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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.

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 ($22 \pm 2\,^{\circ}$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 ($\times$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 ($\times$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 $\times$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.

Cytochrome *c* expression was assessed in cytosolic and mitochondrial renal fractions. Mitochondria homogenates were obtained using the Mitochondria Isolation Kit (Pierce Biotechnology, USA) according to the manufacturer' instructions.

Cytosolic and/or mitochondrial proteins (40 μg) were separated by a 12% SDS-PAGE, blotted on nitrocellulose membranes (Bio-Rad, CA, USA). This was followed by treatment of membranes with 1:500 dilutions of primary anti-EPO-R (M-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-caspase 3 (Cell Signaling Technology, Beverly, MA, USA), anti-Cytochrome *c* (BD-Pharmigen) and anti-β actin (Sigma–Aldrich) antibodies. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Inc, USA). Immunocomplexes were detected by an Opti4CN kit (Bio-Rad, CA, USA). Band optical density (OD) was determined using NIH-image software and results were expressed as the ratio: (protein of interest OD /β-actin OD) × 100.

2.9. Statistical analysis

Results were expressed as mean ± standard error of mean (SEM). Comparisons between groups were performed using one-way analysis of variance (ANOVA) with a post hoc Bonferroni test correction. Data were analyzed with a Prism 4.0 software package (GraphPad Software Inc., San Diego, CA). Differences between groups were considered to be statistically significant at $P < 0.05$.

