

# Erythropoietin Protects the Kidney against the Injury and Dysfunction Caused by Ischemia-Reperfusion

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**Abstract.** Erythropoietin (EPO) is upregulated by hypoxia and causes proliferation and differentiation of erythroid progenitors in the bone marrow through inhibition of apoptosis. EPO receptors are expressed in many tissues, including the kidney. Here it is shown that a single systemic administration of EPO either preischemia or just before reperfusion prevents ischemia-reperfusion injury in the rat kidney. Specifically, EPO (300 U/kg) reduced glomerular dysfunction and tubular injury (biochemical and histologic assessment) and prevented

caspase-3, -8, and -9 activation *in vivo* and reduced apoptotic cell death. In human (HK-2) proximal tubule epithelial cells, EPO attenuated cell death in response to oxidative stress and serum starvation. EPO reduced DNA fragmentation and prevented caspase-3 activation, with upregulation of Bcl-X<sub>L</sub> and XIAP. The antiapoptotic effects of EPO were dependent on JAK2 signaling and the phosphorylation of Akt by phosphatidylinositol 3-kinase. These findings may have major implications in the treatment of acute renal tubular damage.

Erythropoietin (EPO) is the major regulator of proliferation and differentiation of erythroid progenitor cells through its antiapoptotic actions (1). EPO gene expression is under the control of the oxygen-sensitive transcription factor hypoxia-inducible factor (HIF-1), which consists of the regulatory subunit HIF-1 $\alpha$  and the constitutively expressed subunit HIF-1 $\beta$ . Low oxygen tension adverts enzymatic prolyl-residue hydroxylation by prolyl-4-hydroxylase, which, in normoxia, serves as a signal for polyubiquitination and proteosomal degradation, thereby preventing von-Hippel-Lindau (VHL)-dependent HIF degradation, leading to nuclear accumulation of HIF-1 (2). HIF-1 controls the expression of several cytokines that mediate the adaptive response to ischemia, including vascular endothelial growth factor and glucose metabolism. The prolyl-4-hydroxylase requires iron as a co-factor, and cobalt mimics the effect of hypoxia on HIF-1 $\alpha$  activation (3). Cobalt administration to rats caused upregulation of HIF-dependent proteins, including EPO, and diminished the degree of renal injury caused by ischemia-reperfusion (I/R), suggesting the

HIF/EPO pathway may play an important role in ischemic preconditioning (4). EPO is upregulated in the brain and spinal cord after hypoxic stimuli and protects neurones against ischemic or oxidative injury *in vivo* and *in vitro* (5,6). The neuroprotective effects of EPO are dependent on EPO receptor-mediated JAK2 phosphorylation and NF- $\kappa$ B-dependent transcription of antiapoptotic genes, including endogenous inhibitors of apoptosis XIAP and cIAP-2 (7). In the retina, EPO upregulation is essential for hypoxic preconditioning *via* HIF-1 $\alpha$  stabilization. The systemic administration of recombinant EPO also reduces the degree of retinal apoptosis induced by high-intensity light insult (8). EPO receptor-mediated intracellular signaling may involve nuclear translocation of the transcription factor NF- $\kappa$ B and phosphorylation of Akt (protein kinase B) by phosphatidylinositol 3-kinase (PI3K) and the extracellular signal-regulated kinases 1 and 2. Although the peritubular fibroblasts are the major adult site for EPO production, the presence of the EPO receptor in many cell types in the kidney was only recently reported. The EPO receptor is present on proximal tubule epithelial cells, mesangial cells, and the glomerulus (9).

Renal ischemia, whether caused by shock or during surgery or transplantation, is a major cause of acute renal failure. Although reperfusion is essential for the survival of ischemic tissue, there is good evidence that reperfusion itself causes additional cellular injury. Reperfusion initiates a complex and interrelated sequence of events that results in injury and the eventual death of renal cells as a result of a combination of both apoptosis and necrosis (10,11). The degree of cellular

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ATP and GTP depletion plays a crucial role in the determination of mode of cell death. Moderate ATP and GTP depletion favors apoptotic cell death and is associated with DNA fragmentation, p53 induction, and increased cysteine protease activity, whereas profound ATP depletion results in necrosis (12). Apoptotic cell death has been documented in animal models and human biopsies after renal I/R (13), and inhibition of apoptotic signaling and cell death ameliorates the associated injury and inflammation in a murine model (14). Several studies have documented caspase-3 activation after I/R injury in the kidney and in posthypoxic isolated proximal tubules (15,16). Renal I/R in animal models causes mitochondrial swelling and loss of membrane potential, which contributes to both necrotic and apoptotic forms of cell death. *In vivo*, DNA fragmentation and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining, although not wholly specific for apoptotic cell death, are significantly elevated after 2 h of reperfusion (17,18). Ischemic renal injury is associated with an increase in the expression of both the antiapoptotic Bcl-2 family of proteins, Bcl-2 and Bcl-X<sub>L</sub>, and the proapoptotic proteins Bad, p53, FADD, and Bak in the distal and proximal tubules during the first 24 h, with the net effect determining the severity of injury and dysfunction (19).

Here, we report that EPO protects the rat kidney in a model of severe I/R injury, with inhibition of caspase activation and reduced apoptotic cell death. Thus, the systemic administration of recombinant EPO may reduce the renal injury and dysfunction caused by I/R in humans.

