Erythropoietin prevents sepsis-related acute kidney injury in rats by inhibiting NF-κB and upregulating endothelial nitric oxide synthase

Ana Carolina C. Pessoa de Souza,1* Rildo A. Volpini,1* Maria Heloísa Shimizu,1 Talita Rojas Sanches,1 Niels Olsen Saraiva Camara,2,3 Patrícia Semedo,2,3 Camila Eleutério Rodrigues,1 Antonio Carlos Seguro,1 and Lúcia Andrade1

Nephrology Department, University of São Paulo School of Medicine, 2Nephrology Department, Federal University of São Paulo, and 3Immunology Department, University of São Paulo, São Paulo, Brazil

Submitted 15 March 2011; accepted in final form 8 January 2012

Erythropoietin prevents sepsis-related acute kidney injury in rats by inhibiting nuclear factor-κB and upregulating endothelial nitric oxide synthase. Am J Physiol Renal Physiol 302: F1045–F1054, 2012. First published January 11, 2012; doi:10.1152/ajprenal.00148.2011.—The pathophysiology of sepsis involves complex cytokine and inflammatory mediator networks, a mechanism to which NF-κB activation is central. Downregulation of endothelial nitric oxide synthase (eNOS) contributes to sepsis-induced endothelial dysfunction. Erythropoietin (EPO) has emerged as a major tissue-protective cytokine in the setting of stress. We investigated the role of EPO in sepsis-related acute kidney injury using a cecal ligation and puncture (CLP) model. Wistar rats were divided into three primary groups: control (sham-operated); CLP; and CLP+EPO. EPO (4,000 IU/kg body wt ip) was administered 24 and 1 h before CLP. Another group of rats received N-nitro-l-arginine methyl ester (l-NAME) simultaneously with EPO administration (CLP+EPO+l-NAME). A fifth group (CLP+EPOtreat) received EPO at 1 and 4 h after CLP. At 48 h postprocedure, CLP+EPO rats presented significantly higher inulin clearance than did CLP and CLP+EPO+l-NAME rats; hematocrit levels, mean arterial pressure, and metabolic balance remains unchanged in the CLP+EPO rats; and inulin clearance was significantly higher in CLP+EPOtreat rats than in CLP rats. At 48 h after CLP, creatinine clearance was significantly higher in the CLP+EPO rats than in the CLP rats. In renal tissue, pre-CLP EPO administration prevented the sepsis-induced increase in macrophage infiltration, as well as preserving eNOS expression, EPO receptor (EpoR) expression, IKK-α activation, NF-κB activation, and inflammatory cytokine levels, thereby increasing survival. We conclude that this protection, which appears to be dependent on EpoR activation and on eNOS expression, is attributable, in part, to inhibition of the inflammatory response via NF-κB downregulation.

The mortality rates of sepsis and septic shock remain unacceptably high and have not changed in several decades (5, 6, 26, 30). Acute kidney injury (AKI) occurs in ~50% of patients with sepsis (5, 30). In such patients, the development of AKI is predictive of a poor outcome, and the consequent mortality has been reported to be as high as 70% (5). Despite improved strategies for supporting vital organs and resuscitating patients, the incidence and mortality rates of septic AKI remain quite high (5, 6). The prevention of kidney injury in intensive care settings continues to represent a great challenge.

It has been shown that NF-κB mediates the transcription of a large number of genes, the products of which are known to play important roles in septic pathophysiology (24, 31). Mice deficient in those NF-κB-dependent genes are resistant to the development of septic shock and to sepsis-related mortality (31). More importantly, blockade of the NF-κB pathway corrects septic abnormalities. In sepsis, inhibition of NF-κB activation restores systemic hypotension, ameliorates myocardial dysfunction, corrects vascular derangement, inhibits multiple proinflammatory gene expression, diminishes intravascular coagulation, reduces tissue neutrophil influx, and prevents microvascular endothelial leakage (31). Therefore, NF-κB activation plays a central role in the pathophysiology of septic shock (31, 36).

Studies have indicated that downregulation of vascular endothelial nitric oxide synthase (eNOS) contributes to vascular hyporesponsiveness in sepsis (51). In animals submitted to cecal ligation and puncture (CLP), it has been shown that there is a significantly reduction in the extent of the eNOS-immunoreactive positive area in endothelial cells (51). In the CLP model, the decreased eNOS in vascular endothelial cells is at least partially responsible for endothelial cell dysfunction (52). In addition, Ckless et al. (9) suggest that arginase regulates NF-κB via nitric oxide (NO)-dependent redox changes. These findings have implications for the pathophysiology of chronic inflammatory conditions (9).

