Erythropoietin Ameliorates Renal Ischemia and Reperfusion Injury via Inhibiting Tubulointerstitial Inflammation

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Background. Tubulointerstitial inflammation is the characteristics of renal ischemia reperfusion injury (IRI) that is inevitable in kidney transplantation. Erythropoietin (EPO) has recently been shown to have protective effects on renal IRI by anti-apoptosis and anti-oxidation. Here, the effect and mechanism of EPO on renal IRI were further investigated, with a focus on tubulointerstitial inflammation.

Materials and Methods. Male Sprague-Dawley rats were administrated with saline or EPO prior to IRI induced by bilateral renal pedicle clamping. Twenty-four hours following reperfusion, the effects of EPO on renal IRI were assessed by renal function and structure, tubulointerstitial myeloperoxidase (MPO) positive neutrophils, and proinflammatory mediator gene expression. The translocation and activity of NF-κB in renal tissues were also evaluated.

Results. Compared with control groups, the EPO treated group exhibited lower serum urea and creatinine levels, limited tubular necrosis with a lower score of renal histological lesion. MPO positive cells in the tubulointerstitial area were greatly increased by IRI, but significantly reduced by the treatment of EPO. The gene expression of proinflammatory cytokines (IL-1β, IL-6, IL-10, and TNF-α) and chemokine (MCP-1) was also significantly decreased by EPO. In addition, less activation and nuclear-translocation of NF-κB was observed in the kidney treated by EPO as well.

Conclusion. EPO improved renal function and structure in IRI rats via reducing neutrophils in the tubulointerstitium, the production of proinflammatory cytokines and chemokine, as well as the activation and nuclear-translocation of NF-κB. EPO may have potential clinical applications as an anti-inflammation agent clinically for a wide range of injury.

Key Words: erythropoietin; renal ischemia-reperfusion injury; inflammation; NF-κB.

INTRODUCTION

Renal ischemia and reperfusion injury (IRI) is one of the major causes of acute renal dysfunction associated with a high rate of rejection and affects both short- and long-term graft survivals after kidney transplantation [1]. Although some progress has been made in reducing the unfavorable effect [2–4], renal IRI remains a significant clinical challenge and an efficient therapeutic regimen still needs to be established.

Tubular necrosis and interstitial infiltration of inflammatory cells are characteristic pathologic changes in the kidney with IRI. The inflammatory responses following IRI include oxidative stress, increased production of inflammatory cytokines, and infiltration of neutrophils and macrophages [5]. However, the precise mechanisms involving in renal IRI and leading to chronic kidney diseases have not yet been fully elucidated, but may give opportunities for developing new therapeutic methods.

Erythropoietin (EPO) is a hematopoietic hormone produced mainly by the adult kidneys and has been
routinely used in clinic for nearly 20 y in the management of anemia. Apart from its erythropoietic effects, EPO also exhibits powerful tissue-protective effects against IRI in a wide range of organs including kidney [6], heart [7], liver [8], and central nervous system [9]. In an ischemic condition, the EPO receptor will be up-regulated [10–13]. After that, EPO can activate multiple intracellular signaling, including mitogen-activated protein kinase (MAPK), c-Jun N-terminal Kinase (JNK), and phosphatidylinositol 3-kinase activated protein kinase (MAPK), c-Jun N-terminal kinases (JNK), and phosphatidylinositol 3-kinase signaling cascades [14–16], and induces the subsequent transcription of anti-apoptotic [17] and anti-oxidative genes [18]. Based on these properties, EPO has emerged as an efficient renoprotective agent against renal dysfunction and injury caused by hypoxia, oxidative stress, and hemorrhagic shock.

Despite the demonstrated benefits of EPO in renoprotection, there are few data available on its effects on inflammation in renal IRI. This is important, because inflammation has recently been implicated as a critical mechanism responsible for renal IRI injury both in human and animal models. Therapies that target specific inflammatory cell types or effector proteins, such as proinflammatory cytokines [19], chemokines [20] and toll-like receptors [21], can significantly ameliorate renal injury in animal models. These reports highlight the immense therapeutic potential of anti-inflammatory strategy for renal IRI. Interestingly, the anti-inflammatory effect of EPO has been recently revealed in ischemia myocardial [22] and brain injury [23].