## Materials and Methods

### Renal I/R

The model of renal I/R injury in the anesthetized rat and the surgical procedures involved were similar to those described previously (20). Briefly, this study was carried out using 86 male Wistar rats (Tuck, Rayleigh, Essex, UK) that weighed 220 to 300 g and received standard diet and water *ad libitum*. Animals were cared for in accordance with the Home Office Guidance in the Operation of the Animals (Scientific Procedures) Act 1986 (HMSO, London, UK). All rats were anesthetized with sodium thiopentone (Intraval Sodium, 85 mg/kg intraperitoneally; Rhone Merieux, Essex, UK), and anesthesia was maintained by supplementary infusions of sodium thiopentone. The animals were placed onto a thermostatically controlled heating mat (Harvard Apparatus, Kent, UK), and body temperature was maintained at  $38 \pm 1^\circ\text{C}$  by means of a rectal probe attached to a homeothermic blanket. A tracheotomy was performed to maintain airway patency and to facilitate spontaneous respiration. The right carotid artery was cannulated (PP50, I.D. 0.58 mm; Portex, Kent, UK) and connected to a pressure transducer (Sensonor 840, Horten, Norway) for the measurement of mean arterial BP and derivation of the heart rate from the pulse waveform. The right jugular vein was cannulated (PP25, I.D. 0.40 mm; Portex) for the administration of drugs. A midline laparotomy was performed, and the bladder was cannulated (PP90, I.D. 0.76 mm; Portex). Animals were allowed to stabilize for 30 min before they were subjected to bilateral renal occlusion for 45 min, using artery clips to clamp the renal pedicles. Reperfusion commenced once the artery clips were removed. Occlusion was verified visually by change in the color of the kidneys to a paler shade and reperfusion by a blush. Upon completion of surgical procedures, rats were randomly allocated into the following groups: (1) sham +

saline group ( $n = 12$ ), (2) sham + EPO ( $n = 12$ ), (3) I/R + saline ( $n = 12$ ), (4) I/R + EPO 30 min before commencement of ischemia ( $n = 12$ ), (5) I/R + EPO 5 min before the onset of reperfusion ( $n = 12$ ), and (6) I/R + EPO 30 min following reperfusion ( $n = 10$ ). Sham-operated rats (groups 1 and 2) underwent identical surgical procedures but without bilateral renal clamping and were maintained under anesthesia for the duration of the experiment.

At the end of the reperfusion period (6 h in all groups unless stated), blood (1 ml) samples were collected *via* the carotid artery into tubes that contained serum gel. The samples were centrifuged ( $6000 \times g$  for 3 min) to separate serum from which biochemical parameters were measured. All plasma samples were analyzed within 24 h after collection (Vetlab Services, Sussex, UK). Urine was collected throughout the reperfusion period, and the volume was recorded. Activity of urinary N-acetyl- $\beta$ -D-glucosaminidase (NAG), a specific indicator of tubular damage, was also measured (Clinica Medica e Diagnostico Dr Joaquim Chaves, Lisbon, Portugal).

### Histologic Evaluation and TUNEL Staining

Renal sections were prepared as described previously and used for assessment of renal I/R injury (20). Briefly, kidneys were removed from rats at the end of the experimental period and were cut in a sagittal section into two halves, which were fixed in immersion in 10% (wt/vol) formaldehyde in PBS (0.01 M; pH 7.4) at room temperature for 1 d. After dehydration using graded ethanol, pieces of kidney were embedded in Paraplast (Sherwood Medical, Mahwah, NJ) and cut in fine (8  $\mu\text{m}$ ) sections and mounted on glass slides. Sections were then deparaffinized with xylene, counterstained with hematoxylin and eosin, and viewed under a light microscope (Dialux 22; Leitz, Milan, Italy). One hundred intersections were examined for each kidney, and a score from 0 to 3 was given for each tubular profile: 0, normal histology; 1, tubular cell swelling, brush border loss, and nuclear condensation with up to one third nuclear loss; 2, as for score 1 but greater than one third and less than two thirds tubular profiles showing nuclear loss; and 3, greater than two thirds tubular profile showing nuclear loss. The histologic score for each kidney was calculated by addition of all scores, with a maximum score of 300. Sections were assessed quantitatively for apoptotic nuclei and graded for severity and extent of nuclear changes. TUNEL was performed as described previously, with a modification of the detection system using the ApopTag Plus kit (R&D Systems, Abingdon, UK), which adds an amplification step using streptavidin-biotin-immunoperoxidase staining to increase the sensitivity of the technique (21).

### Cell Culture and Reagents

All reagents were obtained from Sigma-Aldrich Co. (Poole, Dorset, UK) unless otherwise stated. HK-2 cells (American Type Culture Collection, Manassas, VA), an immortalized human proximal tubule epithelial cell line, were grown and passaged in 75-cm<sup>2</sup> cell culture flasks that contained DMEM Ham's F12 media (1:1) supplemented with 5% FCS and antibiotics (100 U/ml penicillin G, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 0.25  $\mu\text{g}/\text{ml}$  amphotericin). Subconfluent (80%) HK-2 cells were harvested and seeded into six-well tissue culture plates in 2 ml of growth medium. The cells were allowed to adhere for 18 h in an incubator at  $37^\circ\text{C}$  with 5% CO<sub>2</sub> in 95% air. Immediately before experimental treatments, the medium was replaced with fresh medium. EPO (1 to 100 U/ml) or inhibitors ( $\pm$  vehicle) were added for 60 min preincubation, unless otherwise stated.

### Analysis of DNA Fragmentation and Lactate Dehydrogenase

Monolayers of HK-2 cells were disrupted with a Teflon cell scraper. Cells were centrifuged at  $2000 \times g$  for 5 min, and the pellet was incubated on ice for 30 min in 200  $\mu$ l of lysis buffer (PBS [pH 7.4] containing 10  $\mu$ M digitonin). After centrifugation at  $16,000 \times g$  for 15 min at 4°C, the supernatant was diluted 1:10 with lysis buffer. Diluted lysate (20  $\mu$ l) was assayed in duplicate for microsomal DNA fragments using a commercial ELISA (Cell Death Detection ELISA Plus; Roche Diagnostics, Lewes, UK). Results were expressed as multiples of control corrected to amount of total protein in the lysate (Bradford method).

Lactate dehydrogenase (LDH) release into the supernatant was assayed using a commercial colorimetric method (Cytotoxicity Detection Kit; Roche Diagnostics). Results were expressed as a percentage of total cellular LDH per well measured from cells lysed in 1% Triton-X100 and corrected with appropriate media controls and total protein.