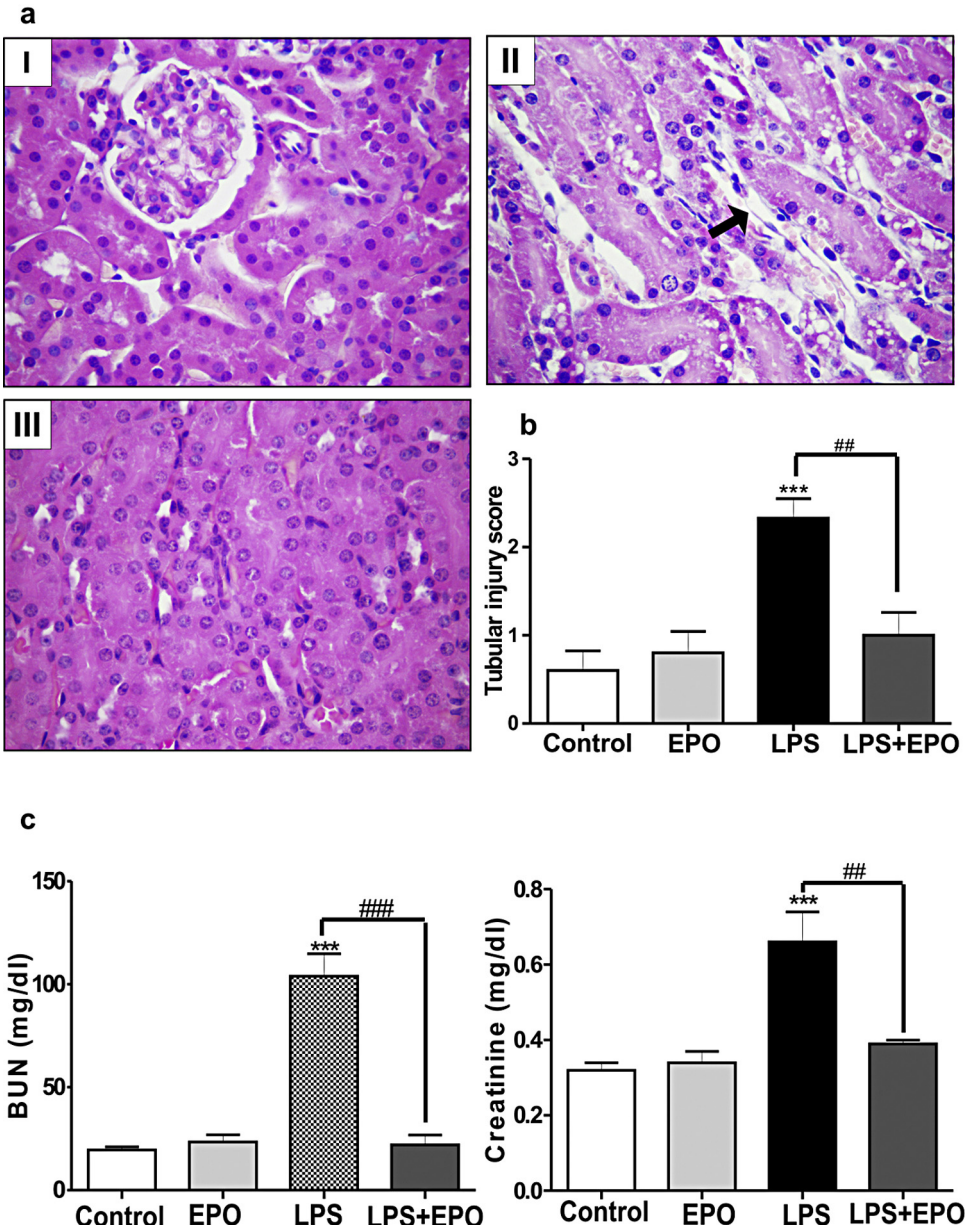

Fig. 1. Effect of EPO on LPS induced tissue damage and changes in renal function. (a) Representative hematoxylin/eosin stained sections. (I) Saline solution treated-control mice show morphologically normal organization of glomeruli and tubules. (II) LPS causes moderate tubular renal injury as manifested by tubular vacuolization (arrow), degeneration and focal tubular dilatation. (III) EPO treatment after LPS administration ameliorates the histopathological changes. Original magnification x 400. (b) Scores of tubular injury. Values are mean ± SEM. Tubular injury increased following LPS treatment compared to the control group. LPS + EPO group showed a significant improvement of tubular damage. (c) BUN (blood urea nitrogen) and serum creatinine. Results are expressed as mean ± SEM ($n = 6$ mice/group). Significantly different from control group at $^{***}P < 0.001$. Significantly different from LPS group at $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ respectively.

3. Results

3.1. Effects of EPO on renal function and histological damage

LPS induced a moderate tubular renal injury. However, injury was prominent in the renal cortex. Renal sections showed significant focal tubular epithelial cell swelling, dilatation and detachment with moderate tubular vacuolization. EPO treatment notably ameliorated the histopathological damage (Fig. 1a), as indicated by the cortical tubular injury scores obtained (0.43 times below LPS group, $P < 0.01$) (Fig. 1b). Kidney histology in the EPO treated group was similar to that of the control group (data not shown).

LPS administration also decreased renal function at 24 h as shown by a remarkable increase in creatinine and blood urea nitrogen (BUN) levels compared to the control group ($P < 0.001$). In contrast, the LPS + EPO group displayed a significant improvement in renal function as indicated by a decrease in the biochemical parameters measured (BUN, about 79% and serum creatinine 41% below LPS group; $P < 0.001$ and $P < 0.01$ respectively, Fig. 1c).

