Apolipoprotein A-I mimetic peptide 4F attenuates kidney injury, heart injury, and endothelial dysfunction in sepsis

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1Division of Nephrology, University of São Paulo School of Medicine, São Paulo, Brazil; 2Heart Institute, University of São Paulo School of Medicine Hospital das Clínicas, São Paulo, Brazil; 3Immunochemistry Department, University of São Paulo, São Paulo, Brazil; and 4Department of Pathology, University of São Paulo School of Medicine, São Paulo, Brazil

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Moreira RS, Irigoyen M, Sanches TR, Volpini RA, Camara NO, Malheiro DM, Shimizu MH, Seguro AC, Andrade L. Apolipoprotein A-I mimetic peptide 4F attenuates kidney injury, heart injury, and endothelial dysfunction in sepsis. Am J Physiol Regul Integr Comp Physiol 307: R514–R524, 2014. First published June 11, 2014; doi:10.1152/ajpregu.00445.2013.—Kidney injury, heart injury, and cytokine-induced vascular hyperpermeability are associated with high rates of morbidity and mortality in sepsis. Although the mechanism remains unknown, apolipoprotein A-I (apoA-I) mimetic peptide 4F reduces inflammation and protects HDL levels, which are reduced in sepsis. We hypothesized that 4F also protects kidneys and hearts in a rat model of cecal ligation and puncture (CLP). We divided Wistar rats into groups: sham-operated (control), CLP, and CLP + 4F rats, baroreflex sensitivity and difference in MAP, the HR was significantly higher in CLP rats than in control and CLP + 4F rats. In CLP + 4F rats, baroreflex sensitivity and cardiac function were completely protected from the effects of CLP, as was glomerular filtration; heart mitochondria morphology was improved; sepsis-induced changes in serum cholesterol, LDL, HDL, and apoA-I were less common; all cytokines were lower than in CLP rats; and expression of Slit2, Robo4, and eNOS was completely restored. Administration of 4F inhibits inflammatory responses and strengthens the vascular barrier, protecting kidneys and hearts in an HDL-dependent manner. To determine the extent of the protective effect of 4F, further studies are needed.

apolipoproteins; cardiac failure; endothelium-derived factors; infection; kidney; nitric oxide synthase

Sepsis continues to be one of the leading causes of death worldwide and the primary cause of mortality in the intensive care unit (3). In many infections, components of the resulting cytokine-induced secondary inflammatory injury can be more toxic than are the invading microbes themselves (38). Inflammatory cytokines destabilize endothelial cell-cell interactions and severely impair vascular barrier function, leading to capillary leakage, tissue edema, organ failure, and death (1, 35). Patients with sepsis typically develop progressive subcutaneous and body cavity edema, suggesting a widespread increase in vascular permeability. A signaling pathway involving the Slit family of secreted proteins, together with their transmembrane receptor, Roundabout (Robo), has been shown to play a role in regulating vascular permeability. London et al. (34) found that those proteins play a role in angiogenesis, and that, in vitro, recombinant Slit can attenuate the endothelial permeability caused by endotoxin activity and cytokines. The authors showed that, by binding the Robo4 receptor, the Slit protein prevents the dissociation of p120-catenin from vascular endothelial (VE)-cadherin, which is a key adhesion molecule, in response to inflammatory mediators, with the result that VE-cadherin remains on the plasma membrane. Thus the disassembly of intercellular junctions is prevented and barrier integrity is maintained.

Plasma HDLs are a family of particles with densities of 1.063–1.21 g/ml. Because HDLs are smaller than are other lipoproteins, they penetrate between endothelial cells, and tissue levels of HDL are high relative to those of other lipoproteins (37). The major apolipoprotein of most plasma HDLs is apolipoprotein A-I (apoA-I), which, in association with phospholipids and cholesterol, envelops a core of cholesterol esters. It has been demonstrated that, in transgenic mice with plasma HDL levels that are twofold higher than normal, plasma levels of tumor necrosis factor alpha are elevated and endotoxin-induced mortality is low (31). The ability of HDLs to bind and inactivate lipopolysaccharide (LPS) is thought to be responsible for its beneficial effects in endotoxemia and sepsis (18). However, it has recently been discovered that HDLs also directly inhibit the expression of E-selectin in human endothelial cells (11, 12). Ex vivo studies have shown that the contractile response to phenylephrine is lower in the aortas of LPS-treated rats than in those of control rats and that the administration of 4F improves vascular contractility and reduces LPS-induced mortality (16). Those benefits were found to be associated with a reduction in plasma endotoxin activity.

