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Amelioration of lipopolysaccharide-induced acute kidney injury by erythropoietin: Involvement of mitochondria-regulated apoptosis

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ABSTRACT

Sepsis remains the most important cause of acute kidney injury (AKI) in critically ill patients and is an independent predictor of poor outcome. The administration of lipopolysaccharide (LPS) to animals reproduces most of the clinical features of sepsis, including AKI, a condition associated with renal cellular dysfunction and apoptosis.

Erythropoietin (EPO) is a well known cytoprotective multifunctional hormone, which exerts anti-inflammatory, anti-oxidant, anti-apoptotic and angiogenic effects in several tissues.

The aim of this study was to evaluate the underlying mechanisms of EPO renoprotection through the expression of the EPO receptor (EPO-R) and the modulation of the intrinsic apoptotic pathway in LPS-induced AKI.

Male inbred Balb/c mice were divided in four experimental groups: Control, LPS (8 mg/kg i.p.), EPO (3000 IU sc) and LPS + EPO. Assessment of renal function, histological examination, TUNEL in situ assay, immunohistochemistry and Western blottings of caspase-3, Bax, Bcl-x_L, EPO-R and Cytochrome *c* were performed at 24 h post treatment. LPS + EPO treatment significantly improved renal function and ameliorated histopathological injury when compared to the LPS treated group. Results showed that EPO treatment attenuates renal tubular apoptosis through: (a) the overexpression of EPO-R in tubular interstitial cells, (b) the reduction of Bax/Bcl-x_L ratio, (c) the inhibition Cytochrome *c* release into the cytosol and (d) the decrease of the active caspase-3 expression.

This study suggests that EPO exerts renoprotection on an experimental model of LPS-induced AKI. EPO induced renoprotection involves an anti-apoptotic effect through the expression of EPO-R and the regulation of the mitochondrial apoptotic pathway.

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1. Introduction

Acute kidney injury (AKI) is a common outcome of sepsis and is responsible for a significant morbidity and mortality in renal patients (Schrier and Wang, 2004). Therapeutics options for treating this condition are limited. Thus, there is a need for experimental and pre-clinical studies to provide new knowledge on the mechanisms involved in septic-related AKI.

Several pathophysiological mechanisms have been proposed for sepsis-induced AKI: (a) Lack of regulation in peritubular capillary network, (b) inflammatory reactions by a cytokine storm, (c) vasodilation-induced glomerular hypoperfusion and (d) tubular dysfunction induced by oxidative stress (Wu et al., 2007a).

In addition, a lipopolysaccharide endotoxin (LPS), a component of the outer cell membrane of Gram-negative bacteria, when administered to animals, reproduces most of the clinical features of sepsis, including acute renal injury (Doi et al., 2009).

It is well known that LPS increases the permeability of the proximal tubular cells and induces structural mitochondrial damage with caspase-mediated apoptosis (Balestra et al., 2009; Langford et al., 2011; Wan et al., 2003; Wang et al., 2005). Cell injury by LPS induces oligomerization of pro apoptotic members belonging to Bcl-2 protein's family, such as Bax, which translocates into mitochondria inducing the formation of pores with release of Cytochrome *c* (Parson and Green, 2010).

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Therefore, it would be of interest to develop therapeutics approaches that can favorably modulate apoptosis in septic-related AKI. One such approach could be the use of human recombinant erythropoietin (rhEPO).

It is known that rhEPO has been used for diminishing renal tubulointerstitial damage in several renal injuries (Esposito et al., 2009; Mohamed et al., 2013; Nakazawa et al., 2013; Vesey et al., 2004).

The anti-apoptotic effect of EPO administration has been shown in both, *in vitro* and *in vivo* conditions. The *in vitro* effects of EPO on the survival of human renal proximal tubular cells in culture have been determined (Salahudeen et al., 2008; Wang and Zhang, 2008). Similarly, the anti-apoptotic properties of EPO were observed *in vivo* using rat models of nephrotoxicity (Salahudeen et al., 2008; Pallet et al., 2010; Kong et al., 2013), renal ischemia-reperfusion injury (Sharples et al., 2004; Yang et al., 2003) and renal damage by hemorrhagic shock (Abdelrahman et al., 2004).

In humans, Song et al. (2009) reported that EPO administration prevented AKI in patients undergoing coronary artery bypass grafting. Moreover, it was observed that rhEPO administered to patients following cardiac surgery would minimize kidney lesions and decrease the incidence of AKI (de Seigneux et al., 2012).

The aim of the study was to evaluate the underlying mechanisms of EPO renoprotection in LPS-induced AKI. In this regard, the role of EPO-R in the modulation of the intrinsic apoptotic pathway was demonstrated.