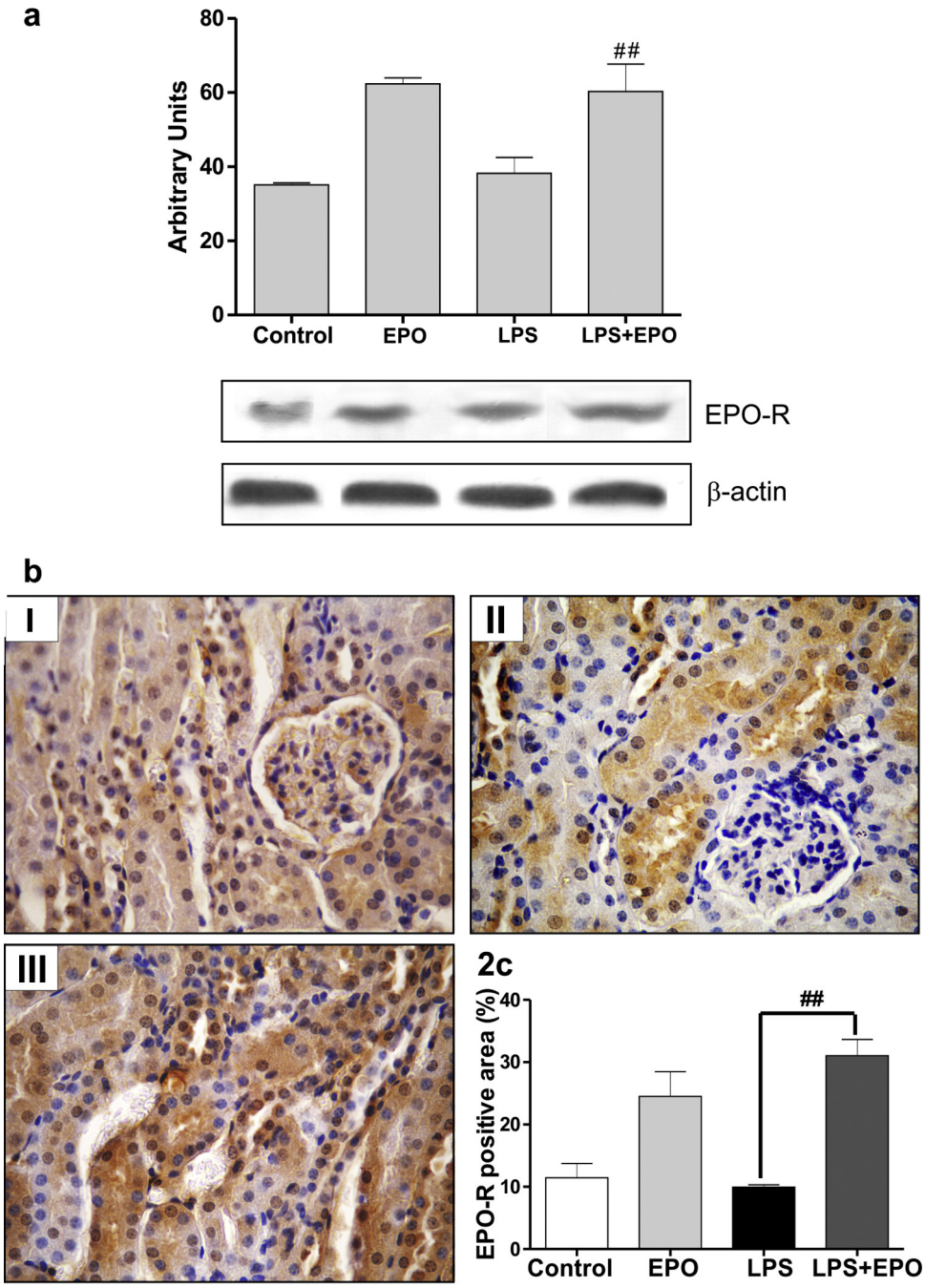

Fig. 2. Effects of EPO on EPO-R expression in renal cortex in LPS-induced AKI. (a) Immunoblottings of EPO-R. EPO treatment caused EPO-R over expression compared to either control group or LPS treated group. Data were normalized to β-actin used as loading control. Values are mean ± SEM of six mice per group. (b) Immunohistochemistry of EPO-R. Representative photomicrographs corresponding to Control (I), LPS (II) and LPS + EPO (III) groups are shown. EPO-R immunostaining is shown on both, plasma membranes and cytoplasm. EPO-R expression is confined mainly to cortical tubules. Original magnification ×400. (c) Quantitative analysis of EPO-R-positive areas. The graph obtained with the results from the morphometric analysis resembles the profile observed with the immunoblotting data [see (a)]. ##Significantly different from the LPS group at $P < 0.01$.