Severely ill patients have low plasma levels of cholesterol and lipoprotein, although triglycerides are often elevated (21, 22). The synthetic apoA-I mimetic peptide 4F (4F) mimics many of the properties of apoA-I, acting as a mediator of cholesterol efflux and thus countering the proinflammatory effects of LDL cholesterol (2). In septic rats treated with 4F, Zhang et al. (52) found that plasma levels of HDL were elevated, which was associated with improved cardiac output and lower mortality. The authors suggested that the protective effects of 4F are related to its ability to prevent the sepsis-induced reduction in plasma HDL. Datta et al. (17) also demonstrated that 4F treatment reduces plasma levels of in-
flammatory mediators and increases left ventricular filling, thereby improving cardiac performance.

We hypothesized that 4F would protect kidneys and hearts in a rat model of sepsis, inhibiting inflammatory responses and strengthening the vascular barrier in an HDL-dependent manner. The objective of the present study was to test that hypothesis.

METHODS

Animals and Experimental Protocol

Male Wistar rats, weighing 180–230 g, were obtained from the animal facility of the University of São Paulo School of Medicine, in São Paulo, Brazil. Animals were fed standard rat chow and given ad libitum access to tap water. Before the experiments outlined below, the rats were randomly divided into three groups: control, consisting of sham-operated rats; cecal ligation and puncture (CLP), consisting of untreated rats submitted to CLP; and CLP + 4F, consisting of rats submitted to CLP and in atraperitoneally injected, 6 h later, with 10 mg/kg body wt of 4F (sequence: Ac-DWKFAYDKVAEKFKEAF-NH₂; Pi Proteomics, Huntsville, AL).

Rats were anesthetized with isoflurane, after which a midline incision was made and the cecum exposed. A 4–0 silk ligature was placed 1.5 cm from the cecal tip. The incision was punctured twice with an 16-G needle and gently squeezed to confirm leakage of cecal contents. The abdominal incision was closed in layers with 3–0 silk sutures. Control animals were submitted to the same procedure, minus the ligation and puncture. To ensure adequate fluid resuscitation, each animal received an injection of 0.15 M NaCl (25 ml/kg body wt ip) immediately after the procedure. Additional fluid therapy (0.15 M NaCl, 25 ml/kg body wt ip) was started at 6 h after the procedure and repeated every 12 h thereafter, as was antibiotic therapy with imipenem/cilastatin (14 mg/kg body wt ip).

Inulin clearance studies. To evaluate renal function, we determined inulin clearance. Before the inulin clearance studies, each animal was anesthetized with an injection of thiopental sodium (50 mg/kg body wt ip). The trachea was cannulated with a PE-240 catheter, and spontaneous breathing was maintained. To control mean arterial pressure (MAP) and allow blood sampling, a PE-60 catheter was inserted into the right carotid artery. To collect urine samples, a suprapubic incision was made, and the urinary bladder was cannulated with a PE-240 catheter. After the surgical procedure, a loading dose of inulin (100 mg/kg body wt at 0.04 ml/min) throughout the experiment. A total of three urine samples were collected at 30-min intervals. Blood samples were obtained at the beginning (0.3 ml) and end of the experiment (4–6 ml). Organs were then perfused with PBS, and the kidneys were immediately removed. Some kidneys were frozen in liquid nitrogen and stored at −70°C for subsequent immunoblotting for Slt2, Robo4, and eNOS. For immunohistochemical analysis, kidneys were immersed in methacarn (60% methanol, 30% chloroform, and 10% acetic acid), after which kidney fragments were embedded in paraffin and cut into 4-μm sections. We determined inulin, in plasma and urine, using the anthrone method. We also measured serum levels of creatinine (the most widely used method for measuring renal function).

Analysis of blood and urine. The volume of each 24-h urine sample was measured gravimetrically. Urine samples were centrifuged in aliquots to remove suspended material, and the supernatants were analyzed. Plasma and urinary levels of sodium and potassium were measured with ion-selective electrodes (NOVA Biomedical, Waltham, MA), whereas kinetic techniques were used to measure serum levels of aspartate aminotransferase, alanine aminotransferase, and LDL. Plasma Levels of creatinine were measured with an automated colorimetric assay. Plasma levels of cholesterol and HDL were measured using colorimetric enzymatic assay. Venous plasma lactate and bicarbonate were measured with a blood gas analyzer (Radiometer Medical, Copenhagen, Denmark).

