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Erythropoietin protects against ischaemic acute renal injury

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Abstract

Background. Erythropoietin (EPO) has recently been shown to exert important cytoprotective and anti-apoptotic effects in experimental brain injury and cisplatin-induced nephrotoxicity. The aim of the present study was to determine whether EPO administration is also renoprotective in both *in vitro* and *in vivo* models of ischaemic acute renal failure.

Methods. Primary cultures of human proximal tubule cells (PTCs) were exposed to either vehicle or EPO (6.25–400 IU/ml) in the presence of hypoxia (1% O_2), normoxia (21% O_2) or hypoxia followed by normoxia for up to 24 h. The end-points evaluated included cell apoptosis (morphology and *in situ* end labelling [ISEL], viability [lactate dehydrogenase (LDH release)], cell proliferation [proliferating cell nuclear antigen (PCNA)] and DNA synthesis (thymidine incorporation). The effects of EPO pre-treatment (5000 U/kg) on renal morphology and function were also studied in rat models of unilateral and bilateral ischaemia–reperfusion (IR) injury.

Results. In the *in vitro* model, hypoxia (1% O_2) induced a significant degree of PTC apoptosis, which was substantially reduced by co-incubation with EPO at 24 h (vehicle $2.5 \pm 0.5\%$ *vs* 25 IU/ml EPO $1.8 \pm 0.4\%$ *vs* 200 IU/ml EPO $0.9 \pm 0.2\%$, $n = 9$, $P < 0.05$). At high concentrations (400 IU/ml), EPO also stimulated thymidine incorporation in cells exposed to hypoxia with or without subsequent normoxia. LDH release was not significantly affected. In the unilateral IR model, EPO pre-treatment significantly attenuated outer medullary thick ascending limb (TAL) apoptosis (EPO $2.2 \pm 1.0\%$ of cells *vs* vehicle $6.5 \pm 2.2\%$, $P < 0.05$, $n = 5$) and potentiated mitosis (EPO $1.1 \pm 0.3\%$ *vs* vehicle $0.5 \pm 0.3\%$, respectively, $P < 0.05$) within 24 h. EPO-treated rats exhibited enhanced PCNA staining within the proximal straight tubule ($6.9 \pm 0.7\%$ *vs* vehicle $2.4 \pm 0.5\%$ *vs* sham $0.3 \pm 0.2\%$, $P < 0.05$), proximal convoluted tubule ($2.3 \pm 0.6\%$ *vs* vehicle $1.1 \pm 0.3\%$ *vs* sham $1.2 \pm 0.3\%$, $P < 0.05$) and TAL ($4.7 \pm 0.9\%$ *vs* vehicle $0.6 \pm 0.3\%$ *vs* sham $0.3 \pm 0.2\%$, $P < 0.05$). The frequency of tubular profiles with luminal cast material was also reduced (32.0 ± 1.6 *vs* vehicle $37.0 \pm 1.3\%$, $P = 0.05$). EPO-treated rats subjected to bilateral IR injury exhibited similar histological improvements to the unilateral IR injury model, as well as significantly lower peak plasma creatinine concentrations than their vehicle-treated controls (0.04 ± 0.01 *vs* 0.21 ± 0.08 mmol/l, respectively, $P < 0.05$). EPO had no effect on renal function in sham-operated controls.

Conclusions. The results suggest that, in addition to its well-known erythropoietic effects, EPO inhibits apoptotic cell death, enhances tubular epithelial regeneration and promotes renal functional recovery in hypoxic or ischaemic acute renal injury.

Keywords: apoptosis; hypoxia; kidney failure, acute; kidney tubular necrosis, acute; kidney tubules; mitosis; regeneration

Introduction

Ischaemic acute renal failure (ARF) is a common, serious condition that culminates in patient death in $> 50\%$ of cases [1]. Its underlying pathophysiological mechanisms are complex, but primarily stem from damage to the proximal straight tubule (PST) and thick ascending limb (TAL) of Henle's loop. Numerous studies have attempted to modify the outcome of ARF by either ameliorating tubular injury (e.g. RGD peptides, anaritide, dopamine or mannitol) or promoting tubular regeneration (e.g. hepatocyte growth factor, epidermal growth factor or insulin-like growth factor-I) [2–6]. However, to date, none of these agents has made any appreciable clinical impact on the high mortality and morbidity rates associated with this condition.

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Recently, erythropoietin (EPO), a widely available treatment for uraemic anaemia, was found to markedly attenuate experimental ischaemic brain injury via inhibition of apoptosis and/or augmentation of cellular regeneration [7–11]. Since EPO receptors are expressed on renal tubular epithelial cells [12], it is possible that the systemic administration of EPO may also provide protection against acute renal damage. Indeed, EPO has been found to ameliorate toxic renal injury caused by cisplatin [13,14]. However, it is critical to establish whether EPO exerts similar renoprotective action in ischaemia–reperfusion renal injury, since this is the most common cause of ARF in the community [15]. The aim of the present study therefore was to determine whether EPO is renoprotective in both *in vitro* and *in vivo* models of ischaemic ARF.