These works prompt us to raise a hypothesis that the therapeutic benefit of EPO in renal IRI might be at least partly due to the influence on the renal inflammatory cascade. Therefore, this study was undertaken to assess whether EPO could efficiently inhibit renal inflammation during IRI, and to explore the possible underlying mechanisms.

MATERIALS AND METHODS

Rat Model of Renal IRI

Male Sprague-Dawley rats weighing 200–250 g were obtained from the experimental animal center of the Chinese Academy of Science (Shanghai, China) and housed in pathogen-free mouse colonies. All animal experiments were performed according to the guidelines of the Care and Use of Laboratory Animals of the Laboratory Animal Ethical Commission of Fudan University with good animal surgical research practices. Rats were randomly divided into three groups: (1) sham group (sham; n = 8); (2) IRI group treated with saline (saline+IRI; n = 8); and (3) IRI group treated with EPO (EPO+IRI; n = 8). EPO (2000 units/kg) was injected intraperitoneally 30 min before clamping the renal pedicle.

The IRI model in rat was similar to that described previously [24]. Rats were anaesthetized with chloral hydrate (0.1 g/kg, intraperitoneally). According to our previous practice, chloral hydrate alone used as an anesthetic method was sufficient for acceptable anesthesia. In addition, several previous studies also used chloral hydrate alone to anesthetize rats for the surgery [25–27]. In this study, the effects of anesthesia induced by chloral hydrate were reliable and satisfying since the rat had no sign of pain during surgical procedures, without using analgesia. Core body temperature was maintained at 37 °C using a homeothermic table during surgery. The abdominal cavity was exposed through a midline incision, and the renal pedicles were carefully isolated. Bilateral renal occlusion for 45 min was performed using nontraumatic vascular clamps, and occlusion was confirmed by observing the color changing of the entire kidney surface. The ischemia of 45 min chosen in this study was based on previous publications [28, 29], and our own preliminary experiments used different ischemia times from 30–60 min. It has been found that 30 min was too short to induce significant renal injury, while 60 min was too long as it caused high mortality of experimental rats. Typical and obvious renal pathologic injuries were evidenced with a lower mortality when 45 min ischemia was applied. After removing the renal clamps, kidneys were observed for 5 min to ensure blood reperfusion. Afterwards, 1 mL of warmed saline was injected intraperitoneally, and the incision was sutured in two layers. As the acute effects of EPO on I/R injury in terms of inflammation, renal function, and structure were emphasized in this study, all animals were euthanized with an overdose of pentobarbitone sodium after 24 h.

Measurement of Biochemical Parameters

Twenty-four hours after IRI, blood samples were collected and centrifuged at 2500 rpm for 5 min, and serum was obtained. Urea nitrogen and creatinine were measured.

Renal Histologic Analysis

The kidneys were removed at 24 h after reperfusion, fixed in 10% (w/v) neutral buffered formalin, sectioned, and then stained with hematoxylin and eosin (H&E). Evaluation of renal injury was performed in a blind manner by a pathologist. Renal sections were scored with a semiquantitative scale designed to evaluate the degree of tubular necrosis and interstitial damage. Injury was graded on a 5-point scale: 0 = normal kidney; 1 = minimal damage (<5% involvement of the cortex or outer medulla); 2 = mild damage (5%–25% involvement of the cortex or outer medulla); 3 = moderate damage (25%–75% involvement of the cortex or outer medulla); and 4 = severe damage (>75% involvement of the cortex or outer medulla).

Immunohistochemical Staining of Myeloperoxidase (MPO)

Immunohistochemical staining of MPO, a marker mainly for neutrophil granulocytes, was undertaken on paraffin sections using a DAKO ChemMate EnVisionTM Detection Kit (DAKO, Glostrup, Denmark). The sections were digested by 40 μg/mL protease K for 15–30 min at 37 °C and blocked by peroxidase-blocking reagent. The sections were labeled by an anti-MPO antibody (1:600 dilution, DAKO) at 4 °C overnight. The antibody binding was revealed by AEC. MPO+ cells in the tubular and interstitial areas were semiquantitatively scored in 20 fields at ×400 magnification separately [30].