Cytokine analysis. To determine plasma levels of interleukin (IL)-6, IL-10, and IL-18, we used a multiplex cytokine assay kit (Bio-Plex Rat 9-Plex; Bio-Rad, Hercules, CA). The assay was read on the Bio-Plex suspension array system, and the data were analyzed with Bio-Plex Manager software, version 4.0 (14).

Immunohistochemistry. Stained sections were handled by technicians who were blinded to the groups. For P-selectin, immunostaining samples were processed in 4-μm paraffinized sections. After deparaffinization, endogenous peroxidase activity was blocked with 0.3% H₂O₂ in water for 10 min at room temperature. Sections were then subjected to incubation overnight at 4°C with an anti-P-selectin antibody (CD62P, 1:100; Abbiotec, San Diego, CA). This was followed by incubation with biotinylated mouse anti-IgG for 30 min at room temperature. The reaction product was detected with the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). The color reaction was developed with 3,3-diaminobenzidine (Sigma), and the sections were counterstained with Harris hematoxylin solution. For all sections, negative controls consisted of replacing the primary antibody with equivalent concentrations of an irrelevant normal rabbit IgG. The sections were examined under light microscopy at a magnification of ×400. For evaluation of P-selectin immunostaining, 30–40 tubulointerstitial grid fields (0.245 mm²) were graded semiquantitatively, and the mean score per kidney was calculated. Each score (0 to IV) mainly reflected changes in the extent, rather than the intensity, of staining and depended on the percentage of grid field showing positive staining: 0, absent or <5%; I, 5–25%; II, 25–50%; III, 50–75%; and IV, >75%.
Histomorphometry. Four-micrometer histological sections of renal tissue were stained with hematoxylin-eosin, and renal tubular damage was examined under light microscopy. We classified the degree of renal tubular damage (cellular debris and cylinders in the tubular lumen, flattening of the epithelium, and desquamation) in terms of the proportion of the microscopic field in which such damage was observed, as follows: < 10%, 10–25%, 26–50%, or > 50%. The morphometric examination was blinded to minimize observer bias, i.e., the observer was unaware of the treatment group from which the tissue originated.

Quantification of eNOS, Slit2, Robo4, and apoA-I. We used a Teflon pestle glass homogenizer to homogenize kidney and liver samples in ice-cold isolation solution (200 mM mannitol, 80 mM HEPES, 41 mM KOH, pH 7.5) containing the protease inhibitor cocktail. The homogenates were centrifuged at low speed (3,000 g) for 15 min at 4°C to remove nuclei and cell debris. The pellets were suspended in isolation solution with protease inhibitors. Protein concentrations were determined by the Bradford assay method (Bio-Rad Protein Assay kit; Bio-Rad).

Membrane fractions from kidney and liver samples were run on polyacrylamide minigels as follows: 8% gels for eNOS, Slit2, and Robo4; and 12% gels for apoA-I. After they were transferred by electroelution to nitrocellulose membranes (PolyScreen, polivinyldene difluoride transfer; Life Science Products, Boston, MA), blots were blocked with 5% milk and 0.1% Tween 20 in Tris-buffered saline for 1 h. Blots were then incubated overnight with an anti-eNOS antibody (1:1,000), Slit2 antibody (1:100), Robo4 antibody (1:500), and apoA-I antibody (1:5,000). Each blot was stripped and reprobed with actin antibody (1:5,000). We visualized the labeling with a horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG, diluted 1:2,000; anti-mouse IgG, diluted 1:2,000; anti-goat, diluted 1:10,000), using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

We scanned the enhanced chemiluminescence films using Image Master VDS software (Pharmacia Biotech, Uppsala, Sweden). We then used densitometry to perform quantitative analyses of the antibodies, normalizing the bands to actin expression.

Phase 2

Autonomic evaluations. For the autonomic evaluations, echocardiographic measurements, and invasive evaluation of left ventricular function, we used 10 animals from each group. Rats were anesthetized with isoflurane, and two catheters filled with 0.06 ml of saline were implanted, one into the femoral artery and the other into the femoral vein. The free catheter ends were tunneled under the skin of the back to the level of the shoulder blades. After CLP or sham surgery, the animals were placed in cages (1 animal per cage) and allowed to recover from surgery. We chose to use small cages to immobilize the animals, thus facilitating the measurement of blood pressure. Fifteen minutes later, the arterial cannula was connected to a strain-gauge transducer (Blood Pressure XDC; Kent Scientific, Litchfield, CT), and arterial pressure signals were recorded over a 30-min period in conscious animals by a microcomputer equipped with an analog-to-digital converter board (WinDaq, 2-kHz; DATAQ, Springfield, OH). The recorded data were analyzed on a beat-to-beat basis to quantify changes in MAP and heart rate (HR), as previously described (18, 19).

