time period. * denotes a significant difference compared with LPS-treated rats ($P < 0.05$). LPS, lipopolysaccharide.

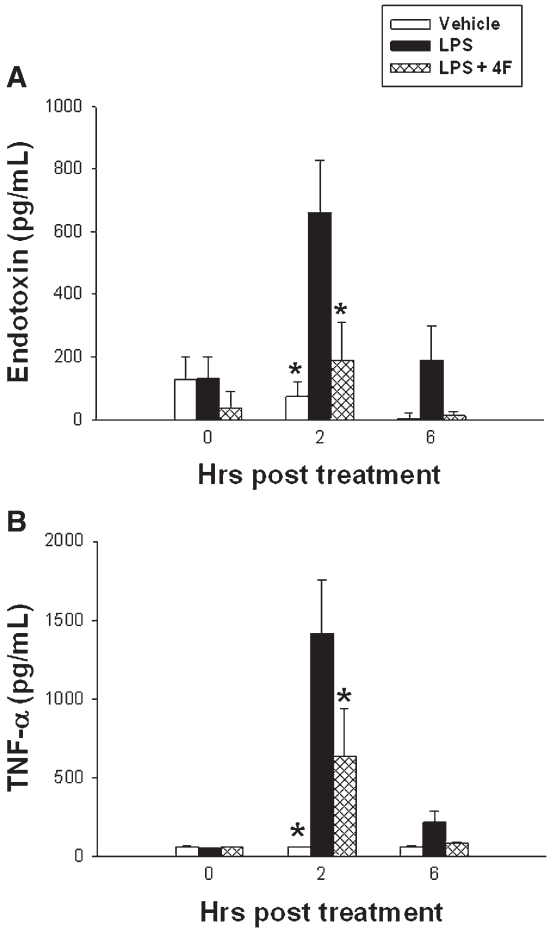

Fig. 7. 4F administration reduces plasma levels of endotoxin and TNF-α. Plasma was collected from rats 0, 2, and 6 h after treatment with vehicle (open bar; n = 8), LPS (filled bar; n = 8), or LPS+4F (hatched bar; n = 8). Plasma LPS levels were determined by measuring endotoxin activity. TNF-α was measured by ELISA. Data are means ± SEM. * denotes a significant difference compared with LPS treatment ($P < 0.05$). LPS, lipopolysaccharide; TNF, tumor necrosis factor.

in HDL cholesterol (28 ± 4 mg/dl; n = 12). HDL concentration in LPS+4F rats was significantly increased (45 ± 5 mg/dl; n = 12, $P < 0.05$) compared with LPS+vehicle treatment.

To determine whether protective effects of 4F in LPS-treated rats were related to the binding/neutralization of endotoxin by HDL, we monitored LPS interactions with plasma lipoproteins fractions. ^{14}C-labeled 4F and Bodipy-labeled LPS were administered by separate intravenous injections to control rats (n = 3). Ten minutes later, a plasma sample was collected and fractionated by FPLC. The collected fractions were assayed for cholesterol, ^{14}C-radioactivity, and Bodipy-dependent fluorescence (λ_{ex} 485 nm, λ_{em} 530 nm) (**Fig. 8**). ^{14}C-4F rapidly localized to a cholesterol fraction containing HDL (Fig. 8A, B). This was confirmed by Western blotting studies showing that this fraction was immunopositive for apoA-I and immunonegative for apoB100 (not shown). ^{14}C-4F localized to a lesser extent to the VLDL fraction and was also associated with a protein- and cholesterol-free fraction (Fig. 8B). In rats treated with ^{14}C-4F, Bodipy-LPS localized principally to the HDL fraction (Fig. 8C). In the absence of ^{14}C-4F treatment, fluorescence associated with Bodipy-LPS was negligible in the HDL fraction (Fig. 8C). Time course studies were performed to determine the clearance of 4F and LPS from the circulation of rats. Analysis of radioactive counts

