Effects of combined erythropoietin and epidermal growth factor on renal ischaemia/reperfusion injury: a randomized experimental controlled study

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OBJECTIVE

To investigate effects of combination of erythropoietin (EPO) and epidermal growth factor (EGF) on renal ischaemia and on reactive oxygen species in a rat model.

MATERIALS AND METHODS

In all, 90 male Sprague-Dawley rats were allocated into five groups of 18, designated: Sham; treated with right nephrectomy only; Control, subjected to left renal ischaemia for 45 min with no treatment; EPO-treated, as the control but with EPO pretreatment; EGF-treated, as the control but with EGF pretreatment; EPO + EGF-treated, as the control but with EPO and EGF pretreatment.

RESULTS

All rats except the controls had a significant improvement in serum creatinine, creatinine clearance and fractional excretion of Na⁺; all three were significantly better in EPO + EGF group than in all other groups. Histopathological examination showed marked structural damage in control rats. The tubular damage was least in the EPO + EGF group. The control group had a significant increase in MDA level and a significant decrease in SOD and GSH, while the EPO + EGF group had a marked significant reduction in MDA and increase in GSH and SOD.

CONCLUSION

The protection against ischaemia/reperfusion injury might be maximal when EPO and EGF are administered concomitantly, and their protective effect might be partly due to their antioxidant effects.

KEYWORDS

kidney, rat, ischaemia/reperfusion injury, erythropoietin, EGF, SOD, GSH, MDA

INTRODUCTION

Renal ischaemia/reperfusion (I/R) injury in clinical practice can occur as a consequence of systemic hypoperfusion (e.g. shock) with subsequent circulatory resuscitations and temporary discontinuation of renal blood supply, e.g. in renal transplantation, partial nephrectomy, and aortic cross-clamping [1]. I/R injury has been correlated with the incidence of acute renal transplant rejection [2], and has been identified as an alloantigen-independent risk factor for chronic allograft nephropathy [3]. The mechanisms underlying renal I/R injury are complex, including ATP depletion, accumulation of intracellular Ca²⁺ and reactive oxygen species (ROS), mitochondrial dysfunction, multiple enzyme system activation and pro-inflammatory cytokine production [4]. Recovery of kidney function after I/R injury depends on the replacement or regeneration of injured cells and protection from programmed cell death (apoptosis) [5,6].

Erythropoietin (EPO) is a cytokine that was originally identified as the major regulator of proliferation and differentiation of erythroid progenitor cells via its anti-apoptotic action [7]. However, increasing evidence suggests that EPO has broader functions independent of its effects on erythropoiesis. Recent in vitro and in vivo studies showed that EPO attenuates renal cell damage [7–10]. During early phases of renal I/R injury, EPO receptor expression is well maintained, whereas EPO expression is markedly down-regulated [11,12].

Epidermal growth factor (EGF) is also a potent cytoprotective and reparative growth factor that is normally synthesized by distal tubular cells, with increasing expression during maturation [13]. Rana et al. [14] reported that there was a remarkable decrease in EGF mRNA levels at the initial sample times of renal injury during I/R. Also, EGF receptor expression is up-regulated at the site of injury [5,6].

Thus we hypothesized that a combination of these two agents, one of them is essential for regeneration of renal tubules (i.e. EGF) and the other with potent anti-apoptotic properties
(EPO) which are deficient in early ischaemia, would be more protective of renal I/R injury than each alone. The aim of the present study was to investigate the effects of EPO and EGF in renal I/R injury in rats, individually and combined. We also evaluated whether the renoprotective effects of these agents are related to the induction of antioxidant systems (superoxide dismutase, SOD, and reduced glutathione, GSH) and the inhibition of lipid peroxidation.

MATERIALS AND METHODS

The study comprised 90 male Sprague-Dawley rats (250–300 g; 3–4 months old) bred and housed in the animal house of the Urology and Nephrology Centre. The rats were housed in individual cages at 20–25 °C with a 12 : 12 h light-dark cycle, and fed standard laboratory chow with free access to tap water. All animal procedures were approved by our local ethic committee of animal care and use.

The pharmacological agents included EPO-α and EGF; EPO-α is a recombinant human EPO, formulated as a sterile colourless liquid in an isotonic sodium chloride/sodium citrate buffered solution in the form of preservative-free vial (4000 IU/0.4 mL). EGF was a human recombinant EGF (97% pure by SDS-PAGE), expressed in Escherichia coli, provided as a lyophilized powder and cell-culture tested, obtained from a 0.2-μm-filtered solution of PBS. The vial was reconstituted using 0.2 μm-filtered 10 mM acetic acid containing BSA to a concentration of ≥20 μg/mL. All chemicals were purchased from Sigma Chemical Co (St. Louis, MO, USA).