### Western Blot Analysis

For *in vivo* experiments, frozen samples of harvested kidneys were washed in ice-cold PBS and homogenized in ice-cold lysis buffer that contained 25 mmol/L EDTA, 20 mmol/L EGTA, 63.2 mmol/L imidazole-HCl, and 10 mmol/L 2-mercaptoethanol (pH 7.3). After centrifugation at  $16,000 \times g$ , the supernatant was aspirated and protein was quantified by the Bradford method. *In vitro*, cell pellets were washed in ice-cold PBS and incubated for 15 min on ice in modified RIPA buffer (150 mM NaCl, 10 mM Tris, 0.1% SDS, 1.0% NP40, 1.0% sodium deoxycholate, 5 mM EDTA, and 100  $\mu$ M sodium orthovanadate [pH 7.2]). Mitochondria-rich cytosolic fractions were obtained by homogenizing cell pellets in extraction buffer (50 mM HEPES [pH 7.5], 1 M mannitol, 350 mM sucrose, and 5 mM EGTA) followed by centrifugation at  $600 \times g$ . The resulting supernatant was centrifuged at  $11,000 \times g$  for 10 min. Twenty to 40  $\mu$ g of protein per lane was electrophoresed on precast graduated (4 to 12%) NuPAGE SDS gel (Invitrogen, San Diego, CA). Proteins were transferred to polyvinylidene difluoride membranes, which were subsequently blocked with Tris-buffered saline/Tween-20 (TBS-T) containing 5% BSA and then incubated for 3 h with antibodies against caspase-3, Bcl-X<sub>L</sub> (Autogen Biotech, Calne, UK), and XIAP (R&D Systems Europe, Abingdon, UK). After washing, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (diluted 1:2000 in TBS-T/1% BSA) and washed, and then immunoreactivity was visualized using enhanced chemiluminescence.

### Caspase Activity

The activity of caspase-3 was measured using the fluorometric substrate Ac-DEVD-AMC as described previously (22). Fifty micrograms of cellular protein was incubated with 50  $\mu$ M substrate in caspase assay buffer (213.5 mM HEPES [pH 7.5], 31.25% sucrose, and 0.3125% CHAPS) for 1 h, and fluorescence was measured on a microplate reader (Fluostar Galaxy; BMG Laboratory Technologies, Aylesbury, UK), with excitation at 380 nm and emission at 460 nm. For each sample, four replicates were assayed with two replicates that contained 50  $\mu$ M of the caspase-3 inhibitor (Ac-DEVD-CHO) and the remaining pair that contained vehicle (DMSO). The activities of caspase-8 and caspase-9 were determined in the same way, using Ac-IETD-AMC and Ac-LEHD-AMC as respective substrates with Ac-IETD-CHO and Ac-LEHD-CHO as inhibitors, respectively. Fluorescence readings from wells that contained inhibitor were sub-

tracted from total fluorescence, and results were calculated as nmol AMC/min per mg protein (Bradford method).

### Statistical Analyses

All values described in the text and figures are expressed as the mean  $\pm$  SEM for the number of observations. Each data point representing biochemical measurements was obtained from up to 10 to 12 separate animals. For histologic scoring, caspase activity assays, and apoptosis scoring, each data point represents analysis of kidneys taken from six individual animals. Statistical analysis was performed using GraphPad Prism 3.01/Instat 1.1 (GraphPad Software, San Diego, CA). Data were analyzed using one-way ANOVA followed by Dunnett *post hoc* test or Kruskal-Wallis ANOVA for nonparametric data.  $P < 0.05$  was considered significant.

## Results

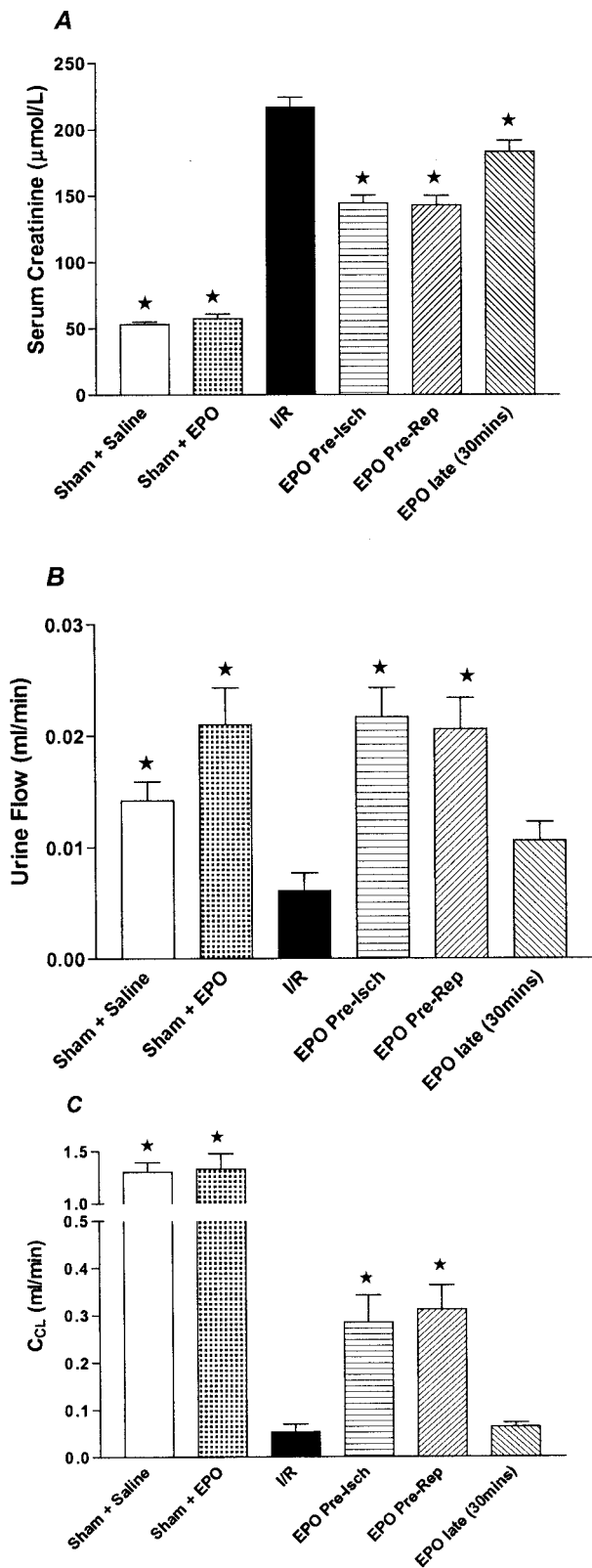
### Effect of EPO on Renal Dysfunction Caused by I/R

In comparison with sham animals, renal I/R produced significant increases in serum, urinary, and histologic markers of renal dysfunction and injury as described in detail below (Figures 1 through 4). Administration of EPO to sham-operated animals did not have any effect on markers of renal function despite an increase in urine flow rate (Figure 1). EPO administration did not have an effect on mean BP in either sham-operated or I/R animals (shown supplementary data, Appendix 1). EPO administration to sham-operated animals had no effect on markers of tubular or reperfusion injury (urinary NAG activity and serum AST; Figure 2), histologic changes (Figure 3), or the number of apoptotic proximal tubular epithelial cells (Figure 4).