Materials and methods

Cell culture

The method for primary culture of human proximal tubule cells (PTCs) is described in detail elsewhere [16,17]. Segments of macroscopically and histologically normal renal cortex (5–10 g) were obtained aseptically from human nephrectomy specimens. Informed consent was obtained prior to each operative procedure, and the use of human renal tissue for primary culture was reviewed and approved by the Princess Alexandra Hospital Research Ethics Committee. Renal cortical tissue was dissected from the medulla, minced, digested with collagenase, and passed through a 100 μm mesh. Filtered cells were resuspended in 45% Percoll and separated into four distinct bands by isopycnic ultracentrifugation. The lowermost band was removed for PTC culture. PTCs were resuspended in serum-free, hormonally defined Dulbecco's modified Eagle's medium (DMEM)/F-12 (Trace Scientific, Melbourne, Australia), supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), human transferrin (5 μg/ml), bovine insulin (0.87 μmol/l), hydrocortisone (0.05 μmol/l), epidermal growth factor (10 ng/ml; Collaborative Research Inc, Bedford, MA), prostaglandin E_1 (50 μmol/l; Sigma, St Louis, MI), selenium (50 nmol/l) and tri-iodothyronine (5 pmol/l) (Sigma). The tubular fragments were plated at a density of 1.5 mg pellet/cm^2 (~5000 fragments/cm^2) in 75 cm^2 flasks. Cells were incubated in a humidified atmosphere of 95% air/5% CO_2 at 37°C and medium changed every 48 h. Confluence was reached ~10 days after plating. Cells were passaged using dispase II (Roche Diagnostics, Indianapolis, IN) and frozen cultures stored in liquid nitrogen.

Cytological examination of PTC preparations from all donors failed to reveal any evidence of cellular atypia. The morphological, biochemical and functional characteristics of these cells have been studied previously in this laboratory and found reproducibly to exhibit the features of PTCs *in vivo* [16]. Fibroblast, mesangial and endothelial cell contamination was negligible, as evidenced by the uniform negative staining of cultures for vimentin, desmin and factor VIII, respectively.

In vitro experimental protocol

All experiments were performed on passage 2 PTCs cultured in 48-well tissue culture plates. Cells were made quiescent by two washes followed by incubation for 24 h in basic medium (DMEM/F12 containing 5 μg/ml transferrin). Cells were incubated with basic medium supplemented with various concentrations of recombinant human EPO (6.25–400 IU/ml; Janssen-Cilag, Sydney) or vehicle [phosphate-buffered saline (PBS) containing 5 mg/ml glycine and 0.13 mg/ml polysorbate 80; a gift from Janssen-Cilag] and simultaneously exposed for 5 or 24 h to 1 or 21% O_2, using a hypoxic tissue culture incubator. In separate hypoxia–reoxygenation experiments, PTCs were exposed to 1% O_2 for 16 h followed by 21% O_2 for 24 h. Cellular apoptosis, proliferating cell nuclear antigen (PCNA) expression, thymidine incorporation and lactate dehydrogenase (LDH) release were then measured.

Apoptosis measurement

For *in vitro* apoptosis measurements, cells were grown on 13 mm Thermanox coverslips (Nalge Nunc International, Napersville, IL) in 24-well plates and treated or not with EPO, as described above, for the indicated times. At the end of the study, cells were washed with PBS, fixed in cold 4% buffered paraformaldehyde for 10 min and stored in PBS at 4°C until processed. Previously defined morphological criteria were used to identify apoptotic cells [18]. These characteristics included cellular rounding and shrinkage, eosinophilic cytoplasm, nuclear chromatin compaction (especially along the nuclear envelope in a crescentic manner), membrane-bound cellular blebbing and formation of apoptotic bodies that may appear in the tubular lumina or be phagocytosed by intrinsic renal cells or invading macrophages.

In situ end labelling (ISEL) nuclear positivity was used for quantification of apoptosis *in vitro* and *in vivo* [19]. Following the methods of Ansari and colleagues [20], paraffin sections were dewaxed, rehydrated, digested in 0.5% pepsin in 0.1 M HCl for 15 min, washed, then labelled using the Klenow fragment of DNA polymerase I (Pharmacia, Australia) and biotin-labelled dUTP (Boehringer-Mannheim, Australia). The reaction was terminated by washing in water, endogenous peroxidase activity was blocked with 0.1% H_2O_2, and biotin-labelled nuclei visualized with horseradish peroxidase-conjugated avidin developed in 3′,3′-diaminobenzidine (DAB) and counterstained with haematoxylin. Negative controls had no Klenow polymerase, and positive controls were rat renal sections from other experiments in which high levels of ISEL were known to correlate with high levels of apoptosis assessed morphologically. A similar method was used for the fixed cultures, except that no pre-treatment with pepsin was carried out. ISEL-positive nuclei were counted at ×400 microscope magnification as the percentage of total cells in a 10 × 10 eyepiece grid.