Phase 3

Survival study. Following another set of the experiments described earlier (CLP, CLP+4F, and control groups; n = 9 animals/group), survival curves were plotted, initially at 6 h after CLP and then every 6–12 h, over a 96-h period.

Statistical Analysis

Differences among the means of multiple parameters were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test. Quantitative data are expressed as means ± SE. For the autonomic evaluations, quantitative data are also expressed as means ± SD. Values of \( P < 0.05 \) were considered statistically significant. The statistical software employed was GraphPad Prism, version 5 (Graph-Pad, La Jolla, CA).

RESULTS

Phase 1 Findings

Renal function. As can be seen in Fig. 1, inulin clearance at 24 h after CLP and sham surgery was lower in the CLP rats than in the control rats (0.50 ± 0.06 vs. 0.79 ± 0.06 ml·min\(^{-1}\)·100
Our immunohistochemical study showed that sepsis induced P-selectin expression in kidney tissue (Fig. 3). Only a few P-selectin-positive cells were seen in control rats (0.34 ± 0.12 cells/0.245 mm²; Fig. 3, A and D). In contrast, CLP induced pronounced expression of P-selectin (CLP: 0.70 ± 0.28 cells/0.245 mm²; Fig. 3, B and D), although treatment with 4F attenuated that response (CLP+4F: 0.35 ± 0.10 cells/0.245 mm²; Fig. 3, C and D).

**Endothelial cell integrity.** Expression of Slit2 was markedly lower in CLP rats than in control rats (68 ± 4.2 vs. 95 ± 1.5%; P < 0.001). As can be seen in Fig. 5, A and B, treatment with 4F completely reversed the downregulation of Slit2 observed in the CLP rats (90 ± 5.2 vs. 68 ± 4.2%; P < 0.001). As can be seen in Fig. 5, C and D, Robo4 protein expression at 24 h postprocedure was markedly lower in the CLP group than in the control group and the CLP+4F group (70 ± 5.3 vs. 100 ± 0.5% and 103 ± 2.0%; P < 0.0001 for both). At 24 h after the surgical procedures, eNOS protein expression was markedly lower in the CLP rats than in the control rats (35 ± 6.7 vs. 97 ± 11%; P < 0.002). Notably, eNOS expression recovered completely after treatment with 4F (CLP+4F: 105 ± 9.0%; P < 0.002 vs. CLP; Fig. 5, E and F). In addition, 4F treatment appeared to protect the microcirculation, plasma lactate being significantly lower in CLP+4F rats than in CLP rats (Table 1).

**Serum cholesterol, LDL, HDL, and apo-A-I.** As can be seen in Table 1, CLP, compared with sham surgery, was associated with a significant reduction in serum levels of cholesterol and HDL. In addition, serum levels of LDL were significantly higher in CLP rats than in control rats. However, in CLP+4F rats, the sepsis-induced alterations in the serum levels of cholesterol, LDL, and HDL were reversed.

We also examined renal and hepatic expression of apo-A-I, which is the dominant protein component of circulating HDL and is predominantly secreted by the liver. As shown in Fig. 5, G and H, we found that renal protein expression of apo-A-I was significantly lower in CLP animals than in control animals.