The rats were taken from the cages, and kept for 30 s in a metal container containing a piece of cotton soaked with 10 mL of halothane. The rats were maintained on sodium thiopental at 12 mg/100 g body weight, injected i.p. After anaesthesia, the rat was fixed supine on a thermoregulated heating board to maintain a body temperature of 37 °C. To induce renal warm ischaemia followed by reperfusion, a modification of the method described by Gupta et al. [15] was used. Through a midline laparotomy, the left renal pedicle was exposed and the left renal artery was dissected from its vein. Then the left renal artery was clamped for 45 min using a vascular Bulldog clamp. The edges of the abdominal incision were approximated to each other and covered by a piece of gauze soaked with warm isotonic saline (37 °C) to prevent undue loss of body fluids. At 5 min before removing the vascular clamp, the right kidney was removed by exposing it and placing a single thread ligature around the renal blood vessels and ureter; the thread ligature was tied securely and the blood vessels were dissected next to the kidney, and then the kidney was removed. After removing the vascular clamp on the left renal artery, the abdomen was irrigated with warm isotonic saline, and then the abdominal incision was closed by continuous stitches using polyglactin 2/0 sutures.

The rats were randomly allocated into five groups of 18 each, as follows: Sham-operated; the rats had a right nephrectomy with no left renal ischaemia and were then injected with 1 mL of sterile saline in the penile vein and 1 mL s.c.; I/R injury (control) group; rats were subjected to right nephrectomy and left renal ischaemia for 45 min and injected with 1 mL of sterile saline in the penile vein and 1 mL s.c.; EPO-treated; as in the control group, but the rats received EPO 500 U/kg body weight in 1 mL saline in the penile vein, 30 min before clamping of the left renal artery; EGF-treated; as in the control group, but the rats received EGF 100 μg/kg body weight in 1 mL saline s.c. 30 min before clamping of the left renal artery; EPO + EGF-treated; as in the control group, but the rats received both EPO and EGF in the same doses as the EPO and EGF groups, respectively.

Blood and urine samples were taken on the day after surgery and at 1, 2 and 7 days after renal ischaemia to estimate the sodium and creatinine levels in both blood and urine. Blood (1 mL) was obtained from the ophthalmic venous plexus using a fine-walled Pasteur pipette. The rat was anaesthetized using halothane inhalation and the pipette was positioned at the inner corner of the eye beside the eyeball, and pushed gently but firmly along the side of the orbit to the ophthalmic venous plexus. Blood was centrifuged and plasma stored at −20 °C. The rats were placed in a metabolism cage for 24 h to collect 24-h urine samples. The rats were anaesthetized again by sodium thiopental i.p. to harvest the left kidney at 1, 2 and 7 days after renal ischaemia (six kidneys per day). The abdomen was opened and the left kidney was perfused briefly with PBS through a cannula inserted into the abdominal aorta, to rinse out the blood. The kidney was removed rapidly and cut into halves by a scalpel; one half was rapidly placed in a container containing 10% neutral buffered formalin for histopathological examination, and the other half was rapidly frozen in liquid N2, and stored at −72 °C until assay of malondialdehyde (MDA), GSH and SOD in kidney tissues.

The concentrations of sodium and creatinine in urine and serum were measured using an auto-analyser (CX 7; Beckman, Foster City, CA, USA). Creatinine clearance and the fractional excretion of Na+(FENa) were calculated from serum, urine creatinine and sodium.

The kidney specimens were processed for paraffin embedding; sections of 3 μm were cut and stained with both haematoxylin and eosin (H&E), and periodic acid-Schiff (PAS), and examined by light microscopy. The kidney sections were submitted for histopathological examination and grading while unaware of origin. Tubulointerstitial injury was defined as tubular atrophy, dilatation, and intratubular casts, as well as thickening of tubular basement membranes, loss of brush border, cellular infiltration, and widening of the interstitium. The degree of tubulointerstitial damage in the cortex and outer medulla was determined using a semiquantitative graded scale [16], where 0 = no abnormality, 1 = minimal damage (involvement of <25% of cortex and outer medulla), 2 = mild damage (involvement of 25–50% of cortex and outer medulla), 3 = moderate damage (involvement of 50–75% of cortex and outer medulla), and 4 = severe damage (involvement of >75% of cortex and outer medulla).