Erythropoietin (EPO) is a growth hormone, and its principal physiological function is the induction of erythropoiesis. For the last two decades, EPO has been used in the management of anemia associated with end-stage renal disease (18). Recent studies have shown that, in addition to its hematopoietic activity, EPO has a cytoprotective effect on many different types of cells and tissues (4, 41). There are EPO receptors (EpoRs) widely distributed throughout a number of tissue types (48). The discovery that EpoRs are expressed in glomerular, mesangial, and tubular epithelial cells in human, rat, and mouse kidneys have prompted studies of the effects of EPO in small-rodent models of AKI (41, 44). Such studies have shown that EPO not only stimulates erythropoiesis but also has anti-inflammatory properties, as well as exerting the following effects (7, 19, 21, 38): reducing apoptosis, oxidative stress, and lipid peroxidation; promoting renal tubular cell regeneration, vascular regeneration, and neoangiogenesis; mobilizing endothelial progenitor cells; and upregulating eNOS expression. It is also known that EPO mediates neuroprotection against axonopathy and apoptosis in the peripheral nervous system by

http://www.ajprenal.org
1931-857X/12 Copyright © 2012 the American Physiological Society

* A. C. C. Pessoa de Souza and R. A. Volpini contributed equally to this work.

Address for reprint requests and other correspondence: L. Andrade, Nephrology Dept., Univ. of São Paulo School of Medicine, Av. Dr. Arnaldo, 455, 3° andar, sala 3310, CEP 01246-903, São Paulo, Brazil (e-mail: luciacan@usp.br).
inhibiting NF-κB activation, indicating rescue from an injury phenotype (44).

In a model of LPS-induced endotoxemia, EPO was found to significantly attenuate renal dysfunction. This EPO-related renal protection was associated with reversal of the effects that the endotoxin has on renal superoxide dismutase (35).

Various preclinical animal models have been developed to evaluate the effect of sepsis on the development of AKI. The CLP model is widely used because the resulting polymicrobial sepsis mimics many features of human sepsis (8, 13, 46, 49).

In the present study, we attempted to determine whether EPO prevents the renal dysfunction seen in the CLP model of sepsis. We also tested whether EPO could be used as a therapeutic agent after the induction of the CLP sepsis. We tested the hypothesis that the mechanism of renal dysfunction in the CLP model of sepsis is mediated, at least in part, by NF-κB upregulation and eNOS downregulation. We further hypothesized that EPO protects renal function by regulating the protein expression of NF-κB, eNOS, and EpRB.

**METHODS**

**Animals and experimental protocols.** Male Wistar rats, 8 wk old and weighing 180–230 g, were obtained from the animal facility of the University of São Paulo School of Medicine. 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 untreated, sham-operated rats; CLP, consisting of untreated rats submitted to CLP; and CLP+EPO, consisting of rats intraperitoneally injected with 4,000 IU/kg body wt (BW) of EPO (Hemax; Biosintética, Uberabinha, Brazil) at 24 and 1 h before CLP.

To analyze the early effects of EPO administration on renal function, we conducted one set of experiments at 24 h after CLP or sham surgery (control, n = 5; CLP, n = 7; and CLP+EPO, n = 7). In this first set of experiments, we measuredulin clearance, mean arterial pressure (MAP), hematocrit, and microvascular perfusion.

In a second set of experiments, the late effects of EPO were analyzed at 48 h after CLP or sham surgery (control, n = 10; CLP, n = 10; and CLP+EPO, n = 10). In this second set of experiments, we quantified renal function by measuring creatinine clearance. This set of experiments also included 12-h metabolic cage studies, as well as immunohistochemical analysis to study the expression of NF-κB.

We also performed immunoblotting studies to determine the expression of eNOS and of the IkB kinase α-subunit (IKK-α). We conducted a third set of experiments to plot the survival curves for the animals submitted to CLP (CLP, n = 9; and CLP+EPO, n = 9).

In a fourth set of experiments, we investigated the therapeutic effect that post-CLP EPO administration has on AKI. In this fourth set of experiments, rats received EPO (4,000 IU/kg BW ip) at 1 and 4 h after CLP (CLP+EPOtreat, n = 4), and we measuredulin clearance at 24 h after the CLP surgery.

An additional group of rats (n = 5), not submitted to CLP, received a single dose of EPO (4,000 IU/kg BW ip) and were evaluated 5 wk later to identify adverse effects such as polyuria and thrombosis, as well as other complications. We compared those animals with a control group (n = 5) in terms of hematocrit levels. In another group of rats not submitted to CLP but receiving EPO (4,000 IU/kg BW ip; n = 7), we measured MAP at 24 h after EPO administration.

To determine whether the beneficial effects of EPO treatment are mediated by NO production, rats were submitted to inhibition of NOS activity, receiving N-nitro-L-arginine methyl ester (L-NAME) simultaneously with EPO administration. At 24 h before CLP, and again at 1 h before CLP, we administered L-NAME (10 mg/kg BW iv; Sigma, St. Louis, MO) and EPO (4,000 IU/kg BW ip). In this group of rats, designated the CLP+EPO+L-NAME group (n = 10), we measuredulin clearance and MAP at 24 h after CLP. The experimental protocols were approved by the Research Ethics Committee of the University of São Paulo School of Medicine Hospital das Clínicas.