Immunohistochemistry

Immunohistochemical staining of PTC for PCNA, an auxillary protein to DNA polymerase δ, was used as a tool for measuring cellular proliferation. Sections were deparaffinized and rehydrated, before staining with the peroxidase–anti-peroxidase method. Non-specific binding of peroxidase or antibodies was blocked with 0.3% hydrogen peroxide (H_2O_2) in methanol, followed by incubation in diluted normal swine serum. The PCNA monoclonal antibody

(PC10, Oncogene Sciences, 1:50 dilution) was applied at 4°C overnight, followed by buffer washes, and the secondary antibody at a dilution of 1:400 in Tris buffer, followed by the ABC–peroxidase reaction for 30 min. The chromogen was DAB in 0.01% H_2O_2, applied for 2–5 min. Sections were lightly counterstained with haematoxylin and then dehydrated to xylene and mounted in Depex.

DNA synthesis

Tritiated thymidine incorporation into cellular DNA, an index of DNA synthesis, was measured according to a previously described method [21]. For the final 4 h of each experiment, 4 μCi (0.15 MBq) of [methyl-^{3}H]thymidine (Amersham-Pharmacia-Biotech, Uppsala, Sweden) was added for each ml of medium. Cells were then washed twice with ice-cold PBS, three times with ice-cold 10% trichloroacetic acid for 20 min, and once with methanol. Monolayers were allowed to dry and then dissolved in 300 μl of 0.3 M NaOH containing 1% SDS. Aliquots of cell lysate were taken for liquid scintillation counting in a β-counter. Results were corrected for cellular protein content. The protein content of the cell lysate was measured by a commercially available protein assay (BioRad, Hercules, CA) using bovine serum albumin as the standard.

Cell viability

PTC viability was assessed using a cytotoxicity detection kit (Roche) which measures LDH release into the culture medium. The manufacturer's protocols were followed.

Ischaemia–peperfusion (IR) renal injury model

Mature Sprague–Dawley rats (200–220 g, $n = 5$–6 per group) were administered either recombinant human EPO (5000 U/kg) or vehicle by intraperitoneal injection. The dose of EPO used was based on that found to be maximally effective in previous studies of acute ischaemic brain injury [7–9]. At 30 min post-injection, the rats underwent either sham operation (controls) or unilateral or bilateral renal artery occlusion for 30 min followed by reperfusion for either 24 or 48 h. At the end of this period, aortic blood samples were collected for determination of urea, creatinine, sodium, LDH and haematocrit. Urine was also collected for measurement of urea, creatinine and sodium. Both kidneys were removed, bisected in an equatorial plane, fixed in buffered formalin at 4°C and prepared routinely for histology and immunohistochemistry. ISEL staining was performed to evaluate apoptosis. Immunohistochemical staining for PCNA was used as a tool for quantitating proliferation.

The percentages of epithelial cells in the proximal convoluted tubule (PCT), PST, distal convoluted tubule and TAL of Henle's loop demonstrating apoptosis, necrosis, mitosis or PCNA staining were determined by counting a minimum of 1000 cells and 10 high power (×400) microscope fields per kidney per experimental condition. The proportion of tubular profiles containing luminal cast material was also measured by assessing a minimum of 40 tubular profiles per section.

Statistical analysis

All *in vitro* studies were performed in triplicate from PTC cultures obtained from at least three separate human donors. Each experiment contained internal controls originating from the same culture preparation. For the *in vivo* studies, each study group consisted of 5–6 rats. Results are expressed as mean ± SEM. Statistical comparisons between groups were made by analysis of variance (ANOVA). Pairwise multiple comparisons were made by Fisher's protected least-significant differences test. Analyses were performed using the software package, Statview version 5.0 (Abacus Concepts Inc., Berkeley, CA). P-values < 0.05 were considered significant.

Results

EPO inhibits hypoxia-induced apoptosis in human PTCs

Exposure of human PTCs to hypoxia for 24 h resulted in a significant increase in apoptotic cell death, which was completely abrogated by EPO (200 IU/ml) (Figure 1). EPO did not have any significant effects on PTC apoptosis under normoxic conditions. The anti-apoptotic effect of EPO on hypoxic PTC was dose dependent (vehicle 2.5 ± 0.5% *vs* 25 IU/ml EPO 1.8 ± 0.4% *vs* 200 IU/ml EPO 0.9 ± 0.2%, $P < 0.05$) and was detectable as early as 5 h (vehicle 0.6 ± 0.2% *vs* 25 IU/ml EPO 0.2 ± 0.1% *vs* 200 IU/ml EPO 0.2 ± 0.1%, $P < 0.05$). At the highest concentrations tested (200 and 400 IU/ml), EPO also stimulated thymidine incorporation by PTCs exposed to hypoxia and to hypoxia followed by reoxygenation (Figure 2). EPO did not significantly affect LDH release by PTCs at any concentration or under any conditions tested (data not shown).