Rats that underwent renal I/R exhibited significant increases in the serum concentrations of creatinine compared with sham-operated animals (Figure 1A) and decreases in urine flow and creatinine clearance (Figure 1, B and C), suggesting a significant degree of glomerular dysfunction. In comparison with I/R animals, the administration of a single intravenous bolus of EPO (300 U/kg), either preischemia or just before the onset of reperfusion, produced a significant reduction in serum levels of creatinine (Figure 1A), associated with maintenance of improved urine flow rate and creatinine clearance (Figure 1, B and C). EPO administration 30 min after the onset of reperfusion still produced a significant reduction in serum creatinine (Figure 1A).

### Effect of EPO on Tubular and Reperfusion Injury Caused by Renal I/R

In comparison with values obtained from sham-operated animals, renal I/R produced a significant increase in urinary NAG activity (Figure 2A), consistent with tubular injury. Renal I/R also produced a significant increase in serum AST, a marker of tubular reperfusion injury (Figure 2B). Administration of EPO caused a significant reduction in urinary NAG activity and serum AST levels (Figure 2), suggesting a marked reduction in the tubular and reperfusion injury associated with renal I/R, respectively.



**Figure 1.** Effect of erythropoietin (EPO) on renal dysfunction caused by ischemia-reperfusion (I/R) injury. Serum creatinine levels (A) and urine flow rate (B) were measured as respective biochemical markers of glomerular dysfunction. Creatinine clearance (C) was calculated as ml/min during the reperfusion period. Data represent mean  $\pm$  SEM. ( $\star P < 0.05$ ;  $\star\star P < 0.01$ ). (A) The increase in serum

### Effects of EPO on Histologic Alterations Caused by Renal I/R

Renal I/R caused marked alterations in renal histology compared with kidneys taken from sham-operated animals. Specifically, this included widespread degeneration of tubular architecture, tubular dilation, swelling and necrosis, luminal congestion with loss of brush border, and an infiltration of polymorphonuclear neutrophils (Figure 3, A and B). In contrast, renal sections obtained from animals that were treated with EPO preischemia or prereperfusion demonstrated marked reduction of the histologic features of renal injury (Figure 3C). EPO administration 30 min after the onset of reperfusion was still associated with a significant reduction in injury, but this reduction was less marked than in the other treatment groups (Figure 3D).

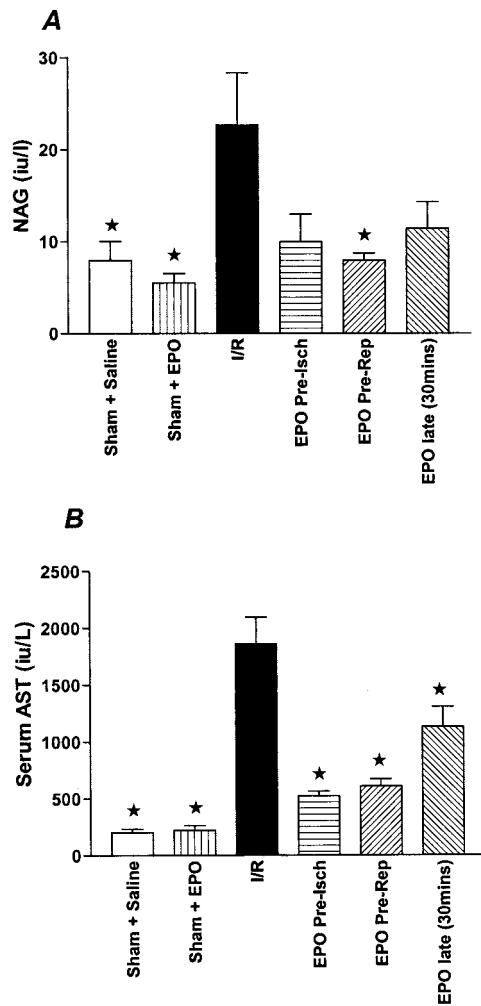
### EPO Administration Reduced Tubular Apoptosis and Necrotic Cell Death

Proximal tubular epithelial cell apoptosis was quantified on renal sections and confirmed by TUNEL staining (data not shown). Kidneys from animals that were subjected to renal I/R showed extensive nuclear changes consistent with apoptotic cell death. In comparison, kidneys from sham-operated animals had no evidence of apoptosis and were negative for TUNEL-positive cells. EPO administration before ischemia and prereperfusion significantly reduced the extent of apoptotic cell death ( $P < 0.05$ ; Figure 4A). EPO administration 30 min after the onset of reperfusion caused only a small reduction in the number of apoptotic cells, but there was a reduction in the degree of necrotic cell death (Figure 4B).