**Table 1. Biochemical parameters at 24 h after cecal ligation and puncture or sham surgery**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>CLP</th>
<th>CLP + 4F</th>
<th>4F</th>
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<tbody>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.16 ± 0.092</td>
<td>0.29 ± 0.274</td>
<td>0.21 ± 0.138</td>
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<td>Water intake, ml/day</td>
<td>16.3 ± 2.1</td>
<td>16.7 ± 4.3</td>
<td>15.0 ± 3.3</td>
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</tr>
<tr>
<td>Urinary output, ml/day</td>
<td>16.4 ± 1.8</td>
<td>9.1 ± 1.45</td>
<td>19.4 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>UVNa, meq/day</td>
<td>1.94 ± 0.16</td>
<td>0.20 ± 0.036</td>
<td>1.31 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>UVK, meq/day</td>
<td>2.13 ± 0.14</td>
<td>3.50 ± 0.554</td>
<td>2.12 ± 0.17</td>
<td></td>
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<tr>
<td>AST, IU</td>
<td>102.0 ± 12.7</td>
<td>317.0 ± 14.46</td>
<td>96.0 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>ALT, IU</td>
<td>45.0 ± 5.0</td>
<td>68.0 ± 6.45</td>
<td>32.0 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>2.1 ± 0.2</td>
<td>3.9 ± 1.09</td>
<td>1.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>67.2 ± 3.2</td>
<td>40.0 ± 4.06</td>
<td>51.1 ± 3.88</td>
<td></td>
</tr>
<tr>
<td>LDL, mg/dl</td>
<td>11 ± 1.4</td>
<td>18 ± 2.75</td>
<td>8.2 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>42.1 ± 2.3</td>
<td>19.3 ± 1.76</td>
<td>34.2 ± 2.99</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8. Control, sham-operated rats; CLP, rats subjected to cecal ligations and puncture (CLP) only; CLP+4F, rats subjected to CLP and treated with apolipoprotein A-I mimetic peptide 4F; UVNa, urinary excretion of sodium; UVK, urinary excretion of potassium; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDL, low-density lipoprotein. *P < 0.001 vs. control. **P < 0.001 vs. CLP+4F. *P < 0.05 vs. control. ****P < 0.05 vs. CLP + 4F.
(28 ± 3.3 vs. 97 ± 7.0%; P < 0.0001). In contrast, renal protein abundance of apoA-I was significantly higher in CLP+4F rats than in CLP rats (97 ± 9.7 vs. 28 ± 3.3%; P < 0.0001). As shown in Fig. 5, I and J, we found that hepatic protein expression of apoA-I in the CLP group was comparable to that observed in the control group (90 ± 3.5 vs. 99 ± 2.7%, not significant). In contrast, hepatic protein abundance of apoA-I was significantly higher in the CLP+4F rats than in the CLP rats (109.7 ± 4.4 vs. 90.3 ± 3.5%; P < 0.01). Taken together, the findings suggest that, in the setting of sepsis, apoA-1 expression is impaired in kidney tissue but not in liver tissue.

Phase 2 Findings

Hemodynamic and autonomic function. Hemodynamic and autonomic evaluations are presented in Fig. 6. As can be seen in Fig. 6A, there was no difference among the groups in the immediate postprocedure period or at 24 h after the procedures in terms of MAP. However, postprocedure HR was significantly higher in the CLP group than in the control and CLP+4F groups (Fig. 6B). Taking these findings together, we postulated that HR increases as a compensatory mechanism to maintain MAP. Baroreflex sensitivity, as evaluated by tachycardic and bradycardic responses, was poorer in the CLP group than in the control group but was completely restored in the CLP+4F group (Fig. 6, C and D).

Sepsis-induced cardiac dysfunction. At 24 h after sham surgery or CLP, cardiac output was significantly lower in the CLP group than in the control group (50.0 ± 6.2 vs. 73.2 ± 2.3 ml/min; P < 0.01; Fig. 7). However, the cardiac output observed in the CLP+4F group (77.0 ± 8.2 ml/min) was comparable to that observed for the control group and significantly different from that observed for the CLP group (P < 0.01). At 24 h after the procedures, the myocardial performance index was significantly higher in the CLP rats than in the control rats and CLP+4F rats (0.64 ± 0.02 vs. 0.45 ± 0.04 and 0.37 ± 0.03; P < 0.001). The left ventricular ejection fraction 4F ATTENUATES SEPSIS-INDUCED ORGAN DYSFUNCTION

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Fig. 2. Effects of apolipoprotein A-I mimetic peptide 4F on serum levels of interleukin (IL)-6 (A), IL-10 (B), and IL-18 (C) at 24 h after CLP or sham surgery (n = 8/group). *P < 0.01 vs. control and CLP+4F.