The renal cortex was separated from the medulla, weighed (75 mg), minced, homogenized in 0.02 M sodium phosphate buffer, pH 7.4 (1 : 4 w/v) using a smooth glass homogenizer with a motor-driven Teflon pestle, and centrifuged at 850 g for 20 min at 4 °C. An aliquot of supernatants was stored at −80 °C until determination of total proteins, MDA, GSH and SOD. Protein content was determined by the method of Lowry et al. [17]. MDA was assayed by the method of Walker and Shah [18], and the results were expressed as nmol/mg tissue protein content. GSH was assayed by the method of Beutler [19] and results expressed as nmol/mg protein. SOD activity was measured according to the method proposed by Marklund and Marklund [20], and the results expressed as U/mg protein.
FIG. 1. Renal function in different groups at different sample times: A, serum creatinine (mg/dl); B, creatinine clearance (ml/min); C, FE Na. All values are the mean (SD). *significant vs control group (P < 0.05), #significant vs EGF + EPO-treated group (P < 0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (SD) at day</th>
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<tbody>
<tr>
<td>Sham</td>
<td>1.17 (0.41)</td>
</tr>
<tr>
<td>Control</td>
<td>4.67 (0.52)</td>
</tr>
<tr>
<td>EPO-treated</td>
<td>3.33 (0.82)*</td>
</tr>
<tr>
<td>EGF-treated</td>
<td>4.00 (0.63)†</td>
</tr>
<tr>
<td>EPO + EGF-treated</td>
<td>2.33 (0.21)*</td>
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One way ANOVA and posthoc Scheffe's s-test for multiple pairwise comparisons; *significant vs control group (P < 0.05); †significant vs EPO + EGF-treated group (P < 0.05).

TABLE 1 The tubulointerstitial damage score in the sham, control, EPO, EGF and EPO + EGF groups at 1, 2 and 7 days after I/R

RESULTS

On the day before surgery there was no statistically significant difference in serum creatinine and creatinine clearance among all groups. Compared with the sham group, the control group had highly significant increase in serum creatinine and significant decrease in creatinine clearance at 1, 2 and 7 days. These changes were significantly attenuated in the EPO-, EGF- and EPO + EGF-treated groups when compared with the control group at the three sample times (Fig. 1A,B). Also, the EPO + EGF-treated group had a significantly lower serum creatinine and significantly higher creatinine clearance than the EPO- and EGF-treated groups at the three sample times, except at 7 days there was no significant difference between the EPO- and EPO + EGF-treated group (Fig. 1A,B).

Compared with sham group, the control group had a significant greater FE Na, at 1, 2, and 7 days; these changes were significantly attenuated in the EPO-, EGF- and EPO + EGF-treated groups when compared with the control group at the three sample times (Fig. 1C). Also, the EPO + EGF-treated group had a significantly lower FE Na than the EPO- and EGF-treated groups at the three sample times, except at 2 and 7 days there were no significant differences between the EPO- and EPO + EGF-treated group (Fig. 1C).

Table 1 shows that there was a significantly higher renal tubulointerstitial damage score in the control group than in the sham group at 1, 2 and 7 days. The EPO-treated group had a significantly lower tubulointerstitial damage score at the different sample times than the control group, while EGF-treated rats had a significantly lower damage score only at 7 days. Moreover, the EPO + EGF-treated rats had a significantly lower damage score than the EGF- or EPO-treated rats. The most severe and pronounced injury was in the cortex and the outer stripe of the outer medulla of the control rats, with an atypical tubular necrosis pattern, which included widespread degeneration of tubular architecture, detachment of epithelial cells from the basement membrane, tubular cell necrosis, intratubular cast formation and luminal congestion with extensive loss of brush border (Fig. 2A) and inflammatory cellular infiltrate mostly neutrophils (Fig. 2B). By contrast, renal sections obtained from the EPO + EGF-treated rats showed a marked reduction in the histological features of renal injury, consisting of mild individual tubular necrosis with an intact brush border (Fig. 2C) and minimal tubular dilatation (Fig. 2D).