EPO enhances tubular epithelial regeneration and inhibits apoptosis in unilateral IR renal injury in rats

Compared with control (sham-operated) animals, rats subjected to unilateral IR injury demonstrated

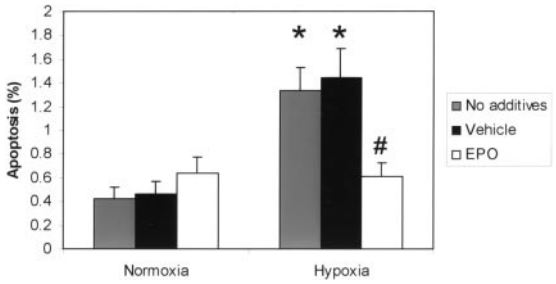

Fig. 1. EPO reduces hypoxia-induced apoptosis in human PTCs. Confluent, quiescent, passage 2 human PTCs were incubated for 24 h under normoxic (21% O_2) or hypoxic (1% O_2) conditions in the presence or absence of vehicle or EPO (200 IU/ml). Apoptosis was assessed by ISEL and expressed as a percentage of the total number of cells. Data represent the mean ± SEM for three separate experiments, each performed in triplicate. *$P < 0.05$ *vs* all normoxic groups; #$P < 0.05$ *vs* the other two hypoxic groups.

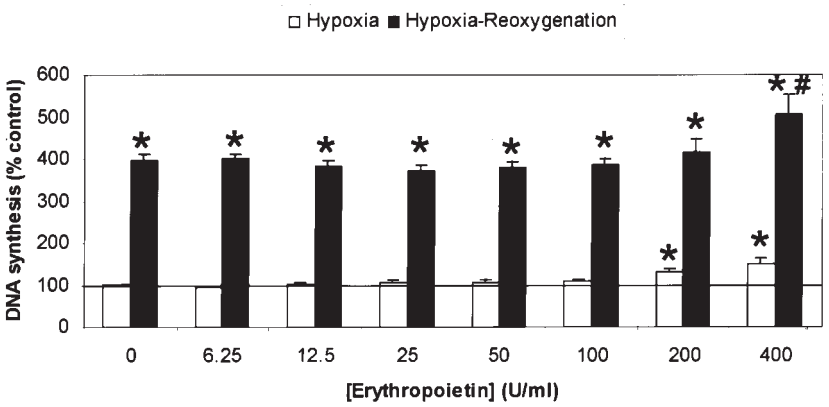

Fig. 2. EPO stimulates PTC mitogenesis. Confluent, quiescent, passage 2 human PTCs were incubated with 21% O_2 for 16 h followed by 1% O_2 for 24 h (hypoxia) or 1% O_2 for 16 h followed by 21% O_2 for 24 h (hypoxia–reoxygenation) in the presence or absence of vehicle or various concentrations of EPO (6.25–400 IU/ml). DNA synthesis was assessed by thymidine incorporation and the results were expressed as a percentage of vehicle-treated hypoxic controls. Data represent the mean ± SEM for two separate experiments, each performed in triplicate. *$P < 0.05$ *vs* vehicle-treated hypoxic control; #$P < 0.05$ *vs* vehicle-treated PTCs exposed to hypoxia–reoxygenation.

significant derangements in renal histology within 24 h, including increased PST necrosis (14.8 ± 1.9% *vs* 0 ± 0% of tubular cells, $P < 0.001$), outer medullary TAL apoptosis (6.5 ± 2.2% *vs* 0.15 ± 0.15%, $P < 0.05$), outer medullary TAL necrosis (10.4 ± 2.8% *vs* 0 ± 0%, $P = 0.01$) and tubular luminal casts (37.0 ± 1.3% *vs* 0 ± 0% of tubular profiles, $P < 0.001$) (Figure 3). These lesions were even more pronounced after 48 h. Compared with vehicle-treated rats subjected to unilateral renal artery occlusion and reperfusion, EPO-treated rats exhibited a significant increase in TAL mitotic figures within 24 h (EPO 1.1 ± 0.3% *vs* vehicle 0.5 ± 0.2% *vs* EPO-treated sham 0.3 ± 0.3% *vs* vehicle-treated sham 0.2 ± 0.1%, $P < 0.05$) (Figure 3). This effect was still observed at 48 h (EPO 1.5 ± 0.3% *vs* vehicle 0.9 ± 0.2% *vs* EPO-treated sham 0.3 ± 0.2% *vs* vehicle-treated sham 0.3 ± 0.2%, $P < 0.05$). Cortical proximal tubule mitotic figures were not altered by EPO at 24 h (0.3 ± 0.1% *vs* vehicle 0.2 ± 0.1% *vs* EPO-treated sham 0.2 ± 0.1% *vs* vehicle-treated sham 0.2 ± 0.1%, P = NS), but were significantly increased in EPO-treated animals at 48 h (1.1 ± 0.2% *vs* vehicle 0.2 ± 0.1% *vs* EPO-treated sham 0.2 ± 0.1% *vs* vehicle-treated sham 0.2 ± 0.1%, $P < 0.05$). The augmentation of proximal and TAL tubular epithelial proliferation by EPO in IR injury was confirmed by PCNA staining (Figure 4).