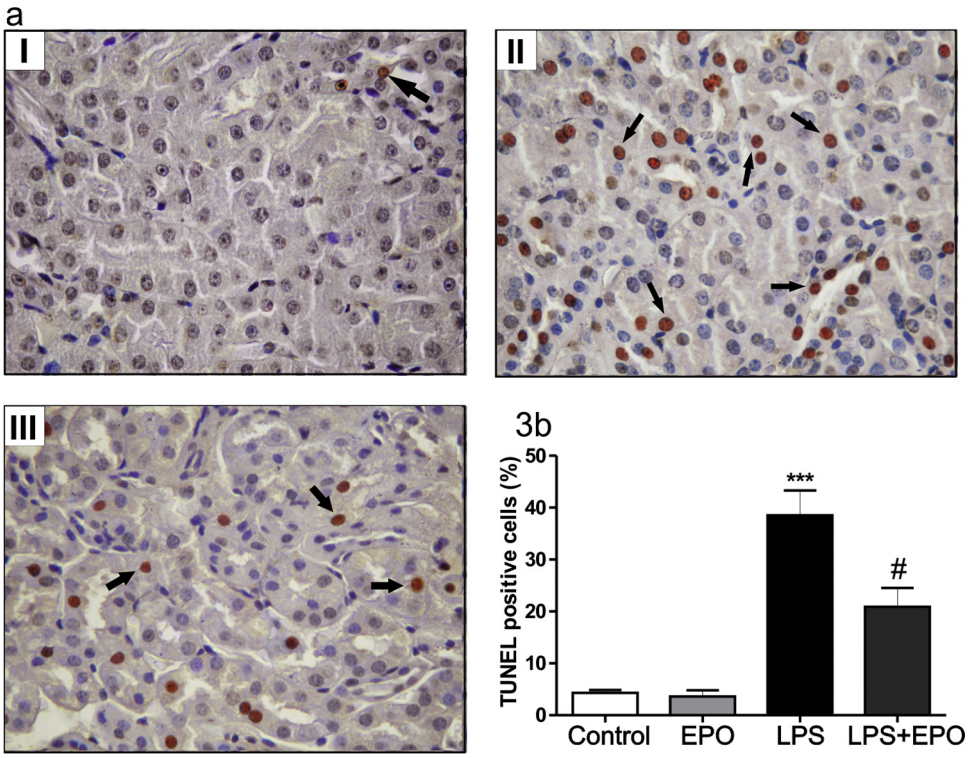

Fig. 3. Effects of EPO on the renal apoptosis observed in LPS-induced AKI. (a) Representative photomicrographs of the TUNEL in situ assay corresponding to control (I), LPS (II) and LPS + EPO (III) groups. Arrows indicate the TUNEL-positive cells in tubulointerstitial areas. Original magnification ×400. (b) Bars represent percentages of TUNEL-positive nuclei. Values are mean ± SEM. Significantly different from control group at $^{***}P < 0.001$. Significantly different from LPS group at $^{\#}P < 0.05$.

3.2. Effects of EPO on the expression of EPO-R

The expression of EPO-R following EPO administration was assessed by immunoblotting and immunohistochemistry.

Western blots revealed that EPO-R expression in the LPS + EPO treated group, exhibited a significant enhancement when compared to the LPS treated group ($P < 0.01$). There was no statistically significant difference in EPO-R expression between LPS and control groups (Fig. 2a).

As it is shown in Fig. 2b, EPO-R immunostaining showed both, membranous and intracellular EPO-R expression, and this expression was mainly confined to the cortical tubular cells. In the LPS treated group, EPO-R immunoreactivity remained similar to the control group. Conversely, EPO treatment showed a notable increment in EPO-R expression in the endotoxemic animals.

Morphometric analyses of EPO-R immunohistochemistry revealed a similar profile to that observed in EPO-R immunoblots (Fig. 2c).

3.3. Effects of EPO on tubular apoptotic cell death

To evaluate whether EPO decreases tubular cell apoptosis in LPS-induced AKI, the proportion of apoptotic cells in renal sections were determined using the TUNEL assay. At 24 h post injury, endotoxemic kidneys showed a substantial number of apoptotic nuclei, predominantly in tubular epithelial cells (Fig. 3a). As shown in Fig. 3b, EPO treatment caused a significant decrease in the number of TUNEL positive cells in endotoxemic mice (nearly two-fold below LPS group, $P < 0.05$).

3.4. Effects of EPO in procaspase-3 cleavage

To further demonstrate whether EPO administration limits the activation of caspase-3, a key mediator in apoptosis, the cleavage of caspase-3 was analyzed by immunoblotting and active caspase-3 expression was evaluated by immunohistochemistry in kidney sections.

As depicted in Fig. 4a, pro-caspase 3 was found to be cleaved whilst active caspase-3 expression increased in the LPS treated group. However, the pro-caspase-3 was slightly less cleaved in the LPS + EPO group when compared to the LPS treated mice ($P < 0.05$). Moreover, EPO treatment caused a significant decrease in caspase-3 active expression ($P < 0.001$) as compared to the LPS treated mice.

Caspase-3 immunohistochemistry was performed to confirm whether the treatment with EPO limits caspase-3 activation, thus, reducing LPS-induced apoptosis in renal tubular cells (Fig. 4b). As shown in Fig. 4c, EPO decreased the percentage of positive cleaved caspase-3 areas in cortical sections (85.7% below LPS group, $P < 0.001$).

3.5. Effects of EPO on the expression of Bax and Bcl-x_L

To interpret the effects of EPO administration on tubular apoptotic cell death regulation; Bax (pro-apoptotic) and Bcl-x_L (anti-apoptotic) expressions were evaluated by immunoblotting of kidney homogenates and by immunohistochemistry of renal sections.

The analysis of the blots revealed that LPS caused a significant over expression of Bax that returned to baseline after EPO administration ($P < 0.001$). On the other hand, and as expected, Bcl-x_L exhibited a notable increase in the LPS + EPO treated group compared to the LPS treated mice (Fig. 5a).

Immunohistochemical studies showed a notable positive immunoreaction of Bax in the cytoplasm of tubular cells in the LPS treated group, which was reduced following EPO administration. The Bcl-x_L immunoreactive areas did not show significant changes between the groups (Fig. 5b and c).