The results of the MDA, SOD and GSH analyses are shown in Fig. 3. There was a significantly higher MDA level in the kidney tissue in the control group at 1 and 2 days after I/R than in the sham group. There was a significantly lower MDA level at 1 and 2 days in the EPO- and EPO + EGF-treated groups, and in the EGF-treated group at 2 days, than in the control group. Moreover, there was a significant difference between EGF- and EPO + EGF-treated groups at 1 and 2 days (Fig. 3A). Figure 3B shows that the GSH level was significantly lower the control group than in the sham group at 1 and 2 days, but not at 7 days, and a significantly higher level in the EPO-, EGF- and EPO-EGF-treated groups than in the control group. Also, GSH was significantly higher in the EPO + EGF-treated group than in the EPO- and EGF-treated groups. The activity of SOD was significantly lower in the control group than in the sham group.
group at 1 and 2 days and significantly higher in the EPO-, EGF- and EPO + EGF-treated groups at 1 and 2 days than in the control group. Also, there was a significant difference between the EPO + EGF- and the EPO- and EGF-treated groups (Fig. 3C).

DISCUSSION

Renal I/R injury is a complex inflammatory process in which the kidney is morphologically and functionally damaged during the ischaemic phase and undergoes further insult during reperfusion. In the present study, we used a rat model of renal I/R injury (45 min) and monitored it by renal function and histopathology over 7 days. The effect of pretreatment with EPO, EGF or both on renal function and histopathology, as well as on MDA (a marker of lipid peroxidation) and SOD and GSH (markers of antioxidants) was investigated.

The present study showed that renal I/R injury caused significant increase in serum creatinine and $\text{FE}_{\text{Cr}}$, and significant decrease in creatinine clearance at 1, 2 and 7 days after I/R. Evidence of tubular injury was also supported by the significant increase in histological damage score in control group. These findings confirm that I/R injury of the kidney causes both glomerular and tubular dysfunctions, and are in agreement with those reported by others [5,6,8]. The involvement of ROS in I/R injury to the kidney and other organs is widely accepted. The main reported sources for ROS production during I/R are endothelial cells, which have hypoxanthine/xanthine oxidase system, and activated neutrophils [21]. In this model of renal I/R injury, renal ischaemia caused a significant increase in MDA content of the kidney tissues (indicating increased lipid peroxidation) and significant reduction of the endogenous antioxidant systems SOD and GSH. These findings confirm the involvement of ROS in renal I/R injury and support findings reported by others [18,21].

This study showed, to the best of our knowledge for the first time, that a combination of EPO and EGF had the maximum protective effect against renal I/R injury. This was supported by the significantly better renal function and histopathological damage scores in rats treated with both EPO + EGF than in rats treated with EPO or EGF alone. In the present study both EPO and EGF produced significantly better renal function and histopathology, but the protective effect of EPO was significantly higher than EGF.

Pre-treatment with EPO as a single dose produced significantly better renal function at the three sample times after ischaemia. Consistent with the laboratory findings, the morphological changes showed that EPO

FIG. 2. Kidney sections stained with PAS and H&E, by light microscopy of four representative samples from two different rats (A and B for untreated control rats and C and D for rats treated with EPO + EGF) killed after 2 days. Untreated control rats had loss of integrity of tubular cell brush border (decreased staining with PAS), tubular dilatation and necrosis (A) and inflammatory infiltrate mostly neutrophils (B). Rats treated with EPO + EGF had relatively well-preserved tubules with an intact brush border (C) and minimally dilated tubules. ×400.

FIG. 3. The effect of pretreatment with the various agents on MDA (A), GSH (B) and SOD (C), in renal I/R injury. Values are the mean (SD). *Significant vs control group ($P < 0.05$); #significant vs EPO + EGF-treated group ($P < 0.05$).
reduced the acute tubular necrosis score at the three times after ischaemia. This beneficial effect of exogenous EPO on the course of I/R injury provides evidence of functional EPO deficiency and support the findings reported by others who showed that EPO treatment improved the functional and morphological tubular injury in rats subjected to I/R injury [7–10]. One potential mechanism of renoprotective action of EPO was the decreased expression of pro-apoptotic Bax protein [7]. Additional protective mechanisms include activation of protein kinase B, induction of heat-shock protein 70, angiogenesis, activation of anti-apoptotic proteins BCL-XI, and increased mobilization of bone marrow-derived stem cells [7–10]. In the present study, EPO before the onset of ischaemia was associated with a significant decrease in MDA and significant increase in the activity of SOD and GSH in kidney tissue in renal I/R injury. These findings verified that EPO might be capable of acting as a direct antioxidant as well by activating antioxidant defence mechanisms. Previous studies established that EPO inhibits lipid peroxidation in oxidative damage in liver, intestine and brain [10,22,23]. The present study, to the best of our knowledge, is the first study to show the effects of EPO in preventing the oxidative stress in I/R-induced renal injury.