### Effect of EPO on Caspase Activity and Protein In Vivo

Caspase-3 activity (nmol/min per mg protein at 37°C) was significantly increased in the homogenates of kidneys at 6 h in kidneys that were subjected to renal I/R (Figure 5A), when compared with sham-operated animals ( $P < 0.01$ ). The elevation in caspase-3 activity was significantly reduced by EPO administration, either preischemia ( $P < 0.05$ ) or just before reperfusion ( $P < 0.05$ ). EPO treatment 30 min after reperfusion was associated with a nonsignificant reduction in caspase-3 activity, consistent with the higher levels of apoptosis observed in these kidneys (Figure 5A). Caspase-3 protein in kidney tissue, determined by Western blot analysis, showed that a 17-kD band, representing the active caspase-3 subunit,

creatinine after I/R is abrogated by EPO (300 U/kg) administered intravenously either preischemia or prereperfusion. EPO administered 30 min after reperfusion still caused a significant reduction in creatinine concentration ( $P < 0.05$ ). There was no difference between shams and shams that were treated with EPO. (B) EPO-treated shams had a nonsignificant increase in urine flow. I/R was associated with a highly significant reduction in urine flow, which was restored by administration of EPO preischemia and prereperfusion. Late EPO treatment was associated with a much smaller improvement in urine flow. (C) Calculated creatinine clearance was significantly higher in EPO-treated animals except in the late treatment group ( $P < 0.05$ ).



**Figure 2.** Effect of EPO on tubular and reperfusion injury caused by I/R. (A) Urinary N-acetyl- $\beta$ -D-glucosaminidase (NAG) concentration correlates with histologic tubular injury and was significantly increased after I/R ( $P < 0.05$ ). EPO administration reduced urine NAG activity to a similar level as that seen in sham animals ( $P < 0.05$ ). (B) Serum AST, a marker for reperfusion injury, was significantly elevated after I/R. EPO (300 U/kg) administration was associated with a significant reduction in AST independent of timing of administration ( $P < 0.01$  all groups against control, ANOVA).

was markedly increased by renal I/R compared with sham-operated animals. The amount of this caspase-3 subunit was diminished in both early EPO treatment groups (Figure 5B). Immunohistochemical staining using a specific antibody to the cleaved active fragment of caspase-3 confirmed these observations. There was widespread positive cytoplasmic and perinuclear staining in both cortical and medullary tubules after I/R, and the severity and distribution of staining was greatly reduced in animals that received EPO either preischemia or prereperfusion but only partially when EPO was administered later in reperfusion (Figure 5C). EPO treatment was associated with significant reduction in the increase in both caspase-8 and caspase-9 activity observed after I/R (supplementary data, appendix 2).

### EPO Prevents Oxidative Stress–Induced Cell Death

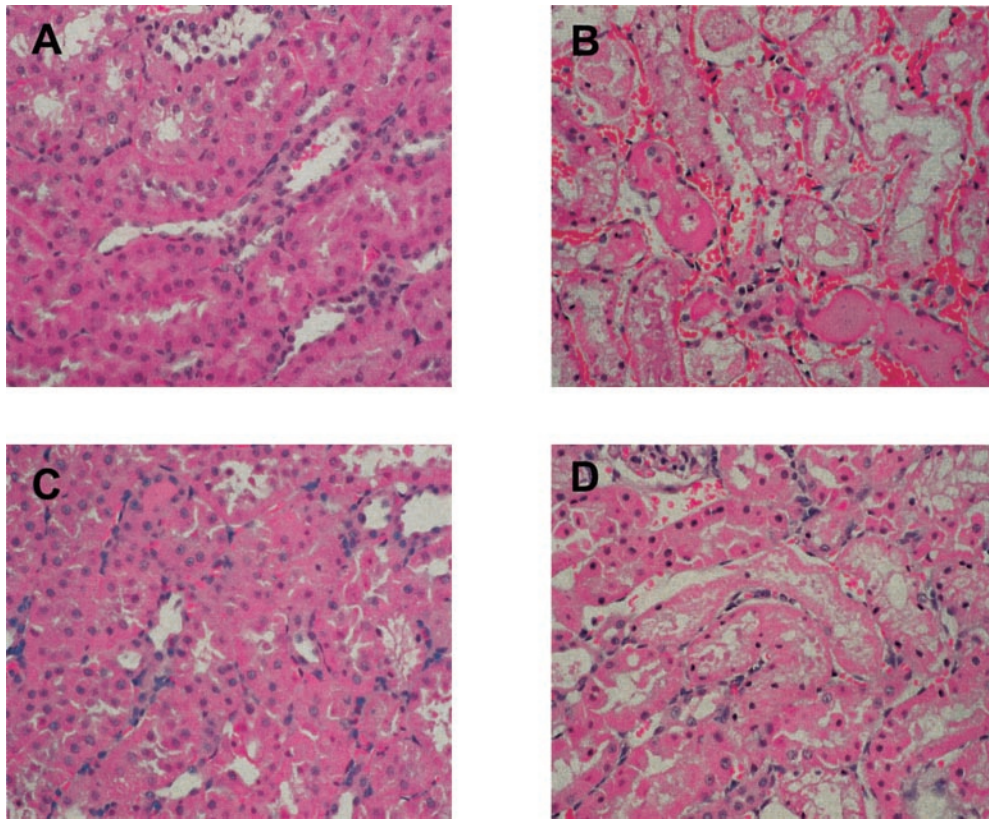
Exogenous hydrogen peroxide was used to induce free radical-mediated oxidant injury in HK-2 cells, an immortalized human proximal tubule epithelial cell line. HK-2 cell injury was quantified by the amount of LDH release into cell culture media. Hydrogen peroxide (1 mM incubated for 4 h) caused significant cell death when compared with controls (Figure 6A). Preincubation with EPO (1 to 50 U/ml) for 1 h significantly protected against hydrogen peroxide-mediated cell death in a dose-dependent manner ( $P < 0.01$ ). Preincubation with EPO (10 U/ml) for 24 h was associated with a similar degree of protection from hydrogen peroxide-induced cell death (data not shown). The antiapoptotic effects of EPO are dependent on the PI3K/AKT pathway in endothelial cells. Co-incubation with LY294002 (2.5 to 10  $\mu$ M) or wortmannin (500 nM), specific inhibitors of PI3K, significantly reduced the protection observed with EPO pretreatment ( $P < 0.01$ ) in a dose-dependent manner (Figure 6B). Tyrphostin (AG490), an inhibitor of JAK2 phosphorylation, was used to determine whether the protection observed with EPO was dependent on EPO receptor activation, as EPO has been reported to have some direct antioxidant activity. Preincubation with AG490 (1 to 10  $\mu$ M) blocked the reduction in cell death observed with EPO in a dose-dependent manner ( $P < 0.05$ ; Figure 6B).