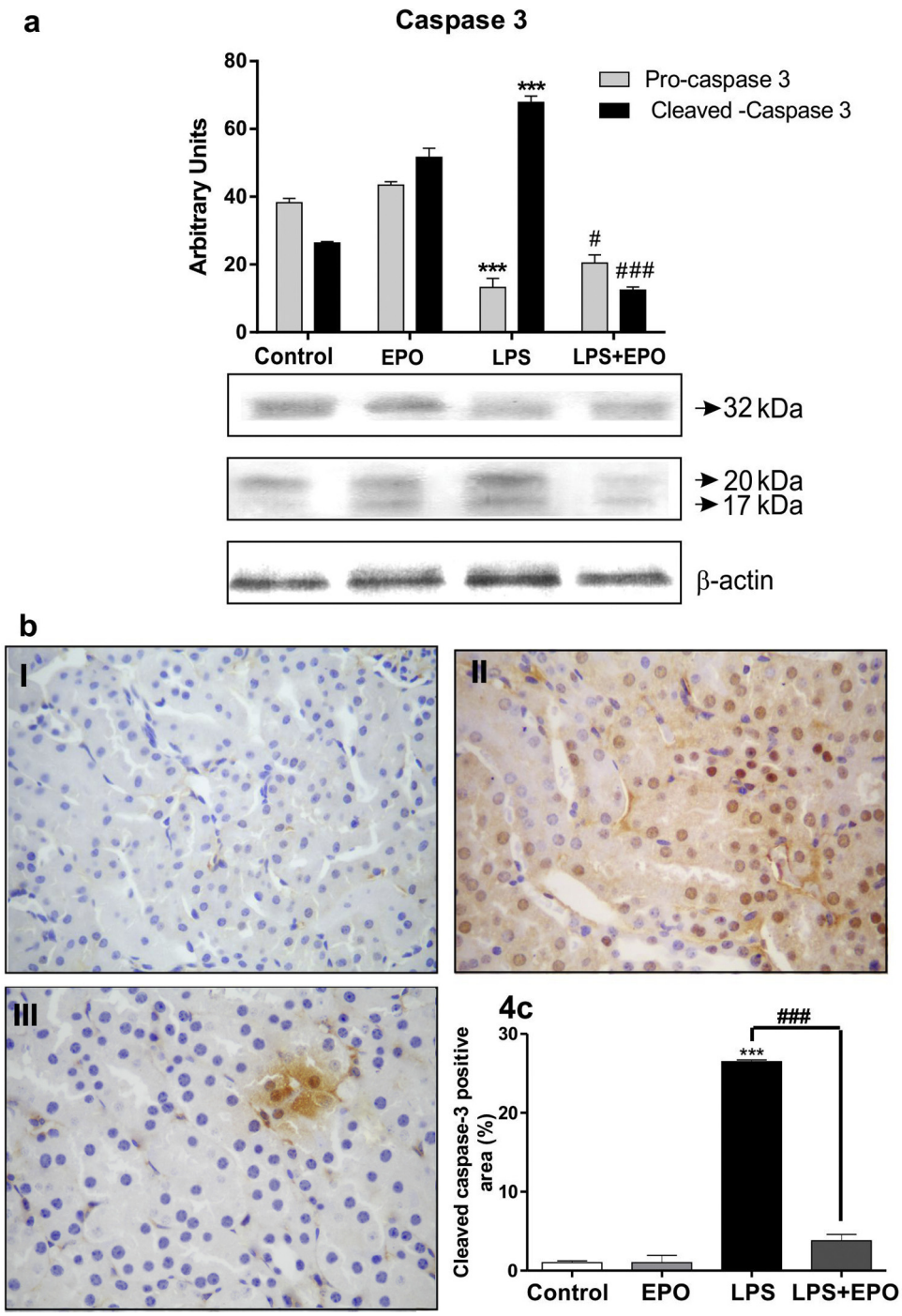

Fig. 4. Effects of EPO on caspase-3 expression in LPS-induced AKI. (a) Immunoblottings of pro-caspase3 and cleaved caspase-3. LPS caused decrease of pro-caspase 3 levels (grey bars) and an over expression of the cleaved active form of caspase-3 (black bars) at 24 h post treatment. Data were normalized to β-actin used as loading control. Values are mean ± SEM of six mice per group. (b) Immunohistochemistry of cleaved caspase-3. Representative photomicrographs of caspase-3 immunoreactivity: control group (I) with low positive immunoreaction; LPS group (II) shows a notable increment of caspase-3 in the cytoplasm of injured tubules; and LPS + EPO group (III) exhibits an evident decrease in caspase-3 immunoreactivity. Original magnification ×400. (c) Quantitative analysis of cleaved caspase-3-positive areas. The graph obtained with the results from the morphometric analysis resembles the profile observed with the immunobloting data [see (a)]. ***Significantly different from control group at $P < 0.001$. Significantly different from LPS group at $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ respectively.