2. Materials and methods

2.1. Animals

Male Balb/c mice (22–25 g, age: 6–8 weeks) were obtained from the animal facility of the National Northeast University, Argentina. Animals were housed in a controlled environment (22 ± 2 °C and relative humidity $55 \pm 15\%$) with a 12-h light/12-h dark cycle. The animals were allowed access to pelleted food and water *ad libitum*. All procedures involving these animals were conducted in compliance to the Guide for the Care and Use of Laboratory Animals (National Institute of Health, Bethesda, MD, USA) and the guidelines established by the Animal Ethical Committee of the Medical School of the National Northeast University.

2.2. Experimental design

Animals were randomly divided into 4 groups of six mice each. They were treated as follows: (I) Control group: sterile saline solution (i.p.), (II) EPO group: 3000 UI/kg of recombinant human erythropoietin (Hemax, BioSidus, Argentina) in 2 subcutaneous (s.c.) doses 12 h apart; (III) LPS group: 8 mg/kg i.p. LPS (*E. coli*, 0127: B8; Sigma, St. Louis, MO); (IV) LPS + EPO group, 8 mg/kg i.p. LPS dose followed by a 3000 UI/kg dose of EPO an hour later, administered as previously described in group (II).

The final dose of LPS was adjusted according to preliminary work with increasing doses (2.5–8 mg/kg). This was done in order to produce a certain level of renal injury. Additionally, the timing and the route of EPO administration were as described by Aoshiba et al. (2009).

Twenty four hours post LPS administration, mice were anesthetized (60 mg/kg pentobarbital i.p.) and bled by heart puncture. After being sacrificed by cervical dislocation, the kidneys were quickly excised and washed in cold saline solution. Renal samples were taken for routine histological, immunoblottings, immunohistochemical and TUNEL assays and serum samples were used in routine biochemical assays.

2.3. Assessment of renal function

Serum creatinine (sCr) and blood urea nitrogen (BUN) were determined by a Synchron CX7 autoanalyzer (Beckman, CA).

2.4. Histopathological studies in the kidney

For routine histological analysis, kidneys were fixed in phosphate-buffered formaldehyde, embedded in paraffin and stained with Hematoxylin and Eosin (H/E). Ten cortical high-power fields (×400) were examined at random by two blinded observers. The tubular injury (e.g. tubular dilatation/flattening and tubular degeneration/vacuolization) was evaluated in H/E sections. Alterations in affected tubules were graded as follows: 0, less than 5%; 1, 5–33%; 2, 34–66% and 3, over 66% (Wu et al., 2007b). Images were taken with an Olympus Coolpix-micro digital camera fitted on a CX-35 microscope (Olympus, Japan).

2.5. Immunohistochemistry

Paraffin-embedded sections were, deparaffinized and rehydrated in graded alcohols using routine protocols, as previously described (Stoyanoff et al., 2012). Briefly, sections were microwaved in citrate buffer (pH 6.0) for antigen retrieval and endogenous peroxidase activity was blocked in 3% H_2O_2 for 15 min. Subsequently, sections were incubated with a rabbit polyclonal anti-Bax (Sigma–Aldrich, dilution 1:400), anti-Bcl-x_L (Santa Cruz Biotechnology CA, USA, dilution 1:200), anti-cleaved caspase-3 (Sigma–Aldrich, dilution 1:1000) or anti EPO-R (H-194, Santa Cruz Biotechnology, CA, USA; dilution 1:100) antibodies for 18 h at 4 °C. Immunostaining was performed using a DAKO LSAB+/HRP kit (Dako Cytomation) followed by the application of a chromogene DAB (DAKO kit) according to the manufacturer's instructions. Negative control samples were processed in PBS. Slides were then counter-stained with hematoxylin and visualized under a light microscope.

2.6. Morphometric analysis

The percentage of positive areas for EPO-R, Bax, Bcl-x_L and caspase-3 was measured using the ImageJ software (National Institutes of Health, Bethesda, MD). Ten randomly selected cortical fields per cross-section were viewed (×400 original magnification). Images were taken using an Olympus Coolpix-microdigital camera fitted on a CX-35 microscope (Olympus, Japan).

2.7. In situ cell death detection (TUNEL assay)

Terminal deoxynucleotidyl transferase-mediated deoxyuridin triphosphate nick end labeling (TUNEL) assay was performed using an In situ Cell Death Detection Kit (Roche, Indianapolis, IN, USA) according to the manufacturer' instructions. Brown labeled TUNEL positive cells were counted under an ×400 magnification. The apoptotic index was calculated as the percentage of TUNEL-positive cells/total number of renal cells.

2.8. Western blot analysis

Expressions of EPO-R, Bax, Bcl-x_L and caspase-3 were determined by immunoblotting of cytosolic renal extracts as previously described (Aquino-Esperanza et al., 2008).Whole kidneys were homogenized and lysed in an ice-cold buffer [10 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.1% IGEPAL (Sigma Co, MO, USA)], supplemented with a protease inhibitor cocktail. Cell lysates were centrifuged at $14{,}000 \times g$ for 20 min and the supernatant (cytosolic fraction) was used for different assays.

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