Quantitative Real-Time PCR

Total RNA was extracted from rat kidney with Trizol reagent (Invitrogen, Shanghai, China) according to the manufacturer’s instructions. Total RNA (3–5 μg) was transcribed into cDNA by Superscript II reverse transcriptase (Invitrogen) and random primer oligonucleotides (Invitrogen). Gene-specific primers for rat TNF-α, IL-1β, IL-6, IL-10, MCP-1, IL-8, IL-4, and GAPDH were designed based on sequences available through the PubMed (for sequences see Table 1). Real-time quantitative PCR was performed in Bio-Rad iCycler iQ system in combination with the Absolute QPCR SYBR Green premix (Takara Bio Inc., Otsu, Shiga, Japan). After a hot start (15 min at
95°C), the parameters for amplification were as follows: 1 s at 95°C, 5 s at 60°C, and 10 s at 72°C for 45 cycles. Expression levels normalized with GAPDH were calculated relative to the housekeeping gene GAPDH using the 2^{△△CT} method.

Electrophoretic Mobility Shift Assay (EMSA)

Nonradioactive EMSA was performed with an EMSA kit according to the manufacturer's instructions (Panomics, Inc., Redwood City, CA). Eight μg of nuclear protein was used to bind biotinylated oligonucleotides containing the NF-κB binding site for 30 min at room temperature. For the blank control, the nuclear extracts were replaced with water. For the competition/cold control, non-biotin-labeled cold probes were added to the reaction. Samples were separated in a 6% nondenaturing polyacrylamide gel with 2.5% glycerol and blotted on a Biodyne B Pre-cut Modified Nylon membrane (Thermo Scientific Protein Pierce Research Products, Rockford, IL). Biotin was labeled with alkaline phosphatase-conjugated streptavidin, and alkaline phosphatase was detected with enhanced chemiluminescence western blotting detection system (Amersham, Arlington Heights, IL). The band was semiquantified by densitometry using the systems as described above.

Statistical Analysis

All data were given as mean ± SD. Statistical analysis of the data was performed with the two-tailed independent Student’s t-test using SPSS version 12.0 (SPSS Inc.). P < 0.05 was considered statistically significant.

RESULTS

EPO Improves Renal Dysfunction Induced by IRI

Rats underwent renal IRI showed a 16-fold increase in the serum creatinine (484.7 µmol/L) and a 9-fold increase in blood urea nitrogen concentration (53.4 mmol/L) compared with the sham group (29.3 µmol/L and 5.9 mmol/L, respectively) (Fig. 1). In comparison with the untreated IRI group, EPO treatment caused mass decrease in serum creatinine and urea nitrogen by approximately 70% and 60%, respectively.

EPO Ameliorates Renal Histologic Damage Following IRI

There were significant tubular changes including loss of brush border, dilation of renal tubules, as well as degeneration and necrosis of renal tubular epithelial cells following IRI compared with the sham group (Fig. 2A). In contrast, EPO treatment significantly ameliorated tubular lesions (Fig. 2A). The semiquantitative assessment of histologic lesion showed a significantly higher score in the IRI group compared with the EPO treated group at 24 h of reperfusion (3.25 versus 1.82, P < 0.05, Fig. 2B).

EPO Ameliorates Neutrophil Infiltration Following Renal IRI

To determine whether EPO could inhibit inflammatory cells infiltration, MPO positive neutrophil infiltration was studied by immunostaining in three different groups. The number of MPO positive cells was significantly increased in the kidneys with IRI, but much less in the kidneys treated by EPO (P < 0.05, Fig. 3), suggesting EPO could significantly inhibit the infiltration of neutrophils, which were recruited in the injured tissues.