Apoptotic cell death induced by unilateral IR injury was significantly abrogated by EPO in the outer medullary TAL at 24 h (Figure 5). Trends towards less apoptosis were observed in both the PCT (EPO 0.0 ± 0.0% *vs* vehicle 0.1 ± 0.1%, P = NS) and PST (EPO 0.5 ± 0.3% *vs* vehicle 0.8 ± 0.3%, P = NS), but these did not achieve statistical significance. The degree of tubule cell necrosis was also not significantly affected by EPO (TAL EPO 9.5 ± 4.1% *vs* vehicle 10.4 ± 2.8%; PCT EPO 3.5 ± 2.3% *vs* vehicle 3.0 ± 1.9%; PST EPO 14.0 ± 2.9% *vs* vehicle 14.8 ± 1.9%). However, the proportion of tubular profiles containing luminal cast material was significantly reduced in EPO-treated animals at 24 h (EPO 32.0 ± 1.6% *vs* vehicle 37.0 ± 1.3%, $P < 0.05$). No or very little (< 0.2%) apoptosis, necrosis or tubular cast material was detected in sham-operated control animals treated with either EPO (5000 U/kg) or vehicle for 24 or 48 h. EPO had no significant effects on any renal histological parameters in controls.

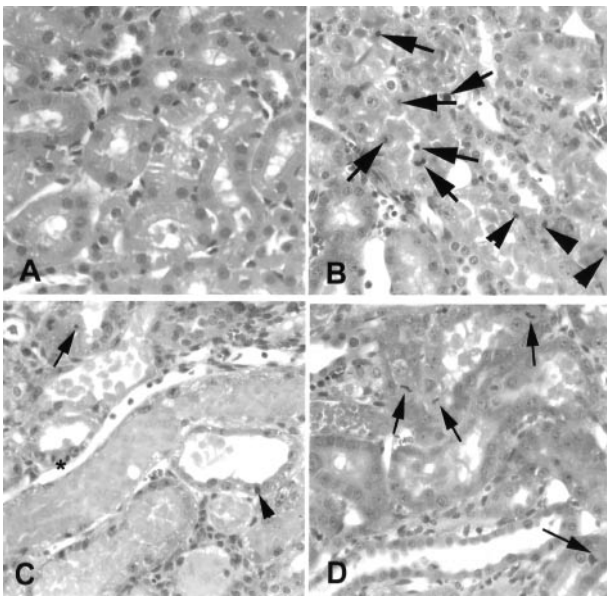

Fig. 3. EPO ameliorates ischaemia–reperfusion injury. Sections of kidneys are demonstrated from control (**A**), unilateral ischaemia–reperfusion at 24 h (**B**), bilateral ischaemia–reperfusion at 24 h (**C**) and bilateral ischaemia–reperfusion with EPO at 24 h (**D**) animals. In (B) and (C), examples of dead cells in the proximal (arrows) and distal (arrow heads) tubular epithelium are indicated. Although the death in (C) appears more extensive than in (B), there remain many flattened and viable cells attached to the basement membranes in most tubules. A mitotic cell in the distal tubular epithelium is shown by an asterisk in (C). In the EPO-treated kidney in (D), many mitotic figures can be seen (arrows) in the damaged epithelium.

Haematocrit was not significantly different between the two groups at either 24 (EPO 38.7 ± 0.7% *vs* vehicle 38.0 ± 0.6%, P = NS) or 48 h (EPO 40.0 ± 1.2% *vs* 38.0 ± 0.6%, P = NS).

EPO ameliorates renal histological damage and functional impairment following bilateral IR renal injury in rats

Compared with the unilateral IR model, rats undergoing bilateral IR demonstrated a similar pattern of renal injury (albeit with somewhat more severe PST injury) and a similar response to EPO. Compared with control (vehicle-treated and sham-operated) animals, rats subjected to bilateral IR injury demonstrated significant derangements in renal histology within 24 h, including increased PST necrosis (47.6 ± 4.0% *vs* 0.0 ± 0.0% of tubular cells, $P < 0.001$), TAL apoptosis (3.7 ± 0.6% *vs* 0.1 ± 0.2%, $P < 0.01$), TAL necrosis (11.4 ± 1.2% *vs* 0.0 ± 0.0%, $P < 0.001$) and medullary tubular casts (67 ± 4% *vs* 0 ± 0%, $P < 0.001$). EPO-treated rats exhibited significant increases in mitotic figures at 24 h compared with all other groups in both the TAL (EPO 2.8 ± 0.3% *vs* vehicle 2.0 ± 0.3% *vs* EPO-treated sham 0.1 ± 0.1% *vs* vehicle-treated sham 0.2 ± 0.2%, $P < 0.01$) and cortical proximal tubule (EPO 1.5 ± 0.2% *vs* vehicle 0.5 ± 0.1% *vs* EPO-treated sham 0.4 ± 0.3% *vs* vehicle-treated sham 0.2 ± 0.2%, $P < 0.01$). These changes were still observed at 48 h (TAL EPO 3.9 ± 0.1% *vs* vehicle 2.7 ± 0.4% *vs* EPO-treated sham 0.2 ± 0.1% *vs* vehicle-treated sham 0.2 ± 0.2%, $P < 0.01$; cortical proximal tubule EPO 1.3 ± 0.3% *vs* vehicle 0.4 ± 0.1% *vs* EPO-treated sham 0.2 ± 0.1% *vs* vehicle-treated sham 0.2 ± 0.1%, $P < 0.001$), but were less pronounced by 4 days (TAL EPO 1.5 ± 0.1% *vs* vehicle 1.4 ± 0.2% *vs* EPO-treated sham 0.1 ± 0.1% *vs* vehicle-treated sham 0.1 ± 0.2%, $P < 0.01$; cortical proximal tubule EPO 0.8 ± 0.2% *vs* vehicle 0.3 ± 0.1% *vs* EPO-treated sham 0.2 ± 0.1% *vs* vehicle-treated sham 0.2 ± 0.1%, $P < 0.05$). Apoptotic cell death in the outer medullary TAL was reduced similarly by EPO compared with vehicle at 24 h (EPO 1.8 ± 0.2% *vs* vehicle 3.7 ± 0.6% *vs* EPO-treated sham 0.0 ± 0.0% *vs* vehicle-treated sham 0.1 ± 0.2%, $P < 0.001$), 48 h (EPO 0.9 ± 0.2% *vs* vehicle 3.3 ± 0.5% *vs* EPO-treated sham 0.0 ± 0.0% *vs* vehicle-treated sham 0.2 ± 0.2%, $P < 0.001$) and 4 days following bilateral IR injury (EPO 0.3 ± 0.1% *vs* vehicle 0.8 ± 0.2% *vs* EPO-treated sham 0.0 ± 0.0% *vs* vehicle-treated sham 0.0 ± 0.0%, $P < 0.01$). EPO had no significant effect on TAL apoptosis in sham-operated rats. No differences were seen in proximal tubule cell apoptosis between EPO- and vehicle-treated rats following IR injury, but overall rates of apoptotic cell death were generally low in this segment (< 0.5%). PCT necrosis was significantly reduced in EPO-treated rats at 48 h (EPO 2.2 ± 0.5% *vs* vehicle 4.5 ± 0.9% *vs* EPO-treated sham 0.0 ± 0.0% *vs* vehicle-treated sham