**CLP procedures.** Rats were anesthetized with 2.5% 2,2,2-tribromoethanol (99%), 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 cecum was punctured twice with an 14-G needle and gently squeezed to confirm leakage of cecal contents. The abdominal incision was closed in two layers with 3-0 silk sutures. Control group 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 ip) immediately after the procedure. Additional fluid therapy (0.15 M NaCl, 25 ml/kg ip) was started at 6 h after CLP and then repeated every 12 h, as was antibiotic therapy with imipenem/cilastatin (14 mg/kg ip; Merck Sharp & Dohme, West Point, PA).

**Inulin clearance studies.** At 24 h after CLP or sham surgery, each designated animal was anesthetized with thiopental sodium (50 mg/kg BW ip). The trachea was then cannulated with a polyethylene (PE)-240 catheter, and spontaneous breathing was maintained. To control 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. A loading dose of inulin (100 ml/kg BW, diluted in 0.9% saline) was then administered through the jugular vein. Constant infusion of inulin (10 mg/kg BW) was then started and continued 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 and the end of the experiment.

**Light microscopy and immunohistochemistry.** The kidneys were flushed with PBS (0.15 M NaCl and 0.01 M phosphate buffer, pH 7.4) injected into the aortic artery. A fragment of the right kidney was immersed in methacarn (60% methanol, 30% chloroform, and 10% acetic acid), where it remained for 24 h, after which the methacarn was replaced with 70% ethanol. Another fragment of the right kidney was immersed in frozen liquid nitrogen and stored at −80°C for Western blotting. After removal of the right kidney, the left kidney was flushed with a 4% paraformaldehyde solution injected into the aortic artery. The left kidney was then removed. A fragment of the left kidney was immersed in 4% paraformaldehyde, where it remained for 2 h, after which it was postfixed in Bouin’s solution for 4 h. Bouin’s solution was then removed, and the tissue fragment was dehydrated in 70% ethanol. The fragments (from the right and left kidney) were then embedded in paraffin and cut into 4-μm sections. The sections were deparaffinized, after which they were subjected to Masson’s trichrome staining for determination of the fractional interstitial area or were used in the immunohistochemical studies.

The fractional interstitial area of the renal cortex was determined by morphometry with a video camera connected to an image analyzer (QWin; Leica, Wetzlar, Germany). In each renal cortex, 20 grid fields (each measuring 37,000 μm²) were evaluated. Interstitial areas were first manually encircled on a video screen and then quantified by computerized morphometry (47).

For ED1 and NF-κB immunostaining, samples were processed in 4-μm paraffin 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-ED1 antibody (1:1,000) or anti-NF-κB antibody (1:80). This was followed by incubation with biotinylated mouse anti-rat IgG for 30 min at room temperature. The reaction product was detected with an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). The color reaction was developed with 3,3′-diaminobenzidine (Sigma), and the sections were counterstained with methyl green.

For all sections, negative controls consisted of replacing the primary antibody with equivalent concentrations of an irrelevant normal
rabbit or normal goat IgG. The sections were examined under light microscopy at a magnification of ×400.

For evaluation of immunoperoxidase staining for NF-κB, each tubulointerstitial grid field was graded semiquantitatively, and the mean score per kidney was calculated. Each score reflected mainly changes in the extent, rather than the intensity, of staining, based on the proportion of the grid field showing positive staining: 0, absent or <5%; I, 5–25%; II, 25–50%; III, 50–75%, and IV, >75%.

To obtain the mean numbers of infiltrating macrophage/monocyte-positive cells in the renal cortical tubulointerstitium, 30 grid fields measuring 0.245 mm² each were evaluated and the mean counts per kidney were calculated.

Light microscopy studies. The kidneys were removed, sectioned transversely, fixed in 4% paraformaldehyde, postfixed in Bouin’s solution for 4–6 h, and processed for paraffin embedding. Histological sections (4-μm thick) were stained with Masson’s trichrome and examined under light microscopy. Tubulointerstitial damage was defined as tubular necrosis, inflammatory cell infiltrate, tubular lumen dilation, or tubular atrophy. Damage was graded on a scale of 0 to 4 based on the proportion of the cortex and outer medulla involved (0 = no involvement; 0.5 = small focal areas; 1 = <10%; 2 = 10–25%; 3 = 25–75%; 4 = >75%), as previously described (42).