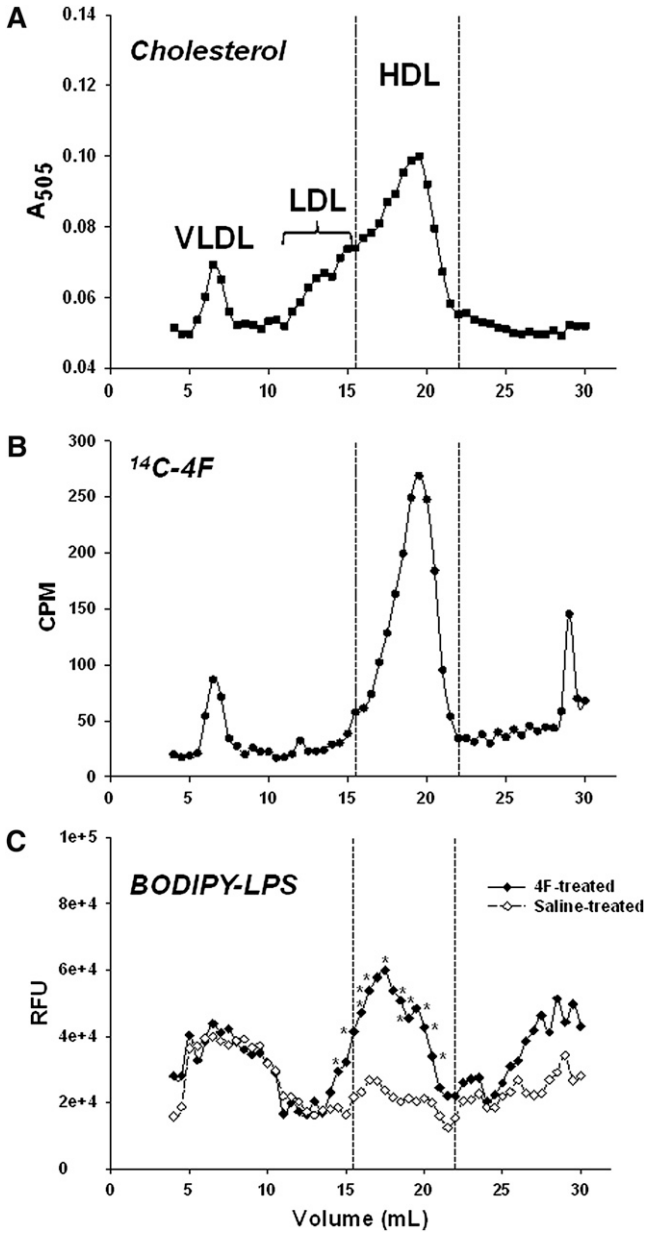

Fig. 8. 4F and LPS colocalize with plasma HDL. Rats (n = 3) received separate intravenous injections with ^{14}C-4F and Bodipy-LPS. After 10 min, plasma samples were collected and fractionated on two Superose 6 columns in tandem. Collected fractions were assayed for cholesterol (A), ^{14}C-4F counts (B), and Bodipy-LPS fluorescence (C). Each panel depicts a composite profile for plasma-associated cholesterol, radioactive counts, and fluorescence for the three rats. These data show that 4F and LPS colocalize predominantly to the HDL cholesterol fraction. In a separate study, three rats were injected with Bodipy-LPS in the absence of ^{14}C-4F (C). There was a significant increase (*$P < 0.05$) in LPS-dependent fluorescence in the HDL fraction of 4F-treated rats compared with those receiving the saline vehicle. LPS, lipopolysaccharide.

in plasma samples showed that the clearance of ^{14}C-4F from plasma was similar in rats treated with either LPS ($t_{1/2}$ = 61 ± 12 min; n = 3) or saline vehicle ($t_{1/2}$ = 86 ± 19 min; n = 4) (**Fig. 9A**). In related experiments, we found that Bodipy-LPS fluorescence was significantly reduced in plasma samples of vehicle-treated rats within 1 h after injection (Fig. 9A). The time course for fluorescence reduction mirrored the clearance of plasma endotoxin in another study (43). In rats that were pretreated with 4F, there was also an initial, rapid reduction in Bodipy-LPS; however, LPS-dependent fluorescence remained significantly elevated in plasma at time points 3 and 4 h after injection (Fig. 9B).