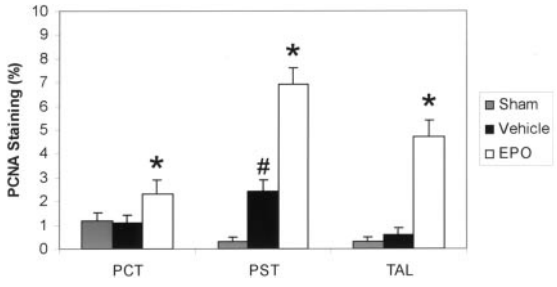

Fig. 4. EPO augments tubular epithelial proliferation in ischaemia–reperfusion injury. Adult Sprague–Dawley rats were administered i.p. vehicle or EPO (5000 U/kg) 30 min prior to sham operation (controls) or unilateral renal artery occlusion followed by reperfusion for 24 h ($n = 5$ per group). Histological sections were stained for PCNA, and positively stained cells were expressed as a percentage of the total number of cells examined. A minimum of 1000 cells and 10 high power (×400) microscope fields were counted per animal per experimental condition for mitosis. $^{*}P < 0.05$ *vs* vehicle-treated animals and sham-operated control. $^{\#}P < 0.05$ *vs* sham-operated controls. PCT, proximal convoluted tubule; PST, proximal straight tubule; TAL, thick ascending limb of Henle's loop.

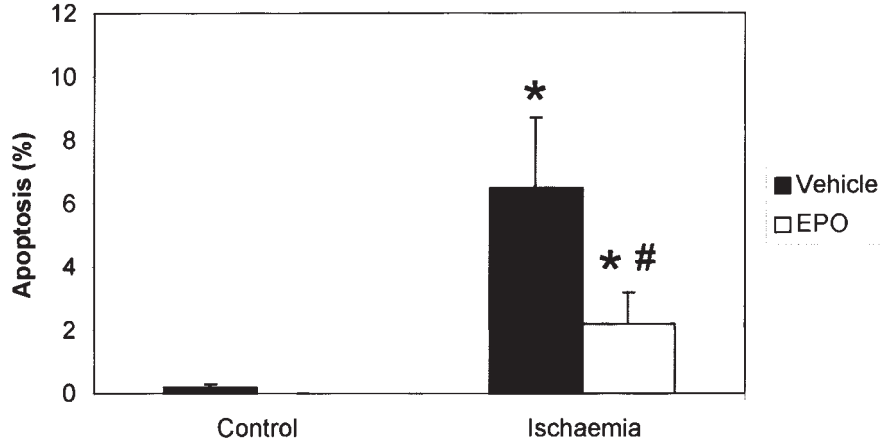

Fig. 5. EPO inhibits outer medulla TAL apoptosis in ischaemia–reperfusion injury. Adult Sprague–Dawley rats were administered i.p. vehicle or EPO (5000 U/kg) 30 min prior to sham operation (controls) or unilateral renal artery occlusion followed by reperfusion for 24 h ($n = 5$ per group). Histological sections of outer medullary TAL were evaluated for apoptosis by ISEL and expressed as a percentage of the total number of cells examined. A minimum of 1000 cells and 10 high power (×400) microscope fields were counted per animal per experimental condition. $^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* vehicle.