Analysis of blood and urine samples. In blood samples obtained 48 h after CLP, plasma levels of creatinine were measured using an automated colorimetric assay. The volume of each 12-h urine sample was measured gravimetrically. Urine samples were centrifuged in aliquots to remove suspended material, and the supernatants were analyzed. Urinary levels of creatinine were also measured using the same techniques. In blood samples obtained at the end of the creatinine clearance study, hematocrit was determined using a microhematocrit capillary tube (Perfecta, São Paulo, Brazil). Serum lactate and bicarbonate were measured with a blood-gas analyzer (Radiometer Medical, Copenhagen, Denmark).

Antibodies. Peptide-derived polyclonal anti-eNOS antibody was obtained from Transduction Laboratories (Lexington, KY). Peptide-derived polyclonal anti-NF-κB and anti-IKK-α antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Peptide-derived polyclonal anti-ED1 antibody was obtained from Serotec (Oxford, UK). Peptide-derived polyclonal anti-EpoR antibody was also obtained from Santa Cruz Biotechnology.

Preparation of membrane fractions. Samples of kidneys were homogenized with a Teflon pestle glass homogenizer (Schmidt and Co., Frankfurt am Main, Germany) in ice-cold isolation solution (200 mM mannitol, 80 mM HEPES, and 41 mM KOH, pH 7.5) containing protease inhibitors (protease inhibitor cocktail; Sigma). The homogenates were centrifuged at low speed (2,000 g) for 15 min at 4°C to remove nuclei and cell debris.

Preparation of cytoplasmic protein extracts. Kidney samples were cut into small pieces, washed with PBS, and centrifuged. The pellet was homogenized using a tissue grinder, and the cytoplasmic protein was extracted with a commercial kit (Ne-Pe Nuclear and Cytoplasmic Protein Extraction Kit; Pierce Biotechnology, Rockford, IL), in accordance with the manufacturer’s instructions.

Electrophoresis and immunoblotting. Samples of membrane fractions or kidney samples were run on 10% polyacrylamide minigels (for eNOS, EpoR, and IKK-α). After transfer by electrophoresis to nitrocellulose membranes (PolyScreen, PVDF Transfer; Life Science Products, Boston, MA), blots were blocked with 5% milk and 0.1% Tween 20 in TBS for 1 h. Blots were then incubated overnight with the antibodies anti-eNOS (1:2,000), anti-EpoR (1:500), and anti-IKK-α (1:1,000). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Piscataway, N J).

Quantification of renal expression of eNOS, EpoR, and IKK-α. The ECL films were scanned using Image Master VDS software (Pharmacia Biotech, Uppsala, Sweden). Quantitative analysis of antibodies was performed using densitometry. The bands were normalized to actin protein expression.

Survival study. Following another set of the experiments described above (CLP and EPO administration), survival curves were plotted, initially at 6 h after CLP and then every 6–12 h, for a total of 60 h.

Measurement of renal tissue levels of inflammatory cytokines. Renal tissue levels of cytokine were measured and analyzed with the Bio-Plex system. A Bio-Plex rat 9-Plex cytokine assay kit (Bio-Rad Laboratories, Hercules, CA) was used to test samples for the presence of IL-1α, IL-1b, IL-2, IL-4, IL-6, IL-10, colony-stimulating factor, IFN-γ, and TNF-α. The assay was read on the Bio-Plex suspension array system, and the data were analyzed using Bio-Plex Manager software, version 4.0. Standard curves ranged from 32,000 to 1.95 pg/ml. Data are expressed as cytokine level (pg/ml) per protein level of renal tissue extract (μg/ml).

Statistical analysis. All quantitative data are expressed as means ± SE. Differences among the means of multiple parameters were analyzed by ANOVA followed by the Student-Newman-Keuls test. Differences between two parameters were analyzed either by unpaired t-test or by nonparametric methods (Mann-Whitney test). Survival analyses were compared by a log-rank test. Values of P < 0.05 were considered statistically significant.

RESULTS

Early post-CLP EPO effects on inulin clearance. As can be seen in Table 1, inulin clearance at 24 h after CLP or sham surgery was lower in the CLP rats than in the control rats (CLP: 0.43 ± 0.08 vs. 0.83 ± 0.04 ml·min⁻¹·100 g BW⁻¹, P < 0.01), as would be expected. At the same time point, inulin clearance was also significantly lower in the CLP rats than in the CLP+EPO rats (0.43 ± 0.08 vs. 0.99 ± 0.12 ml·min⁻¹·100 g BW⁻¹, P < 0.001), although there was no significant difference between the CLP+EPO rats and the control rats (Fig. 1A).

Early post-CLP EPO effects on hematocrit values. At 24 h postprocedure, hematocrit values were markedly lower in CLP rats than in control rats, whereas there was no such decrease in hematocrit levels in the CLP+EPO rats (Table 1).