The next step in this work was to investigate the effect of EGF on renal I/R injury; s.c. EGF 30 min before ischaemia, produced a significant improvement in renal function at the three sample times after I/R. Consistent with the laboratory findings, the morphological changes showed that EGF reduced the acute tubular necrosis score only at 7 days after I/R. These findings suggest that the administration of EGF before the onset of ischaemia reduces both the glomerular and the tubular injury and dysfunction caused by severe I/R injury. However, the nephroprotective effect offered by EGF against renal I/R injury was lower than that by EPO. This dramatic effect of a single 100-pg/kg dose of EGF in acute tubular injury could be attributed to the heightened sensitivity to EGF in acute tubular injury due to an increase in the number of EGF receptors and/or affinity [5,6]. EGF receptors are present in the proximal tubular cells of the kidney, and are activated during I/R injury, resulting in enhanced regeneration of the injured segments of the kidney [24]. The protective effect of EGF on renal I/R injury might be attributed to its mitogenic action and enhanced regeneration of tubular cells [25], and by interfering with the endothelin-mediated vasoconstrictor mechanism, or by favouring the production of vasodilator agents such as nitric oxide by endothelial cells [26].

For ROS, the present study showed that EGF administration before the onset of ischaemia caused a significant decrease in MDA only at 2 days, and significant increase in the activity of SOD and GSH at 1 and 2 days after ischaemia in kidney tissue. In vitro and in vivo evidence converges to support our findings that the cytoprotective ability of EGF is expressed by reducing lethal tissue damage and the lipid peroxidation process. These findings include renal I/R injury [27], carbon tetrachloride-mediated liver necrosis [28], and apoptosis by hyperoxia in cultured neurones [29], and cerebral injury by ischaemia [29].

We also investigated the effect of combined EPO and EGF on renal I/R; the combination significantly increased creatinine clearance and significantly reduced the serum creatinine and FEmCr on various days after I/R. Consistent with the laboratory findings, this combination improved the renal tubular damage score at the three sample times. These findings suggest that a combination of EPO and EGF before the onset of ischaemia is protective in renal I/R injury, and reduces both glomerular and the tubular dysfunction. Moreover, the protective effect of EPO and EGF together was significantly better than either agent alone, especially in 1 and 2 days after ischaemia. There might be a synergistic effect of EPO and EGF to increase the GFR; this synergistic action of EPO and EGF could be caused by interfering with the endothelin-mediated vasoconstrictor mechanism, or by favouring the production of vasodilator agents such as nitric oxide by endothelial cells. In agreement with this hypothesis, Haug et al. [26] concluded that EGF reduced endothelin-1 synthesis in endothelial cells, and Cherian et al. [30] reported that EPO might up-regulate or stimulate endothelial nitric oxide synthase.

We also showed that the combination of EPO and EGF significantly decreased MDA levels, and increased GSH and SOD level, but adding EGF to EPO did not significantly decrease MDA by more than EPO alone, while adding EGF to EPO significantly improved the endogenous antioxidants, especially SOD activity. In agreement with these findings Baranano and Snyder [31] reported that EPO increased the production of radical scavengers, and Akisu et al. [32] showed that EPO inhibited the iron-catalysed reactions for generating free oxygen radicals. Also, Price et al. [33] reported that EGF mainly up-regulated the cellular antioxidant enzymes. The interaction between EGF and EPO in vivo seems to be too complex to be described by stimulatory/inhibitory categories. The present study was designed to address the clinical and histopathological effect of EPO, EGF and their combination on I/R injury. Further studies will be needed to clarify the mechanisms of this synergistic action between EGF and EPO.

In conclusion, the present study confirms the protective effects of EPO and EGF on rat kidney in a model of severe I/R injury. The cytoprotective action of EPO and EGF might be partly due to their antioxidant properties. Moreover, concomitant administration of EPO and EGF produced the maximum protective effect. Further clinical studies are recommended to test the combined effect of EPO and EGF before changing surgical procedures or technique which results in renal I/R injury, such as renal transplantation, partial nephrectomy, renal artery angioplasty, enucleation of renal cell carcinoma, aortic bypass surgery, accidental or iatrogenic traumatic renal injury repair, and elective urological operations.

CONFLICT OF INTEREST
None declared.

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Abbreviations: IR; ischaemia/reperfusion; FENa, fractional sodium excretion; ROS, reactive oxygen species; EPO, erythropoietin; EGF, epidermal growth factor; SOD, superoxide dismutase; GSH, reduced glutathione; MDA, malondialdehyde; H&E, haematoxylin and eosin; PAS, periodic acid-Schiff.