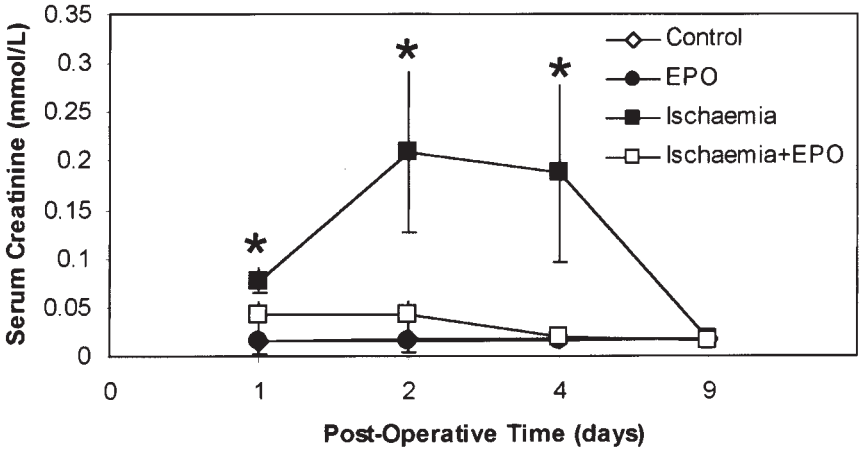

Fig. 6. EPO ameliorates renal functional impairment following bilateral ischaemia–reperfusion injury. Sprague–Dawley rats were administered i.p. vehicle or EPO (5000 U/kg) 30 min prior to sham operation (controls) or bilateral renal artery occlusion followed by reperfusion for 24 h. Aortic blood samples were collected for determination of serum creatinine concentration at day 1, 2, 4 or 9 following ischaemia–reperfusion injury ($n = 6$ per group for each time period). *$P < 0.05$ *vs* vehicle-treated, sham-operated control.

$0.0 \pm 0.0\%$, $P < 0.05$), but this difference was not observed at any other time point. By 48 h, EPO-treated rats also displayed significantly fewer casts in outer medullary (EPO $44 \pm 3\%$ *vs* vehicle $67 \pm 4\%$ *vs* EPO-treated sham $0 \pm 0\%$ *vs* vehicle-treated sham $0 \pm 0\%$, $P < 0.05$) and cortical tubular profiles (EPO $6 \pm 2\%$ *vs* vehicle $37 \pm 8\%$ *vs* EPO-treated sham $0 \pm 0\%$ *vs* vehicle-treated sham $0 \pm 0\%$, $P < 0.001$). No or very little (?0.2%) apoptosis, necrosis, mitosis or tubular cast material was detected in sham-operated control animals treated with either EPO (5000 U/kg) or vehicle for 24, 48 or 96 h.

EPO administration prior to bilateral IR renal injury in rats significantly abrogated subsequent renal functional impairment (Figure 6). Plasma creatinine elevations peaked at day 2 following bilateral IR injury and were significantly lower in EPO- compared with vehicle-treated rats (EPO 0.04 ± 0.01 *vs* vehicle 0.21 ± 0.08 mmol/l, $P < 0.05$). EPO-treated rats subjected to bilateral IR injury had peak plasma creatinine concentrations that were not significantly different from those of sham-operated controls (EPO-treated IR injury 0.04 ± 0.01 *vs* EPO-treated sham 0.02 ± 0.00 *vs* vehicle-treated sham 0.02 ± 0.00 mmol/l, $P =$ NS). Similar results were seen for plasma urea concentrations, which also peaked on day 2 (EPO-treated IR injury 12.6 ± 3.0 *vs* vehicle-treated IR injury 40.6 ± 10.9 mmol/l *vs* EPO-treated sham 4.1 ± 0.6 *vs* vehicle-treated sham 4.4 ± 0.1 mmol/l, respectively, $P < 0.05$). EPO had no effect on renal function in sham-operated controls. No significant differences were seen between EPO- or vehicle-treated rats subjected to bilateral IR injury with respect to plasma LDH concentration, fractional sodium excretion or body weight (data not shown).

Discussion

In this study, EPO was found to have significant anti-apoptotic and mitogenic effects in human PTCs exposed to hypoxia *in vitro*. Cellular regeneration following hypoxia and subsequent reoxygenation was also augmented by EPO. These results were extended in an IR animal model of ARF by additionally demonstrating that EPO administration enhanced proximal and TAL tubular epithelial regeneration, ameliorated tubular cast formation and inhibited TAL apoptosis (at 24 h). These EPO-induced improvements in the histological appearances of IR injury were accompanied by significant amelioration of renal functional impairment following bilateral IR injury.

Although there have been no previous published studies of the effect on EPO on tubular cellular recovery following acute ischaemic renal injury, a recent investigation by Nemoto and associates [22] found that rats treated with EPO (3000 U/kg) prior to severe IR renal injury enjoyed superior survival rates compared with controls. No changes were seen with respect to serum creatinine levels, but the follow-up was relatively short (3 days) and the number of animals studied was small ($n = 3$ per group). Moreover, the haematocrits of EPO-treated animals were significantly increased after 48 h, thereby making it difficult to determine whether the mortality benefitis of EPO were related to anaemia correction or to other actions.