Early post-CLP EPO effects on hemodynamic and metabolic acid-base status. At 24 h after CLP or sham surgery, MAP (mmHg) was completely preserved (at normal levels) in CLP+EPO rats (Table 1). We also found no significant difference between the rats not submitted to CLP or sham surgery but receiving EPO and the control (sham-operated only) rats in terms of the MAP at 24 h after EPO administration (107 ± 6.6 mmHg vs. 110 ± 5.2 mmHg, respectively).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Inulin clearance, ml·min⁻¹·100 g BW⁻¹</td>
<td>0.82 ± 0.04</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>112 ± 2.5</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>43.0 ± 1.0</td>
</tr>
<tr>
<td>Bicarbonate, meq/l</td>
<td>26 ± 1.3</td>
</tr>
<tr>
<td>Base excess</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>1.4 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE. CLP, cecal ligation and puncture; EPO, erythropoietin; BW, body weight.ᵇP < 0.01 vs. control.ᶜP < 0.001 vs. CLP+EPO.ᵈP < 0.01 vs. control.ᵉP < 0.01 vs. CLP+EPO.ᶠP < 0.05 vs. control.ᵍP < 0.05 vs. CLP+EPO.
vs. 112 ± 2.5 mmHg). In addition, EPO administration prevented the systemic hemodynamic abnormalities seen in the CLP rats (Table 1). Furthermore, EPO administration completely sustained serum levels of lactate and bicarbonate, as well as the base excess. It is known that sepsis increases serum levels of lactate and can cause metabolic acidosis. Serum levels of lactate were significantly lower in CLP/EPO rats than in CLP rats (Table 1).

_Late post-CLP EPO effects on creatinine clearance and MAP._ At 48 h after CLP or sham surgery, creatinine clearance was significantly higher in CLP+EPO rats than in CLP rats (0.98 ± 0.08 vs. 0.56 ± 0.1 ml-min⁻¹·100 g BW⁻¹, *P* < 0.001). As shown in Fig. 1B, there was no significant difference between the CLP+EPO rats and the control rats (0.98 ± 0.08 vs. 1.14 ± 0.1 ml-min⁻¹·100 g BW⁻¹). EPO administration completely prevented the drop in MAP seen in the untreated animals at 48 h after CLP (CLP: 76 ± 7.5 mmHg; CLP+EPO: 97.5 ± 3.1 mmHg, *P* < 0.05). As expected, MAP was significantly lower in the CLP group than in the control group (76 ± 7.5 vs. 109 ± 3.0 mmHg, *P* < 0.01), although there was no statistical difference between the CLP+EPO and control groups.

_Late post-CLP EPO effects on fractional interstitial area._ As can be seen in Fig. 2, A–C, there were marked histological differences between the CLP group and the CLP+EPO group at 48 h after CLP. In the CLP group, the fractional interstitial area was significantly greater than that observed in the control group (Fig. 2D). However, at 48 h post-CLP, the fractional interstitial area was markedly lower in the CLP+EPO group than in the CLP group. Nevertheless, there was no significant difference between the CLP+EPO and control groups in terms of the fractional interstitial area.

_Late post-CLP EPO effects on renal macrophage infiltration._ As can be seen in Fig. 3, A–C, the number of cells presenting ED1 staining for macrophages/monocytes in the tubulointerstitium at 48 h after the surgical procedures was significantly higher in

---

_Fig. 1. Renal function measured by inulin clearance at 24 h after CLP surgery (A) and creatinine clearance at 48 h after cecal ligation and puncture (CLP; B) or sham surgery in control rats, rats subjected to CLP only, and rats subjected to CLP/receiving erythropoietin (CLP+EPO rats)._
the CLP group rats than in the control group rats (23.2 ± 3.7 vs. 7.3 ± 0.74 cells/0.245 mm², P < 0.001). However, the numbers of infiltrating macrophages/monocytes were significantly lower in the CLP+EPO rats than in the CLP rats (7.5 ± 0.8 vs. 23.2 ± 3.7 cells/0.245 mm², P < 0.001).

Late post-CLP EPO effects on NF-κB activation. At 48 h postprocedure, staining for NF-κB in the tubulointerstitium of the renal cortex was more pronounced in CLP rats than in control rats (1.32 ± 0.08 vs. 0.67 ± 0.06, P < 0.001). As shown in Fig. 4, EPO administration attenuated the NF-κB staining seen in CLP rats (CLP: 1.32 ± 0.08; CLP+EPO: 0.81 ± 0.09, P < 0.001).

Late post-CLP effects on IKK-α protein expression. As can be seen in Fig. 5, cytoplasmic IKK-α protein expression at 48 h postprocedure was markedly higher in the CLP group rats than in the control group rats (207.5 ± 7.5 vs. 99.3 ± 0.7%, P < 0.001). Although such expression was higher in the CLP group than in the CLP+EPO group (207.5 ± 7.5 vs. 142.5 ± 4.8%, P < 0.001), it was also higher in the CLP+EPO group than in the control group (142.5 ± 4.8 vs. 99.3 ± 0.7%, P < 0.001).