3.6. *Effects of EPO on Cytochrome c expression*

To elucidate whether EPO treatment affects the mitochondrial apoptotic pathway in LPS-induced AKI, Cytochrome *c* expression was evaluated in cortical mitochondrial and cytosolic homogenates by immunoblotting.

As shown in Fig. 6, Cytochrome *c* baseline levels were detected in the mitochondrial homogenates of the control group, whereas they were absent in the cytosolic extracts; suggesting the integrity of the mitochondrial membranes. On the other hand, LPS administration induced Cytochrome *c* release from the mitochondria into the cytosol. EPO treatment clearly prevents this translocation and,

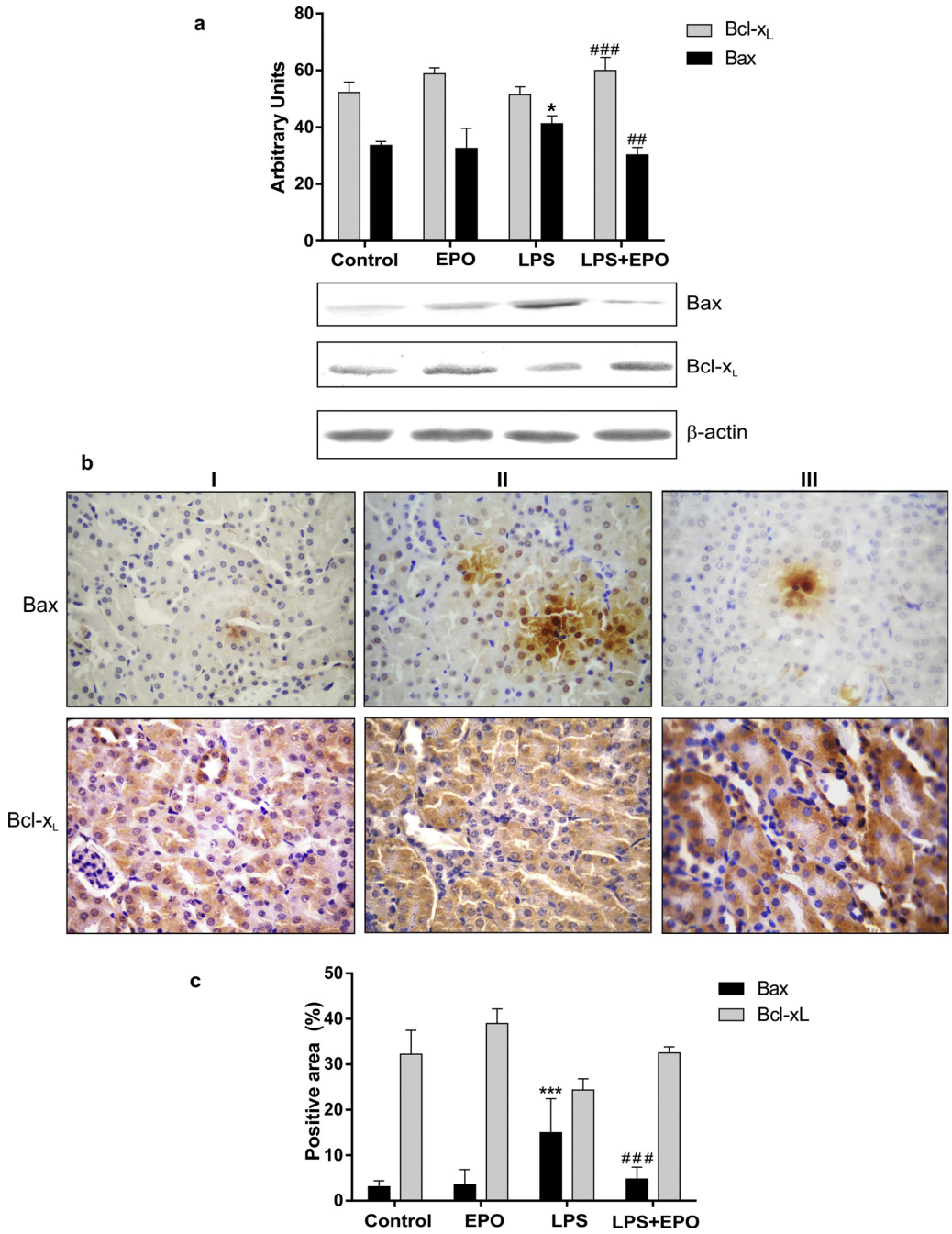

Fig. 5. Effects of EPO on Bcl-x_L and Bax expressions in LPS-induced AKI. (a) Western blottings of Bcl-x_L (grey bars) and Bax (black bars). Values are mean ± SEM of six mice per group. Representative immunoblots of Bcl-x_L and Bax are shown. Data were normalized to β-actin used as loading control. (b) Immunohistochemistry of Bax and Bcl-x_L. Representative photomicrographs of Bax and Bcl-x_L immunoreactivities: control mice (I) showing weak baseline immunoreactivity of Bax and evident positive immunoreactivity of Bcl-x_L in the cytoplasm of most tubular cells; (II) LPS group reveals a remarkable Bax positive immunoreactivity whereas Bcl-x_L remains unchanged. (III) LPS + EPO group exhibits a marked decrease in Bax expression associated with a Bcl-x_L enhanced immunoreactivity. Original magnification ×400. (c) Quantitative analysis of Bcl-x_L and Bax-positive areas. Significantly different from control group at $^{*}P < 0.05$ and $^{***}P < 0.001$ respectively. Significantly different from LPS group at $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ respectively.

as shown above, avoids a series of biochemical reactions that result in caspases activation with a subsequent LPS–induced tubular apoptosis.

4. Discussion

It is now widely accepted that EPO is not solely a hormone involved in the regulation of proliferation and differentiation of erythroid progenitor cells, but it also has cytoprotective effects in many non-hematopoietic tissues.