### EPO Prevents Apoptosis after Serum Starvation

In cultures of HK-2 cells deprived of serum, DNA fragmentation was increased at 24 h when compared with control (5% FCS) wells ( $P < 0.05$ ). Co-incubation with EPO (10 U/ml) significantly reduced the amount of apoptotic cell death (Figure 7A). Dose-response experiments (EPO 1 to 100 U/ml) reversed the reduction in cell viability caused by 24-h incubation in serum-free conditions. EPO at doses  $>10$  U/ml caused a significant degree of proliferation despite serum deprivation (Figure 7B). The lysates in which DNA fragmentation was measured were used to examine the protein expression of intermediates of apoptotic signaling. Serum starvation for 24 h caused activation of caspase-3 and was associated with a reduction in expression of antiapoptotic proteins Bcl- $X_L$  and XIAP. EPO treatment prevented the activation of caspase-3 and was associated with increased expression of Bcl- $X_L$  and XIAP (Figure 8C).

### Confirmation of Phosphorylation of AKT by EPO

HK-2 cells were incubated for 6 h with 20 U/ml EPO and in the presence of LY294002 (10  $\mu$ M). Membranes were probed with specific monoclonal antibodies for total AKT and phosphorylated AKT (serine 473). Incubation with EPO caused increased expression of phosphorylated AKT, which was completely prevented by co-incubation with LY294002, confirming activation of AKT by EPO in a PI3K-dependent manner. EPO also increased the activation of extracellular signal-regulated kinases 1 and 2, which was not dependent on PI3K (Figure 8).



**Figure 3.** EPO attenuates the morphologic changes of I/R injury. A renal section taken from a sham-operated rat (A) is compared with that from a rat that was subjected to renal I/R (B). Renal sections from a rat that was subjected to renal I/R after administration of EPO preischemia (30 min; C) and EPO postreperfusion (30 min; D) are also presented. Hematoxylin and eosin–stained kidney sections. Figures are representative of at least three experiments performed on different days ( $n = 6$  for all groups). Magnification,  $\times 150$ .

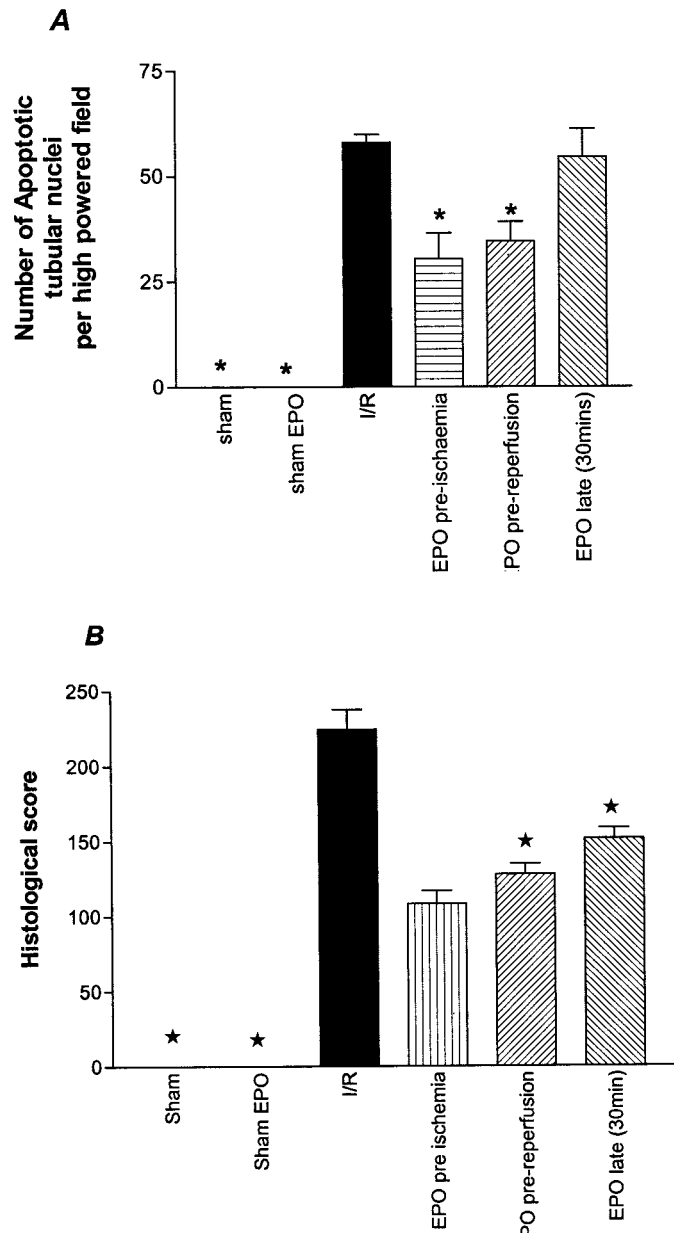
## Discussion

In the present study, we present the novel finding that recombinant human EPO protects the kidney against I/R injury. We demonstrate that EPO reduces both the glomerular and the tubular injury and dysfunction (as assessed by biochemical and histologic parameters) caused by severe (45 min) ischemia and reperfusion in the rat. EPO reduced I/R injury when administered as a single intravenous bolus injection either before the onset of ischemia (30 min pretreatment) or when given just before the onset of reperfusion. Most notably for clinical relevance, EPO even partially protected the kidney against I/R injury when given as late as 30 min after the onset of reperfusion. Thus, our study provides convincing evidence that EPO reduces I/R injury of the kidney *in vivo* (22).