Late post-CLP EPO effects on eNOS protein expression. At 48 h after the surgical procedures, the CLP group rats demonstrated markedly lower eNOS protein expression (% of normal) compared with the control rats (28.3 ± 6 vs. 93 ± 3.3%, P < 0.001). It is of note that EPO administration completely prevented the downregulation of eNOS expression (CLP+EPO: 90 ± 6.0%; CLP: 28.3 ± 6%, P < 0.001), as can be seen in Fig. 6.

Renal effects of coadministration of EPO and l-NAME. Of the 10 rats in the CLP+EPO+l-NAME group, only 4 survived until 24 h after the surgical procedure. At 24 h after CLP, inulin clearance was significantly lower in CLP+EPO+l-NAME rats than in CLP+EPO rats (0.64 ± 0.05 vs. 0.99 ± 0.12 ml-min⁻¹·100 g BW⁻¹, P < 0.05), although there was no significant difference between the CLP+EPO+l-NAME rats and the CLP rats (0.64 ± 0.05 vs. 0.43 ± 0.08 ml-min⁻¹·100 g BW⁻¹). As expected, MAP was significantly higher in the CLP+EPO+l-NAME group than in the CLP and CLP+EPO groups (155.5 ± 4.1 vs. 74 ± 7.5 and 99 ± 2.3 mmHg, P < 0.001 for both). The difference between the CLP+EPO and CLP groups, in terms of MAP, was also statistically significant (P < 0.01).

Late post-CLP EPO effects on EpoR protein expression. Expression of EpoR was markedly lower in CLP rats than in control rats (27.5 ± 4.5 vs. 100 ± 0.6%, P < 0.001). As can be seen in Fig. 7, EPO administration completely averted the downregulation of EpoR observed in the CLP rats (94.2 ± 3.2 vs. 27.5 ± 4.5%, P < 0.001).

Late post-CLP EPO effects on histological lesions. By 48 h after CLP, the CLP and CLP+EPO rats had developed interstitial lesions. The degree of interstitial damage in the CLP group was statistically different from that observed for the control group (2.0 ± 0.29 vs. 0.19 ± 0.09, P < 0.001). In the CLP+EPO group, the interstitial damage did not progress and the histological score was significantly lower than that calculated for the CLP group (1.0 ± 0.16 vs. 2.0 ± 0.29, P < 0.01).
However, the extent of the histological lesions seen in the CLP/EPO group was higher than that seen in the control group ($P < 0.01$).

**EPO administration decreases cytokine levels in kidney tissue.** Using a multiplex assay in renal tissue extracts, we demonstrated that CLP rats presented elevated renal protein levels of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α compared with extracts of renal tissue obtained from control animals at 48 h after CLP. We also attempted to determine whether pre-CLP administration of EPO can affect the renal levels of these cytokines in this model. As shown in Table 2, EPO administration restored cytokines in kidney tissue to levels comparable to those observed in control animals.

**EPO effects on survival in polymicrobial sepsis.** Mortality was significantly lower in the CLP/EPO group than in the CLP group (Fig. 8). Among the rats receiving a single dose of EPO and not subjected to CLP, hematocrit levels at 5 wk after EPO administration were comparable to those seen in the control group (42.2 ± 1.5 vs. 39.6 ± 2.2%). None of those rats developed thrombi or showed any clinical manifestations consistent with stasis, thrombosis, or other complications, all remaining clinically well throughout the evaluation period.

**EPO treatment 4 h post-CLP procedure rescues rat kidney from AKI.** In clinical settings, practitioners rarely have the chance to anticipate sepsis-induced AKI and are typically confronted with the syndrome after it has become established. Therefore, to be worthwhile, any AKI treatment given after the development of sepsis must be efficacious. To test whether EPO treatment after CLP might still exert therapeutic effects on AKI, we administered EPO 1 and 4 h after CLP. Administration of EPO was effective in restoring renal function.
rats than in the control rats (0.43 ± 0.08 vs. 0.83 ± 0.04 ml·min⁻¹·100 g BW⁻¹; P < 0.01). At the same time point, inulin clearance was also significantly lower in the CLP rats than in the CLP+EPO rats (0.43 ± 0.08 vs. 0.82 ± 0.08 ml·min⁻¹·100 g BW⁻¹; P < 0.01). There was no significant difference between the CLP+EPO rats and the control rats.

**DISCUSSION**

In rats, the experimental model of CLP, which mimics the clinical features of bowel perforation and mixed bacterial infection of intestinal origin in humans, is a realistic model of polymicrobial sepsis (12). Using the CLP model, we evaluated the effects of EPO on sepsis-induced AKI. Based on a previous report by Holly et al. (23), animals were treated with fluid resuscitation and broad-spectrum antibiotics, which makes this model even more clinically relevant. To mimic the delay typically seen in the presentation of human sepsis, fluid resuscitation and antibiotic treatment were not started until 6 h after surgery, although some fluid was administered immediately after the induction of sepsis to replace initial surgical losses.