Several studies have shown that both, erythropoietin (EPO) and its receptor (EPO-R), are functionally expressed in many tissues where EPO exerts a remarkable cytoprotection against different type of injuries (Jelkmann and Wagner, 2004).

Endogenous EPO is known to be mainly produced by renal cortical fibroblasts in response to hypoxia and it is found in blood at a low concentration (1–7 pmol/L, reviewed in Jelkmann, 2007). In LPS-induced AKI, serum levels of EPO are controversial (Frede et al., 1997; Mitra et al., 2007). In addition, the local concentration of EPO necessary for tissue-protective effects is higher than that needed

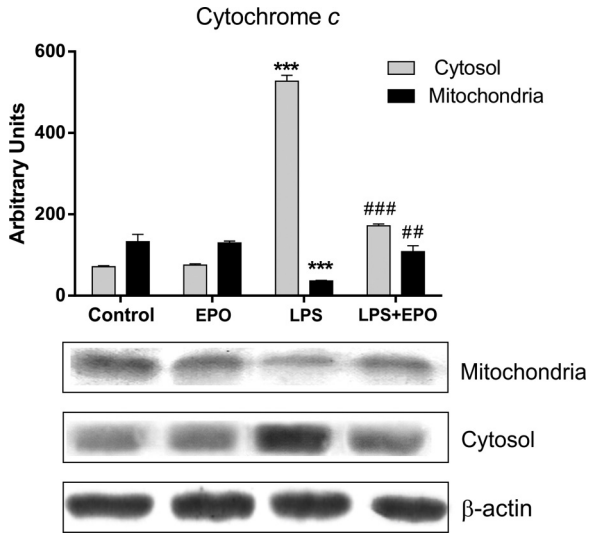

Fig. 6. Effects of EPO on Cytochrome *c* expression in LPS-induced AKI. Western blottings of Cytochrome *c* in cytosolic (grey bars) and mitochondrial fractions (black bars). LPS enhances Cytochrome *c* traslocation into the cytosol whereas EPO administration suppressed its release from mitochondria. Values are mean ± SEM of six mice per group. Representative blots are shown. Data are normalized to β-actin used as loading control. Significantly different from control group at $^{***}P < 0.001$. Significantly different from LPS group at $^{##}P < 0.01$ and $^{###}P < 0.001$ respectively.

for its hormonal effects (Brines, 2010). Furthermore, EPO receptor expression is induced by TNF-α, while EPO synthesis is suppressed (Nagai et al., 2001; Brines and Cerami, 2008).

The present study demonstrates that EPO has a renoprotective effect on a model of endotoxemic-related AKI by the modulation of both, the intrinsic apoptotic pathway and the expression of EPO-R.

The results extend data of earlier studies showing that LPS induces a functional change and a histopathological damage in the kidney (Cunningham et al., 2002; Wu et al., 2007a). Furthermore, in agreement with previous observations (Aoshiba et al., 2009; Mitra et al., 2007), exogenous EPO administration causes an improvement in renal function during endotoxemia, as indicated by significant decreases in BUN and creatinine serum levels. This was accompanied by histopathological improvement of the alterations caused by LPS.