What, then, is the mechanism(s) by which EPO protects the kidney against I/R injury? One could argue that an increase in renal blood flow may contribute to or even account for the observed protective effects of EPO. This is unlikely, however, as we were unable to demonstrate any significant hemodynamic effect of acute administration of EPO. Although EPO increased urine flow rate in sham-operated rats, suggesting a possible effect on cortical perfusion and intraglomerular pressure, creatinine clearances were similar and there was no difference in tubular function. This observation is backed by the study by Huang *et al.* (23), who demonstrated no effect on

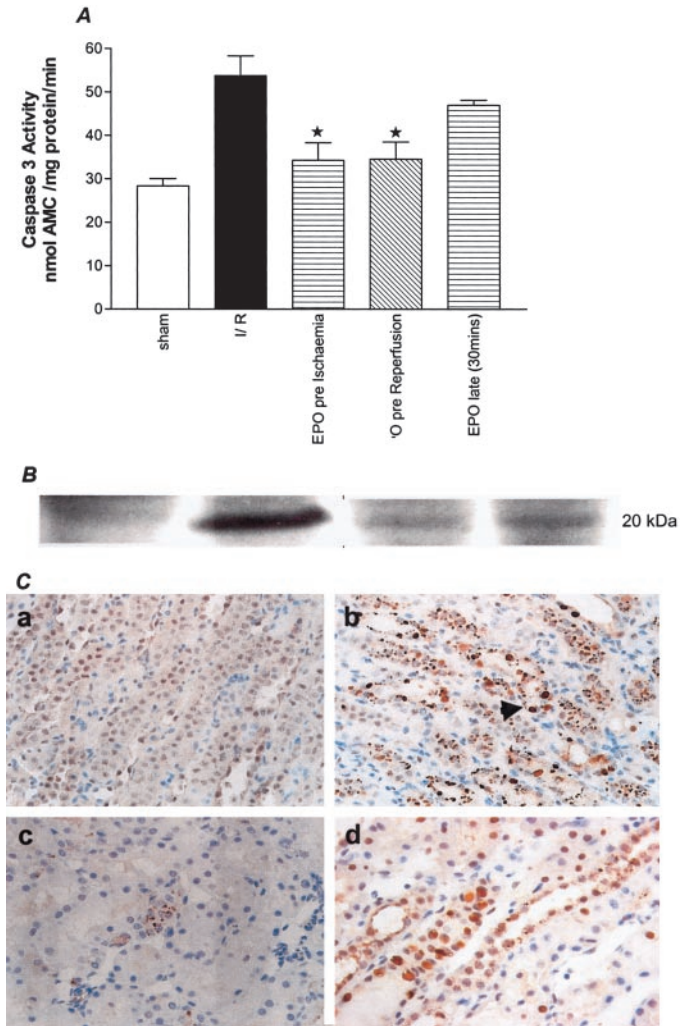
cortical and papillary perfusion in normal rats after acute EPO administration.

We propose here that EPO acts directly on proximal tubule epithelial cells against I/R injury. This proposal is based on the following key findings: EPO protected human proximal tubule epithelial cells against the cell injury and death caused by serum starvation and oxidative stress (hydrogen peroxide), the latter of which is most likely to simulate reperfusion injury. We then investigated whether the protection by EPO of proximal tubule epithelial cells is secondary to the activation of EPO receptors and due to an antiapoptotic effect of EPO in these cells. We demonstrated that the protection of proximal tubule epithelial cells by EPO was attenuated by an inhibitor of JAK2 signaling. Thus, the activation of the EPO receptor is essential in the observed protective effect of EPO. Activation of JAK2 by EPO leads (in endothelial cells and neuronal cells) to the activation of PI3K and Akt phosphorylation (24). Once activated, Akt activates multiple targets with antiapoptotic effects, including phosphorylation of Bad, Bax, caspase-9, and GSK-3 $\beta$ , maintenance of mitochondrial membrane potential and preservation of glycolysis and ATP synthesis (25). Inhibition of PI3K abolishes the protective effects of EPO in human proximal tubule epithelial cells and inhibited the specific serine-473 phosphorylation of AKT induced by EPO. Thus, we suggest that EPO protects proximal tubule cells against injury



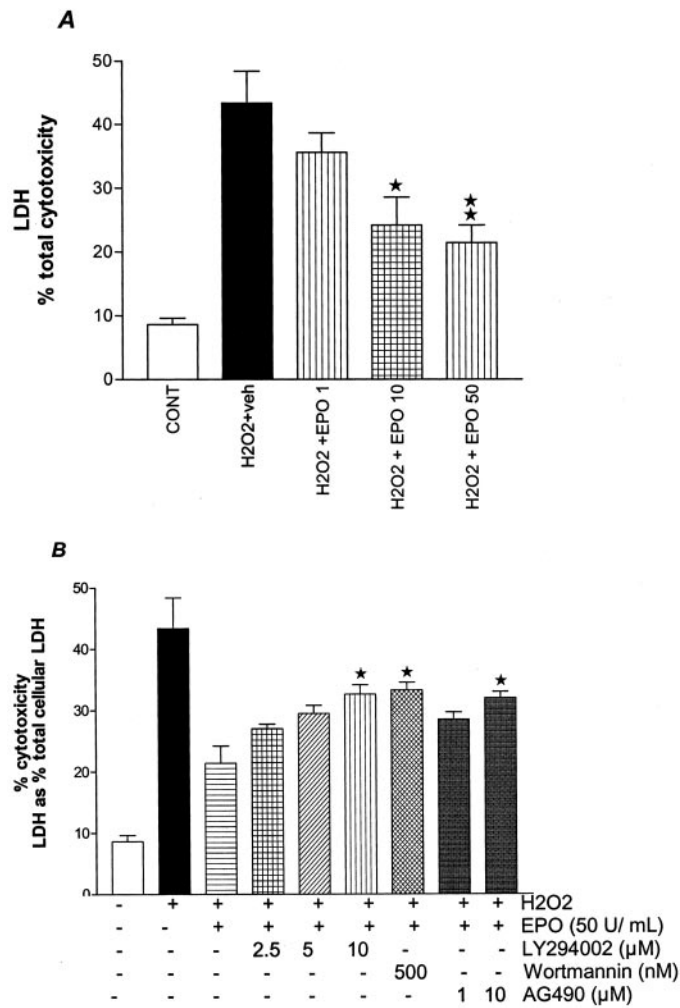
**Figure 4.** EPO reduces tubular apoptosis and necrotic cell death caused by I/R. (A) The degree and severity of tubular cell apoptosis were assessed on morphologic criteria by two blinded observers. I/R injury was associated with a significant increase in the number of apoptotic cells per high-powered field. EPO treatment preischemia and prereperfusion attenuated the amount of apoptosis ( $P < 0.05$ ). EPO treatment after 30 min of reperfusion did not significantly reduce the number of apoptotic tubule cells. (B) Histologic sections were scored for histologic changes of acute tubular necrosis (tubular cell swelling, loss of brush border, nuclear condensation) out of a total of 300 (see Materials and Methods) by a pathologist who was blinded to treatment group. I/R injury caused severe necrotic injury compared with sham animals. EPO administration preserved tubule morphology even when administered 30 min after the onset of reperfusion ( $P < 0.05$ , respectively).