EPO Suppresses Proinflammatory Cytokines and Chemokine Expression in Kidneys with IRI

To determine the effect of EPO on inflammation in renal IRI, the expression of major proinflammatory cytokines and chemokines was examined. Compared with

### TABLE 1

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<th>Gene</th>
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| GAPDH  | Upper: AGTTCACAGGCACAGTGAAG  
        | Lower: TACTCAGACACAGTCAGCA            |
| IL-1β  | Upper: GCTGACAGACCGCAAGAGAT  
        | Lower: TGGTGGACGGATGTCTGAGA           |
| IL-6   | Upper: GCCCTTCAGGAGAGCTGATGA  
        | Lower: TGTCACACCATGCTCAGCCAGGA       |
| TFN-α  | Upper: CCTTATCATCCTCAGGTTCTGCA  
        | Lower: GAGGTCTGACTTCTCCTGGTTATG       |
| IL-10  | Upper: GTGACACGTCAGCGCA  
        | Lower: GTCAGCATTTTCAGGAGACTGGAA       |
| IL-4   | Upper: ACACTTTGAAACAGGTGACAG  
        | Lower: CAAGCAGCGGAGGTACATCAG          |
| MCP-1  | Upper: GATCCTCTCTTCTCCAGCAGCATG  
        | Lower: GAAATAGTACAGCAGCGCTGAGCT         |
| IL-8   | Upper: CATTAAATTTTAACAGATGCTGATACGGTTTCA  
        | Lower: GCCCTACATCTTTAAAATGCAGCAAT       |

FIG. 1. Effect of EPO pretreatment on renal function. Serum creatinine (A) and urea nitrogen (B) in sham-operated, IRI and EPO-treated groups were measured at 24 h after reperfusion. Data were shown as mean ± SEM. *P < 0.05.
the sham group, the transcription of inflammatory cytokines (IL-1β, IL-6, TNF-α, and IL-10) and chemokine (MCP-1) was greatly up-regulated in the kidneys with IRI, but were limited in the kidneys treated by EPO (P < 0.05, Fig. 4), suggesting that EPO treatment could significantly reduce the production of inflammatory cytokines and chemokines.

EPO Inhibits the Nuclear Translocation of NF-κB Following Renal IRI

The expression of numerous proinflammatory cytokine and chemokine genes requires NF-κB nuclear translocation and activation [31]. To determine whether EPO could inhibit IRI mediated inflammation by inhibiting NF-κB translocation and activation, we analyzed the nuclear lysates of renal tissues by electrophoretic mobility shift assay (EMSA), which measures the binding activity of NF-κB to labeled DNAs. In the kidneys of sham-operated rats, there was low-grade nuclear translocation for NF-κB (Fig. 5A). In response to IRI, the translocation of NF-κB significantly increased up to 12-fold higher than the normal level; this elevation was remarkably suppressed by EPO treatment (8.13 versus 12.10, P < 0.05) (Fig. 5B), indicating that EPO may perform...
its anti-inflammation effect in renal IRI by inhibiting NF-κB activation and nuclear translocation.

**DISCUSSION**

In the past decades, renal IRI response has been studied in great detail and has shown to be a highly orchestrated process [32]. In addition to acute tubular necrosis, massive interstitial infiltration of neutrophils and macrophages is a characteristic pathologic change in IRI. At the initial phase, hypoxic and anoxic cell injuries occur and result in the local robust synthesis of proinflammatory cytokines [33]. These cytokines can launch defensive physiologic activities to isolate and restrain tissue damage; or further aggravate organ injury and dysfunction by inducing free radical production and recruiting inflammatory cells. Several lines of evidence have reported that the expression of proinflammatory cytokines is significantly increased in ischemia myocardial, hepatic, and renal diseases, and shows a positive correlation with the severity of organ damage and dysfunction [34–36].

The data from this study demonstrate that EPO treatment is improved renal function compared with those in the IRI control group, with decreased tubular necrosis and reduced MPO positive neutrophils. These results suggest the intriguing possibility that EPO effectively blocked neutrophil infiltration or promoted neutrophils clearance in postischemic renal tissues in some ways, which could be associated with tubular epithelial preservation and renal function recovery.