EPO-mediated renoprotection results from the direct interaction of EPO with its receptor EPO-R (Brines et al., 2004) which is present in human, rat and mouse kidney (Westenfelder et al., 1999). In this study, both Western blotting and inmunohistochemistry results showed a significant increase in EPO-R expression post EPO treatment, whereas LPS treatment did not affect either the control levels of the receptor or its enhanced expression by EPO. These results are in agreement with those of Pessoa de Souza et al. (2012) who reported EPO-R expression in a cecal ligation and puncture (CLP) animal model of a sepsis related to AKI. These data also suggest that, in agreement to De Beuf et al. (2009), the cytoprotective effect of EPO is associated with increased EPO-R levels in tubular cells.

Previous studies have also shown that LPS causes a severe disruption of cortical peritubular perfusion and renal failure (Legrand et al., 2011). Although hemodynamic factors might play a role in the pathogenesis of endotoxemic-AKI, other mechanisms are likely to be involved. Among these, apoptosis may be a dominant one (Duranton et al., 2010).

In this regard, the present results support the role of apoptosis in LPS-induced acute renal failure. The TUNEL in situ assay and the active caspase-3 expression confirmed that apoptotic events occur mostly in tubular cells following LPS administration.

EPO treatment significantly reduced the caspase-3 cleavage and the number of TUNEL-positive cells ($P < 0.05$) in the kidneys of endotoxemic mice. These findings suggest that the renoprotective effect of EPO in LPS-induced AKI occurs at least, in part, by reducing apoptosis. These observations are in agreement with previous studies on the anti-apoptotic effect of EPO in other models of acute renal injury (Salahudeen et al., 2008; Sharples et al., 2004).

There are a number of signaling pathways by which EPO may exert its anti-apoptotic effects on renal cells. The activation of the PI3-kinase/AKT pathway promotes cell survival and anti-apoptotic effects by: (a) inactivation of the pro-apoptotic protein Bad, (b) the activation of protein Bcl-x_L, (c) inactivation of caspases, and (d) prevention of Cytochrome *c* release. Moreover, the activation of EPO-R also triggers a cross-talk between JAK2/STAT and NF-kB signaling pathways (Moore et al., 2011). The contribution of each signaling cascade downstream EPO-R activation in renoprotection has not been clearly elucidated and may differ in different models of AKI. A particular mechanism of interest is the modulation of the apoptotic mitochondrial pathway which involves the disruption of the mitochondrial membrane with a subsequent release of Cytochrome *c* into the cytosol (Martinou and Green, 2001).

The present results show Cytochrome *c* in kidney cytosolic fractions of LPS treated mice concomitant with caspase-3 over expression; thus, suggesting the involvement of the mitochondrial pathway of apoptosis. Furthermore, EPO treatment prevents LPS induced mitochondrial membrane changes and Cytocrome *c* release into the cytosol, blocking a series of biochemical reactions that result in caspase-3 activation and subsequent apoptosis. These results are in line with previous observations of EPO protection on the cerebral vascular system (Chong et al., 2003).

The inhibitory/promoting actions of the Bcl-2 family members are involved in the release of Cytochrome *c* (Parson and Green, 2010). A previous study has shown that LPS produces an increase in pro-apoptotic proteins, such as Bax, and a decrease in anti-apoptotic proteins, such as Bcl-x_L (Wan et al., 2003). Similarly, the present work shows that LPS causes an over expression of the pro-apoptotic protein Bax in cortical renal cells. Furthermore, TUNEL results show that EPO administration causes in both, control and LPS treated mice, an increase in the Bcl-x_L/Bax ratio, which is indicative of a pro-survival effect. The increment in Bcl-x_L levels observed in LPS-induced AKI, following the administration of EPO may be the result of an overexpression of the EPO-R.

EPO effects on Bcl-2 family in renal studies usually include assessment of the Bcl-2 anti-apoptotic member (Aoshiba et al., 2009; Sharples et al., 2004; Yang et al., 2003). In the present findings, the over expression of Bcl-x_L in the LPS + EPO treated animals, when compare to treatment with LPS alone, indicates a role of EPO downstream events leading to renal cell survival.

In conclusion, our data show that EPO ameliorates the renal apoptosis triggered by LPS. This protective effect depends on EPO-R over expression, a subsequent increase of the Bcl-x_L/Bax ratio and the inhibition of the release of Cytochrome *c* from mitochondria into cytosol.

The results presented here also provide new insights into the anti-apoptotic mechanisms of EPO in endotoxemic-AKI and suggest further EPO clinical applications.

Conflicts of interest statement

The authors declare no conflict of interest.

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