DISCUSSION

Synthetic peptides, whose structures are based on the helical repeating domains of apoA-I, represent an emerging area of HDL therapy (32, 46, 47). ApoA-I mimetic peptides have been shown to improve HDL quality/function by increasing its antioxidant activity, inhibiting lesion formation in dyslipidemic mice, and stimulating the formation of small HDL particles (pre β-HDL) with high cholesterol uptake capacity (32, 44, 46, 47). The rationale for testing effects of an apoA-I mimetic in LPS-treated rats is underscored by the observation that plasma HDL is significantly reduced by sepsis and that increasing plasma apoA-I and HDL improves outcomes in septic rodents and humans (22–24). Further, we previously reported that the apoA-I mimetic 4F significantly inhibits the induction of proinflammatory mediators by LPS in cultured endothelial cells (31). The goal of the current study was to extend these observations by defining the mechanism(s) of action of the peptide in improving vascular contractility in a rodent model of endotoxemia.

Endotoxemia and bacterial sepsis are associated with severe blood pressure reduction, tissue hypoperfusion, and multiple organ injury (6, 15, 23). Proinflammatory cytokines and secondary metabolites play an important role in cardiovascular depression (9, 23, 25). In the context of sepsis, cytokines induce the expression of NOS2 and COX-2, which are thought to reduce peripheral vascular