Fig. 3. Immunohistochemical localization of intercellular adhesion P-selectin in rat kidney sections at 24 h after CLP or sham surgery. A: control (sham-operated) rats (n = 8); B: rats subjected to CLP only (n = 8); C: CLP+4F rats (n = 8). Sections were incubated overnight at 4°C with primary goat polyclonal anti-rat P-selectin antibody (1:100). D: bar graph representing values obtained in each of the groups. Values are means ± SE.
fraction was significantly higher in CLP+4F rats than in CLP rats (79 ± 2.0 vs. 67 ± 2.8%; \(P < 0.03\)). As shown in Fig. 6, there was no significant difference between the CLP+4F rats and the control rats in terms of the left ventricular ejection fraction (79 ± 2.0 vs. 75 ± 2.7%). We found it interesting that the left ventricular end-diastolic diameter was significantly lower in the CLP group than in the control group (0.59 ± 0.03 vs. 0.67 ± 0.01 cm; \(P < 0.02\)). There was no statistical difference between the CLP+4F and control groups (0.66 ± 0.01 vs. 0.67 ± 0.01 cm).

**Fig. 4.** Histological sections of renal tissue stained with hematoxylin-eosin and examined under light microscopy to quantify renal tubular damage after CLP or sham surgery. A: sham-operated (control) group. B: CLP group. C: CLP+4F group.

**Fig. 5.** Semiquantitative immunoblotting of kidney fractions and densitometric analysis of samples from sham-operated (control) rats (\(n = 8\)), CLP rats (\(n = 8\)), and CLP+4F rats (\(n = 8\)). A: immunoblots reacted with anti-Slit2 revealing a 200-kDa band. C: immunoblots reacted with anti-Robo4 revealing a 140-kDa band. E: immunoblots reacted with anti-endothelial nitric oxide synthase (eNOS) revealing a 140-kDa band. G and I: immunoblots reacted with anti-apo-A-I revealing a 28-kDa band. B, D, F, H, and J: densitometric analysis of all samples from control, CLP, and CLP+4F rats. Differences among the means were analyzed by ANOVA followed by the Student-Newman-Keuls test. *\(P < 0.001\) vs. sham and CLP+4F. #\(P < 0.0001\) vs. sham and CLP+4F. \&\(P < 0.002\) vs. sham and CLP+4F. &\(P < 0.01\) vs. CLP+4F.
In accordance with the data related to left ventricular end-diastolic diameter, invasive evaluation of left ventricular function demonstrated that 4F administration completely prevented the drop in left ventricular end-diastolic pressure seen in the untreated animals at 24 h after CLP (8.0 ± 0.6 vs. 4.6 ± 0.45 mmHg; \( P < 0.0003 \); Fig. 7). Left ventricular end-diastolic pressure did not differ between the CLP and control rats (8.0 ± 0.6 vs. 7.5 ± 0.55 mmHg).

Phase 3 Findings

Sepsis-induced heart mitochondrial dysfunction. In the CLP group, we observed alterations in mitochondrial morphology (Fig. 8), including swelling, as well as disruption of cristae and of the internal mitochondrial structure, especially in the cristae with balloon-like expansions, which may be indicative of pore formation. In some mitochondria, these alterations were only partial; others showed complete disruption of their internal structure. In the treated animals, there was significant improvement in the morphology, with partial recovery of the structure.

Phase 4 Findings

Survival in polymicrobial sepsis. Mortality was significantly lower in the CLP+4F group than in the CLP group (Fig. 9).

DISCUSSION

In this study, we demonstrated that, in the CLP model of sepsis, there is a significant increase in the serum levels of cytokines, leading to damage of the microvascular endothelium. These changes in the endothelium can lead to increased vascular permeability and vascular leak, as well as to accumulation of parenchymal and interstitial fluid. We confirmed the above by demonstrating decreased expression of Slit2 and Robo4, proteins responsible for the intercellular junction assembly and for maintaining the integrity of the endothelial barrier, as is eNOS, the expression of which is also decreased in the CLP model. It is possible that the decreases in left ventricular end-diastolic diameter and left ventricular end-diastolic pressure resulted from this phenomenon (impaired left ventricular filling induced by vascular leak). In support of that hypothesis, we observed a decrease in the urinary excretion of sodium and an increase in the urinary excretion of potassium, suggesting activation of the renin-angiotensin-aldosterone system. We also observed extensive fluid accumulation in the peritoneal cavity of CLP rats. We have also demonstrated a defect in the baroreceptor response.

As we expected, sepsis induced multiorgan dysfunction, with impaired renal, cardiac, and hepatic function. We find it...
interesting that 4F-induced increases in the levels of HDL and apoA-I resulted in lower cytokine levels; higher expression of Slit2, Robo4, and eNOS; normalization of the baroreceptor response; complete restoration of renal, cardiac, and hepatic function; and lower mortality.