Two studies in a model of toxin-induced ARF (cisplatin nephrotoxicity) have supported our findings that EPO enhances histological and functional recovery. Bagnis *et al.* [13] observed that EPO administration (100 U/kg/day i.p. for 9 days) was associated with a significantly greater number of PCNA-positive cells in the cortical and corticomedullary regions of rat kidneys following cisplatin exposure. These histological appearances, similar to those of our study, suggested enhanced tubular cell regeneration and were accompanied by significantly higher inulin clearance rates at day 9. Although apoptosis was not studied, the authors hypothesized that EPO may have directly acted as a growth factor on tubular cells in the cortex and outer medulla. Vaziri *et al.* [14] similarly reported enhanced

renal cortical thymidine incorporation in EPO-treated rats with acute cisplatin tubulopathy. The beneficial effect appeared to be independent of haemoglobin levels, since similar results were also observed in EPO-treated rats subjected to daily phlebotomies.

In the present study, the beneficial effects of EPO on ischaemic renal histopathology were demonstrable in the absence of a significant change in haemoglobin levels, suggesting that the therapeutic effects of EPO in IR renal injury were independent of its haemopoietic actions. It was also unlikey that EPO engendered any favourable haemodynamic changes in view of the fact that acute and chronic studies of EPO therapy in animal models have not reported any measurable changes in renal blood flow or other haemodynamic parameters during the first few weeks of regular administration [23].

Since EPO receptors have recently been identified in human and rat kidney tissue (cortex, medulla and papilla) and in renal cell lines (PTCs, mesangial and collecting duct cells) [12], the possibility that EPO exerted direct renoprotective effects on tubular epithelial cells was additionally investigated in this study using primary cultures of human PTCs. In keeping with the findings of Westenfelder *et al*. [12], EPO stimulated PTC mitogenesis. This occurred under conditions of both hypoxia and hypoxia–reoxygenation. Similarly, tubular epithelial regeneration following IR renal injury in rats was also augmented by prior EPO administration. EPO-treated PTCs additionally demonstrated a marked reduction in hypoxia-induced apoptosis compared with cells incubated with vehicle. A trend towards decreased proximal tubule apoptosis was further observed in the IR injury model, although this reduction did not achieve statistical significance. The reason for the latter negative finding was most likely to be attributable to the relatively minor degree of apoptosis observed in PTCs compared with TAL cells in this particular *in vivo* model, but could conceivably have reflected a difference between examining single cell *vs* whole animal effects.

The cytoprotective and mitogenic effects of EPO on proximal and distal tubular epithelial cells may explain the observation of a modest reduction in tubular cast formation in EPO-treated rats following IR renal injury. Decreased cell sloughing and cast formation may have, in turn, contributed to a reduction in intratubular obstruction and a subsequent amelioration of renal functional impairment.

Other studies have also shown that EPO exerts significant mitogenic and/or anti-apoptotic actions in non-renal and non-erythroid cells, including endothelial cells [24], gastric mucosal cells [25], myoblasts [26], Leydig cells [27], vascular smooth muscle cells [28], gynaecological cancers [29] and cortical neurons [30]. There is a substantial body of evidence that systemic EPO administration (5000 U/kg) markedly attenuates ischaemic cerebral injury by up to 75% [7–11], as well as experimental brain damage complicating blunt trauma, autoimmune encephalomyelitis and neurotoxin exposure [9,31]. The proposed mechanism responsible for the observed cytoprotective effects of EPO in the brain is Jak2 phosphorylation of IκB and subsequent NF-κB-dependent transcription of neuroprotective genes [32]. Additional potential protective mechanisms that might be activated downstream from the EPO–receptor–Ras–mitogen-activated protein kinase and EPO–receptor–phosphatidylinosine 3 kinase interactions include antioxidation [8], direct neurotrophic effects [8], angiogenesis [8], activation (phosphorylation) of Bcl-xl [33,34] and inhibition of nitric oxide (NO) production [10,35]. Similar mechanisms could conceivably be operating in the renoprotective actions of EPO in ischaemic ARF. The EPO doses used in the present study were based on those previously demonstrated to provide effective neuroprotection. However, since there is no equivalent of the blood–brain barrier in the kidney, it is possible that renoprotection could be afforded by considerably lower dosages, thereby reducing therapy costs. Nevertheless, it should be noted that the doses of EPO needed to promote maximal anti-apoptotic and mitogenic effects *in vitro* were quite high (200–400 IU/ml).

In addition to the issue of optimal dosing, the present study did not answer the question of whether administering EPO some time after the noxious renal insult would still afford some degree of renoprotection. Extrapolation from trials of EPO administration to rats with ischaemic brain injury suggests that the drug may still be capable of ameliorating cell injury when injected some time after the injurious event [9]. However, even if EPO was only effective at preventing acute renal injury when administered prior to the inciting event, there are many common clinical situations where pre-emptive EPO treatment could potentially prove to be of enormous clinical benefit. Such clinical scenarios include renal transplantation, aortic or coronary artery surgery, and radiographic contrast administration to diabetic or chronic renal failure patients.

In conclusion, the present study demonstrated that EPO pre-treatment had a direct cytoprotective action on human PTCs *in vitro* and was capable of augmenting histological and functional recovery from ischaemic acute renal injury *in vivo*. This raises the possibility that EPO may have potential clinical applications as a novel renoprotective agent for patients at risk of ischaemic ARF. Further studies are warranted to ascertain the lowest dose and the latest dose timing which still permit effective renoprotection.

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