Many investigations indicate that neutrophils are important mediators in renal IRI. Inflammatory cascades that are initiated by endothelial dysfunction can be augmented dramatically by the generation of a number of potent proinflammatory cytokines and chemokines by the ischemic proximal tubule. Given the pivotal roles of proinflammatory mediators in IRI, the effects of EPO on the expression of proinflammatory cytokines and chemokines were further investigated in this study. The kidney specimens post 24 h reperfusion were collected, and the transcription level of certain cytokines and chemokine in these kidneys were measured by real-time PCR. EPO treatment induced substantial reduction in the gene expression of major proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokine (MCP-1) in the renal tissue. The reduced cytokine and chemokine levels in EPO-treated kidneys were associated with much less MPO positive neutrophil infiltration and less tubular necrosis. These results reveal that EPO ameliorated inflammation and renal histologic damage in IRI due to minimizing the production of proinflammatory mediators. The present results are consistent with some previous studies. Chang et al. [37] reported that EPO performed a protective action in rat unilateral ureteral obstruction partially by inhibiting TNF-α production. Lieutaud et al. [38] described that the systemic administration of EPO significantly lowered the concentration of IL-1β and MIP-2 in a rat brain trauma model, confirming the potential anti-inflammation activity of EPO. Interestingly, a new research by Solling et al. [39] showed that EPO can improve glomerular filtration rate after IRI via immunomodulatory effects that were manifested as decreased TNF-α and IL-10 in renal biopsies and reduced TNF-α in plasma.

However, some controversial studies have also been reported. In a human model of acute systemic...
low-grade inflammation, EPO administration significantly increases the levels of cytokines, e.g., TNF-α and IL-6, after LPS challenges [40]. In an in vitro culture system, EPO also did not inhibit the LPS-mediated proinflammatory activation of microglial cells. These phenomena indicate that the effect of EPO on the inflammation response is very complicated and may highly rely on the type of cells or tissues as well as the cause, degree, and stage of injury.

NF-κB is regarded as one of the most important and best-characterized transcription factors. In most cell types, NF-κB exists as a latent complex binding to its inhibitor in the cytosol. Upon stimulation, the inhibitor of NF-κB becomes degraded, and NF-κB releases and translocates to the nucleus where NF-κB binds to its consensus sequence in target genes. NF-κB up-regulates the expression of many genes, most of which encode proteins that play crucial and often determining roles in the processes of inflammation [31]. It has been reported that activation of NF-κB results in the up-regulation of adhesion molecules and chemokines in vascular endothelial cells and within the tissues. NF-κB also plays an essential role in the transcriptional activation of proinflammatory cytokines and apoptosis-associated proteins. Recently, the contribution of NF-κB in ischemia/hypoxia-induced inflammation has been gradually appreciated [33, 41]. It has been reported that NF-κB activation occurred during ischemia and reached its peak after 15 min of reperfusion, suggesting that NF-κB plays a major role in the initiation of inflammation [42]. In the present study, EPO treatment significantly inhibited NF-κB nuclear translocation and activation, which may be associated with the down-regulated gene expression of cytokines and chemokine detected in this study. This result, therefore, also illustrated another possible one of the anti-inflammatory mechanisms of EPO. This finding is also in accordance with previous studies that showed the inhibitory functions of EPO on NF-κB signaling in ischemia myocardial and brain injury [22, 23].

There is accumulating evidence that the therapeutic benefits of EPO go above and beyond ameliorating anemia and accompanying tissue hypoxia. In this study, EPO protected kidneys against IRI partially by decreasing MPO positive neutrophils and suppressing the expression of proinflammatory cytokines and chemokines. The anti-inflammation effect of EPO was also likely mediated by inhibiting the NF-κB signaling pathway. Therefore, the anti-inflammatory effect of EPO may play an important role in tissue protection in addition to its well documented anti-apoptotic effect. This study provides an extra evidence that EPO may have a potential application clinically as a novel anti-inflammation agent for IRI.

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REFERENCES