It is known that lipoprotein metabolism is strongly influenced by inflammation, infection, and sepsis (7, 27). Patients with sepsis frequently manifest decreased serum lipid levels and can develop hypolipoproteinemia (39). In addition, lipoprotein infusion is protective against lethality in endotoxemia (49), and HDL receptor-deficient mice manifest increased mortality following CLP (29). It has been suggested that HDL plays a role in innate immunity and is an anti-inflammatory molecule (45). One of the anti-inflammatory effects of HDL is

Fig. 7. Transthoracic echocardiography and invasive evaluation of left ventricular end-diastolic pressure at 24 h after CLP or sham surgery in control rats (n = 10), CLP rats (n = 10), and CLP + 4F rats (n = 10). A: cardiac output (CO). B: myocardial performance index (MPI). C: left ventricular ejection fraction (LVEF). D: left ventricular end-diastolic diameter (LVIDA). E: invasive evaluation of left ventricular end-diastolic pressure (LVEDP). #P < 0.01 vs. sham and CLP + 4F. *P < 0.001 vs. sham and CLP + 4F. &P < 0.0003 vs. sham and CLP + 4F.

Fig. 8. Morphology of mitochondria in left ventricular tissue 24 h after CLP or sham surgery in sham-operated rats (control; n = 3; A and D); in rats subjected to CLP only (n = 3; B and E); in CLP rats and CLP + 4F rats (n = 3; C and F). Arrows, deformation of mitochondrial cristae (red) with balloon expansion (yellow). Magnification: ×24,000 (A, B, and C) and ×50,000 (D, E, and F). Bar: 0.05–1 μm.
neutralizing gram-negative and gram-positive microorganisms by binding lipoteichoic acid to LPS (23, 51). Another important HDL mechanism involves stimulation of eNOS production and protection of LDL against peroxidation, which inhibits the expression of endothelial cell adhesion molecules (such as selectins and the intercellular adhesion molecule family proteins), and preventing the release of proinflammatory cytokines (10, 11). In our study, the treated animals presented significant increases in serum HDL and eNOS protein expression, together with decreases in serum LDL and protein expression of E-selectin. In addition, post-CLP treatment with 4F resulted in cytokine levels that were lower than those observed in the untreated animals submitted to CLP. Gupta et al. (24) reported that 4F antagonizes the inflammatory effects of LPS in cell culture models.

The soluble protein Slit2, interacting with its transmembrane receptor, Robo, was first described in Drosophila as a neuronal and axonal repellent during development of the central nervous system (8, 28). Cell types that have been shown to produce Slit2 include arterial endothelial cells and vascular smooth muscle cells (33, 50). The results of a recent in vitro study conducted by London et al. (34) indicate that recombinant Slit, by interacting with Robo4, prevents endotoxin- and cytokine-induced endothelial permeability. The authors suggested that this protective effect is mediated by greater localization of VE-cadherin to the cell membrane, given that endotoxin activity and the release of cytokines displace VE-cadherin from the cell membrane to the interior of the cell, which creates gaps between endothelial cells, leading to increased permeability. In our CLP model of sepsis, we demonstrated that 4F increased the expression of Slit2 and Robo4, perhaps by decreasing the levels of cytokines. Further studies are needed to determine whether 4F and apolipoproteins have direct effects on the protein expression of Slit2 and Robo4.

Patients with sepsis typically develop subcutaneous and body cavity edema, which suggests greater vascular permeability. Parenchymal and interstitial fluid accumulation could impair organ function by increasing the diffusion distance of oxygen and by increasing interstitial pressure, thus impairing microvascular perfusion (30). It is known that fluid overload has a negative effect on outcomes in critically ill patients (5). Decreases in left ventricular diameter in diastole and in left ventricular end-diastolic pressure, resulting in a defect in cardiac performance, might be due to impaired left ventricular filling. The results of another study in rats suggested that a decrease in cardiac output after CLP is associated with a reduction in plasma volume (46). Impaired cardiac performance, as was observed in our CLP rats, contributes to adverse outcomes in patients with sepsis (13). We found that treatment with 4F restored cardiac performance (cardiac output and left ventricular ejection fraction) to normal levels, as has been demonstrated by Zhang et al. (52).

Cardiac dysfunction is an important component of multiorgan failure induced by severe sepsis (39). In the heart, mitochondrial dysfunction, such as impaired metabolism and altered energy generation, has been implicated in promoting sepsis-associated myocardial injury (47). In our study, the treated animals showed significant improvement in mitochondrial morphology, with partial recovery of the structure of the heart mitochondria.