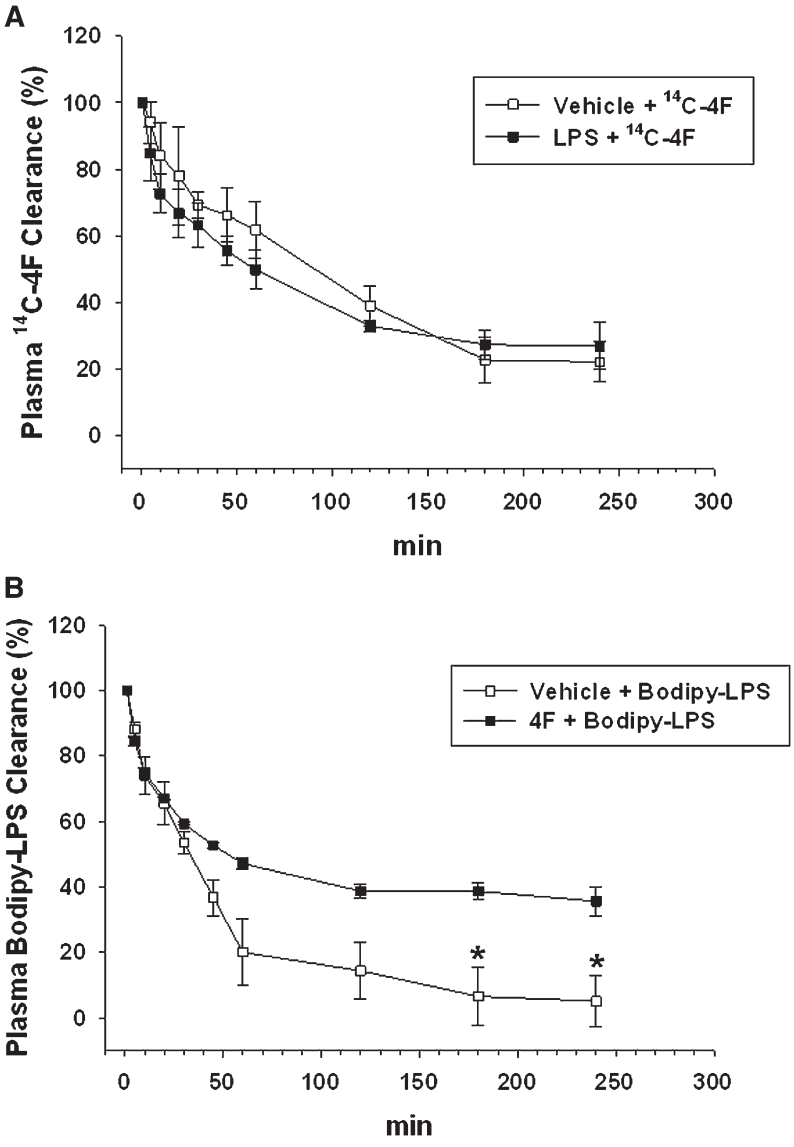

Fig. 9. Time-dependent clearance of 4F and LPS from plasma. A: Rats received intravenous injections with saline vehicle (n = 4) or LPS (10 mg/kg; n = 3), followed immediately by injection with ^{14}C-4F (10 mg/kg). Blood samples were collected over a 4 h study period. Radioactive counts for each time point were calculated as a percentage of counts present in the 1 min sample. B: Rats received intravenous injections with saline vehicle (n = 5) or 4F (10 mg/kg; n = 3), followed immediately by injection with Bodipy-LPS (10 mg/kg). Blood samples were collected over the 4 h study period, and plasma fluorescence was measured. * denotes a significant difference compared with Bodipy-LPS+4F treatment ($P < 0.05$). LPS, lipopolysaccharide.

resistance and arterial pressure via the formation of NO and vasoactive prostanoids, including prostacyclin (PGI_2) (9–13, 48). Results of the current study show that LPS administration to rats caused a reduction in SBP by 6 h post treatment. Further, this response was attenuated by the apoA-I mimetic peptide 4F. In contrast, administration of the control peptide Sc-4F, which lacks an amphipathic α-helical structure, did not influence blood pressure changes in LPS-treated rats. These results underscore the critical requirement of a class A amphipathic structure for the protective action of 4F.

To gain insight into the protective mechanism of 4F action, we performed ex vivo studies of vascular function in control- and LPS-treated rats. LPS significantly reduced the sensitivity of isolated aortic ring segments to PE (ED_{50} = 459 ± 83 nM) compared with vehicle-treated controls (ED_{50} = 57 ± 6 nM). Further, in vivo treatment with 4F resulted in normalization of this response to PE (ED_{50} = 60 ± 9 nM). As noted above, 4F treatment provided highly significant protection against the LPS-induced hypotensive response, while completely normalizing the contractile response to PE in isolated blood vessels. The reason for this differential response to 4F under in vivo and ex vivo conditions is not clear. Arterial pressure regulation, especially in the context of sepsis, is a complex response that is modulated by numerous factors, including blood volume, sympathetic nervous system activity, and the action of cytokines and humoral factors (49). While our data suggest that inhibition of NOS2 expression in aorta is an important response to 4F, effects of the peptide on other physiological mechanisms of blood pressure regulation are unclear.

Endothelium-dependent relaxation was similar in all treatment groups studied, suggesting that changes in vascular reactivity were not related to alterations in endothelial NOS3 activity. COX-2 and NOS2 have been implicated as mediators of vascular dysfunction in sepsis (9–14). The relative contribution of each enzyme to the development of hypotension, however, has been the subject of debate. Accordingly, one of the goals of this study was to determine whether 4F modulates vasorelaxant properties of COX-2 and/or NOS2. NS-398 and indomethacin failed to improve the contractile response to PE ex vivo, suggesting that COX does not play a major role in the regulation of vascular function in this model. Our observations are supported by previous findings that the COX-2 inhibitor NS-398 does not prevent the development of hypotension in LPS-treated rats or septic rats subjected to cecal ligation and puncture (CLP) injury (49–51). Strong support of a role for NOS2 in LPS-induced hypotension and the attenuation of vascular contractility has been obtained in recent years (9, 10, 12). To test whether the impaired contractile response observed in our study was associated with increased NOS2-derived NO, we administered LPS and the specific NOS2 inhibitor 1400W concurrently to rats. 1400W increased the sensitivity of isolated aortic ring segments to PE (ED_{50} = 19 ± 3 nM) compared with ring segments from rats treated with LPS alone (ED_{50} = 459 ± 83 nM). Further, 1400W induced a significant leftward shift in the PE dose-response profile compared with vehicle-treated controls (Fig. 4). These data suggested that the impaired response to PE in ring segments of LPS-treated rats was associated with increased NO production. This is underscored by findings that vascular contractility induced by catecholamines, $PGF_{2\alpha}$, and TxA_2 analogs is impaired by LPS treatment in wild-type mice but normal in NOS2-deficient animals (10, 12). Our data, therefore, suggest that 4F treatment in LPS-treated rats prevents the development of vascular hyporeactivity by limiting NOS2 protein expression and NO formation. Furthermore, these changes were associated with a significant reduction in plasma endotoxin activity.