We found that EPO administration improved survival and renal function. In the CLP+EPO rats, the systemic hemodynamic and metabolic acid-base status remained unchanged at 24 h after CLP. At 48 h after the surgery, CLP+EPO rats presented stable kidney function, as well as lower renal NF-κB activation, a decrease in fractional interstitial area, less renal macrophage infiltration, and greater renal eNOS protein expression, compared with control rats.

The response to EPO administration, in terms of renal function (as quantified by measuring creatinine clearance), was homogeneous at 48 h after surgery, translating to a complete prevention of the decreased glomerular filtration seen in response to sepsis. In the CLP+EPOtreat rats, renal function was completely restored by 24 h after surgery. In the picric acid assay (the most common method of determining creatinine...
clearance), chromagens in serum have been shown to result in a overestimation of serum creatinine (32, 50). In addition, reduced production of creatinine limits its use as a marker of kidney injury in sepsis (14). Therefore, at 24 h after surgery, we performed inulin clearance studies to determine whether EPO protects against sepsis-related AKI. As expected, inulin clearance was reduced in the CLP group. Two previous studies in mice have shown that EPO has a renoprotective effect on renal function in sepsis. The first was performed using the LPS model of sepsis (35). The authors found that EPO, given 30 min before LPS administration, significantly attenuated sepsis-related renal dysfunction, as assessed by inulin clearance. In that study, the renal dysfunction observed during endotoxemia was found to be associated with a decrease in renal superoxide dismutase, and the EPO-related renal protection was associated with the reversal of that effect. The second of those two studies employed LPS and CLP models (3). The authors administered EPO at different time points after the induction of sepsis. In the LPS model, serum levels of creatinine were reduced in the EPO-treated endotoxemic mice.

The cytokine EPO has emerged as having cytoprotective effects, including the ability to protect many tissues, such as the brain, heart, and kidneys, against ischemia and traumatic injury. It has been reported that treatment with EPO protects the kidney against ischemia-reperfusion injury (27, 37, 40, 45). It has also been shown that EPO is stimulated during hypoxia and that it upregulates erythroid progenitor cell proliferation and differentiation by inhibiting apoptosis (29). The major physiological function of EPO is the induction of erythropoiesis (18).

Anemia is quite common in critically ill patients, especially in those with AKI. In such patients, erythropoiesis is impaired as a consequence of blunted EPO production and the direct inhibitory effects of inflammatory cytokines (15). In a study involving patients admitted to a long-term acute care facility, weekly administration of EPO resulted in significantly less exposure to allogeneic red blood cell transfusion and significantly higher hemoglobin levels than did a placebo (43).

We demonstrated protection of hemodynamic parameters in animals receiving EPO and subjected to CLP. Two recent studies found that EPO administration attenuates hemorrhagic and zymosan-induced shock in rats (1, 10).

It is now known that EpoRs are expressed on vascular endothelial cells (2, 48), which suggests that the vasculature is a biological target of EPO. It has been demonstrated that EPO prevents LPS-induced vascular hyporeactivity and endothelial dysfunction (11), and that the vascular-protective effects of EPO are critically dependent on the activation of eNOS (16). In the present study, we observed a profound decrease in eNOS protein expression in septic animals and that decrease was prevented in rats receiving EPO before CLP, the latter presenting levels of eNOS protein expression comparable to those seen in the sham-operated animals. In addition, as was the case for pre-CLP administration of EPO alone, the pre-CLP coadministration of EPO and l-NAME (an inhibitor of NO activity) had no apparent protective effect on renal function, as measured by inulin clearance, indicating that the beneficial effects of EPO treatment are mediated, in part, by NO production. Another interesting finding was that, although MAP was higher in the CLP+EPO+l-NAME group than in the CLP and CLP+EPO groups, the addition of l-NAME also had no apparent protective effect on renal function. We can therefore conclude that the EPO-related protection of renal function was not mediated by increased blood pressure.

We found that arterial lactate levels were markedly elevated in the CLP group, an alteration that was effectively prevented by EPO administration. In addition, EPO administration preserved bicarbonate levels, which typically decrease during sepsis. Although it is often accompanied by hemodynamic instability, hyperlactatemia can also occur under stable hemodynamic conditions, in which case it is considered to be due to occult hypoperfusion (25). In a study employing a murine model of CLP-induced sepsis, EPO was given in a smaller dose than that used in the present study (28). The authors found that EPO had no effect on blood pressure or lactate levels but produced an increase in capillary perfusion in skeletal muscle. In a cohort study of 830 adults admitted to the emergency room with severe sepsis, the initial serum lactate level was associated with mortality, independent of organ dysfunction and shock (34).