It is of note that there was no difference between pre- and post-CLP MAP in any of our groups when the animals were conscious. Some authors have suggested that there is a reduction in MAP in rats at 24 h after CLP (4, 20). We found that to be true only if MAP was determined while the animals were anesthetized. In the present study, treatment with 4F protected against the post-CLP drop in MAP, even when the animals were under anesthesia. We also observed an increase in HR at 24 h after CLP. We hypothesized that HR increased as a compensatory mechanism, to maintain MAP within a normal range. We found that post-CLP treatment with 4F normalized HR.

The baroreflex system is devoted to maintaining cardiovascular homeostasis and preserving blood flow to vital organs (9). In sepsis, there appears to be a direct relationship between baroreflex sensitivity and survival time, reduced survival time having been reported in the setting of reduced baroreflex function (42, 43). It is known that eNOS activity is impaired in endothelial cells exposed to native LDL cholesterol (19). In addition, oxidized LDL particles can independently alter eNOS abundance (32). Decreased HR variability and increased blood pressure variability, determined in part by nitric oxide-dependent endothelial dysfunction, are correlated with an unfavorable prognosis in cardiovascular disease. Pelat et al. (40) demonstrated, in vivo, that rosuvastatin decreases expression of caveolin-1 (an inhibitor of eNOS) and promotes NOS function in apoE-/− dyslipidemic mice, with concurrent improvements in the variability of HR and blood pressure. In the present study, we demonstrated impaired baroreflex function in rats submitted to CLP. Further studies are warranted to determine whether the 4F-induced improvement in baroreflex sensitivity was due to the increase in eNOS expression or to the decrease in serum cytokine levels. Such improvement might also be attributable to the fact that treatment with 4F was found to increase serum HDL and decrease serum LDL.

The rationale for testing the effects of 4F in CLP-treated rats is based on previous studies demonstrating that plasma HDL is significantly lower in sepsis, as well as that increasing plasma apoA-I and HDL improves outcomes in septic rodents and humans (16, 17, 52). In an elegant study, Dai et al. (16)
examined whether the protective effects of 4F in LPS-treated rats were related to the binding or neutralization of endotoxin by HDL. The authors monitored LPS interactions with plasma lipoprotein fractions. They administered [14C]4F and boron dipyrromethene (Bodipy)-labeled LPS to control rats by separate intravenous injections. They found that the [14C]4F rapidly localized to a cholesterol fraction containing HDL. Those authors also performed time-course studies, involving radioactive counts in plasma samples, to investigate the clearance of 4F and LPS from the circulation of the rats. They showed that the clearance of [14C]4F from plasma in rats treated with LPS was similar to that observed for rats receiving saline vehicle alone. However, they found that Bodipy-LPS fluorescence was significantly reduced in plasma samples of vehicle-treated rats within 1 h after injection. In rats pretreated with 4F, the authors also observed an initial, rapid reduction in Bodipy-LPS, although LPS-dependent fluorescence in plasma remained significantly elevated at 3 h and 4 h after injection.

In intensive care unit patients with sepsis and concomitant acute kidney injury, mortality can be as high as 70% (6). We recently published two studies demonstrating that, in the CLP model of sepsis, the use of erythropoietin or continuous erythropoietin receptor activator improve renal function by increasing protein expression of eNOS and decreasing cytokine levels (41, 44). In the present study, the response to 4F treatment, in terms of renal function (insulin clearance), was homogeneous at 24 h after surgery, translating to complete prevention of the sepsis-induced decrease in glomerular filtration.

In conclusion, treatment with 4F exhibited anti-inflammatory effects; improved cardiac, hepatic, and renal function; and provided a significant survival benefit in rats subjected to CLP. We have also demonstrated that, in this CLP model of sepsis, baroreflex function is impaired. In the setting of sepsis, treatment with 4F, in addition to increasing serum HDL, might have a protective effect on endothelial function, attributable to 4F-induced increases in the protein expression of eNOS, Slt2, and Robo4. Administration of 4F inhibits inflammatory responses and strengthens the vascular barrier, protecting kidneys, liver, and heart in an HDL-dependent manner.

Perspectives and Significance

On the basis of our results and those of other studies, we can speculate that further clinical trials with 4F might prove fruitful in reducing the unacceptably high mortality rates seen among patients with sepsis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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