A reduction in HDL/apoA-I accompanies the acute phase response to bacterial infection and overt sepsis (6, 22, 23). In the current study, we found that HDL levels were modestly reduced in LPS-treated rats compared with baseline measurements. 4F treatment, however, resulted in a significant increase in plasma HDL in LPS-treated rats compared with animals treated with LPS+vehicle. This response may be explained by previous studies showing that acute treatment of mice with an apoA-I mimetic peptide induces the formation of lipid-poor HDL particles in plasma within several minutes after peptide treatment (52). Increasing plasma HDL has been shown to reduce complications associated with endotoxemia (24). In transgenic mice that overexpress apoA-I, plasma HDL concentration is increased 2-fold; this increase is associated with increased protection against the deleterious effects of exogenously administered LPS (24). It has been proposed that LPS is neutralized by HDL via insertion of its hydrophobic lipid A component into the lipid leaflet of the particle (24). Other data suggest that apoA-I administered as reconstituted HDL reduces inflammatory injury by inhibiting expression of the LPS receptor CD14 and TNF-α release in human monocytes (53).

Our results show that acute treatment of rats with 4F and LPS resulted in the rapid colocalization of these molecules in a plasma cholesterol fraction that was composed of HDL. These data are in agreement with previous studies showing that 4F associates with HDL in plasma of $apoE^{-/-}$ and $LDLR^{-/-}$ mice (52, 54). However, our results contrast with a recent report that biotinylated 4F does not bind HDL (55). Results of the current and previous studies show that radiolabeled 4F associates with HDL within minutes after injection and then degrades to form a product associated with hemoglobin (56). The association of biotinylated 4F with HDL was tested at later time points, and it is possible that an initial interaction between these metabolites was missed (55). Alternatively, biotinylation of 4F may alter its structure, thus increasing its susceptibility to degradation (56). Under these conditions, a reduced association of 4F with HDL would be expected.

^{14}C-4F clearance studies showed that the plasma $t_{1/2}$ for the peptide was similar in rats receiving saline vehicle (86 ± 20 min) or LPS (61 ± 12 min). In other studies, we found that Bodipy-LPS was rapidly cleared from the circulation of both vehicle- and 4F-treated rats. At later time points, however, LPS-dependent fluorescence was significantly elevated in plasma of 4F-treated rats compared with rats

receiving Bodipy-LPS+vehicle. 4F treatment in LPS-treated rats was also associated with a reduction in plasma endotoxin activity. Collectively, these data suggest that 4F reduces LPS-induced inflammatory responses by directing endotoxin to the HDL particle where LPS activity becomes neutralized. Alternatively, 4F may interact with circulating lipoproteins to form small HDL-like particles that directly bind and neutralize LPS. These results mirror our previous finding that 4F or a 4F-lipid complex physically interacts with LPS and inhibits endotoxin-induced inflammatory responses in cultured endothelial cells (31). It is proposed that the 4F-mediated neutralization of LPS prevents the induction of proinflammatory mediators, including TNF-α and NOS2, prevents defects in vascular function, and improves long-term survival in LPS-treated rats. We recently reported that 4F administration, after sepsis induction, also reduces inflammatory injury and improves survival in rats that underwent cecal ligation and puncture surgery (57). Results of these studies suggest that the apoA-I mimetic peptide 4F, which is currently undergoing clinical evaluation, may be effective in reducing CV complications and inflammatory injury associated with sepsis in humans.

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