We found that EPO exerts certain anti-inflammatory effects, such as preventing the increases in fractional interstitial area and in the number of infiltrating macrophages/monocytes. We also demonstrated that treatment with EPO maintained cytokines in renal tissue extracts at sham levels. In this model of sepsis, EPO also inhibited NF-κB expression, which is activated by a variety of pathogens known to cause septic shock syndrome (31). Cytoplasmic IKK-α protein expression was upregulated in the CLP group animals, an effect that was minimized in the in the CLP+EPO group animals. It is known that, in the cytoplasm, the inhibitory protein IkB maintains the transcription factor NF-κB in an inactive form. Activation of NF-κB and its migration to the nucleus requires that IkB be phosphorylated on specific serine residues (Ser), which results in targeted degradation of IkB. The interaction between IKK-α and IkB-α results in specific phosphorylation of IkB-α at Ser 32 and Ser 36, the sites that trigger its degradation. In addition, IKK-α appears to be critical for NF-κB activation in response to increased production of proinflammatory cytokines (20). In patients with sepsis, higher levels of NF-κB activity are associated with increased mortality rates and worse clinical outcomes, since NF-κB mediates the transcription of an exceptionally large number of genes, the products of which are known to play important roles in the pathophysiology of sepsis (31). Inhibition of NF-κB activation restores systemic hypotension, ameliorates septic myocardial dysfunction and vascular derangement, inhibits multiple proinflammatory gene expression, diminishes intravascular coagulation, reduces tissue neutrophil influx, and prevents microvascular endothelial leakage (31). Studies using a CLP model have shown that inhibition of NF-κB activation significantly improves survival (22, 30, 33).

The anti-inflammatory effects of EPO have been well described. In a recent study employing a rat model of LPS-induced endotoxemia, it was demonstrated that EPO attenuates pulmonary inflammation and suppresses overproduction of TNF-α, which was partially mediated by NF-κB inhibition (39). In addition, treatment with SN-50, a specific inhibitor of the p50 subunit of NF-κB, in combination with antibiotic therapy, results in a significant survival benefit in the CLP model (36). Furthermore, NF-κB is responsible for a broad spectrum of responses, including inflammatory/immunoregulatory functions, apoptosis, adhesion molecule production, and...
cell cycle regulation (31). Our data indicate that EPO protects renal function and that this renoprotective affect is, in part, attributable to inhibition of the inflammatory response via downregulation of NF-κB activation. We hypothesized that EPO decreases NF-κB protein expression and consequently decreases inflammatory cytokine expression via EpoRs.

Endre et al. (17) conducted a controlled trial, designated the EARLYARF study, to determine whether a high dose of EPO (up to 50,000 U) would prevent AKI in intensive care unit patients if administered early (within 6 h of injury). To select patients for treatment, the authors measured urinary levels of the proximal tubular brush-border enzymes γ-glutamyl transpeptidase and alkaline phosphatase, both of which are biomarkers of kidney injury. Patients in whom the levels of those biomarkers exceeded the thresholds were randomized to receive either placebo or two doses of EPO. Although early intervention with high-dose EPO was found to be safe, it did not alter outcomes. Although elevated levels of γ-glutamyl transpeptidase and alkaline phosphatase were efficient in predicting poorer outcomes, they were hardly predictive of AKI, with a receiver operating characteristic curve of 0.54. In addition, the primary outcome measure was an increase in the mean creatinine level relative to baseline over 4–7 days. Consequently, AKI was identified through post hoc analysis. It is obvious that such diverse criteria can affect diagnostic accuracy.

Our results clearly indicate that the beneficial effects of EPO treatment are mediated, in part, by the higher production of NO, as evidenced by the higher levels of eNOS protein expression. We have demonstrated that, in a rat model of polymicrobial sepsis that mimics human sepsis, EPO provides a benefit due to its anti-inflammatory properties and its ability to upregulate eNOS protein expression.

In conclusion, treatment with EPO exhibited anti-inflammatory effects, improved systemic hemodynamics, and provided a significant survival benefit in rats subjected to CLP. We have also demonstrated that, in this CLP model of sepsis, EpoR expression is downregulated.

In the setting of sepsis, EPO administration, in addition to downregulating NF-κB activation, might have an endothelial protective effect, attributable to an EPO-induced increase in eNOS protein expression.

GRANTS

Financial support for this study was provided by the Brazilian Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Foundation for the Support of Research in the State of São Paulo), the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Foundation for the Support of Research in the State of São Paulo), and the Laboratórios de Investigação Médica (LIMs, Medical Investigation Laboratories) of the Faculdade de Medicina da Universidade de São Paulo (FMUSP, University of Sao Paulo School of Medicine). The American Society of Nephrology (ASN) is grateful for the Amgen Inc. and the Amgen Research Foundation for support of this meeting.

DISCLOSURES

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

AUTHOR CONTRIBUTORS


REFERENCES