by activating the EPO receptor, resulting in activation of PI3K and ultimately Akt. We demonstrated that EPO inhibits apoptotic cell death in proximal tubule epithelial cells (as deter-



**Figure 5.** EPO inhibits caspase-3 signaling *in vivo*. (A) In homogenates of kidneys from rats that were reperused for 6 h, caspase-3 activity was significantly increased compared with sham-operated animals. EPO administration preischemia ( $P < 0.01$ ) and prereperfusion significantly reduced mean caspase-3 activity ( $P < 0.01$ ). EPO administered 30 min after reperfusion caused a small reduction in caspase-3 activity. (B) Kidney specimens were homogenized, separated by SDS-PAGE, and immunoblotted with rabbit anti-caspase-3 antibody. The expression of the active p20 subunit of caspase-3 was elevated in I/R. (C) Immunohistochemical staining for active fragment of caspase-3 was performed. (a) Sham kidneys stained negative for active caspase-3. (b) I/R caused severe and widespread staining (arrow) in both cortical and medullary tubules and loop of Henle. (c) EPO preischemia and prereperfusion (shown here) significantly reduced positive staining. (d) EPO 30 min after reperfusion was associated with minor reduction in staining (representative photos from  $n = 5$ /group).

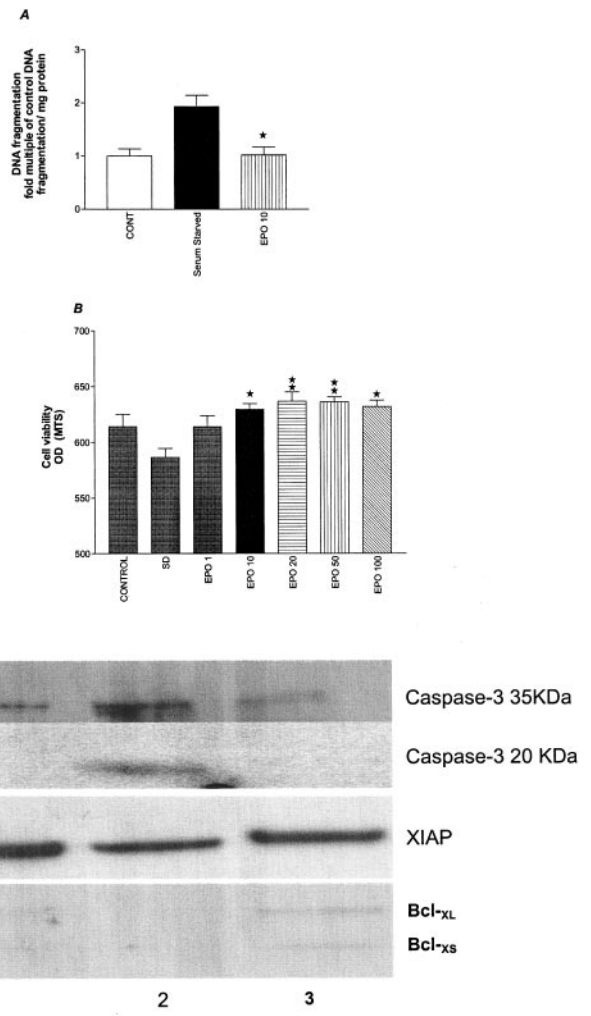
mined by DNA fragmentation) and in higher doses causes significant proliferation despite serum-free conditions. Most notably, EPO administration was associated with the upregulation of Bcl-X<sub>L</sub> and XIAP and reduction of caspase-3 activation. XIAP may be an important mediator of EPO's protective effects in I/R injury, because it acts on both death receptor



**Figure 6.** EPO prevents oxidant-induced cell death. (A) HK-2 cultures undergo necrotic cell death when exposed to 1 mM hydrogen peroxide for 4 h. EPO significantly abrogated cell death measured by Lactate dehydrogenase release in a dose-dependent manner ( $P < 0.001$ ). (B) LY294002 (2.5 to 10  $\mu\text{M}$ ) and Wortmannin (500 nM), inhibitors of PI3-kinase (PI3K), significantly decreased the protective effect of EPO on oxidative cell death in a dose-dependent manner ( $P < 0.01$ ). Blocking JAK2 phosphorylation with AG490 (1 to 10  $\mu\text{M}$ ) inhibited EPO-induced protection from oxidative insult ( $P < 0.001$ ).

(Fas-FasL) mediated and mitochondrial pathways of caspase activation, as well as directly inhibits the activation of caspase-3, -7, and -9 (28). XIAP may also have an indirect antiapoptotic effect by inducing p21cip1, leading to cell cycle arrest at G1/S transition, which has been shown to be protective in renal I/R (29,30).

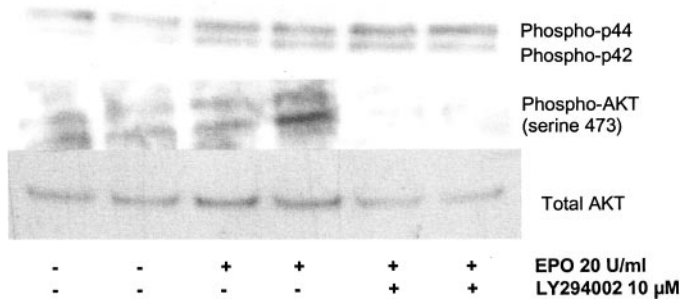
Taken together, these *in vitro* findings support the conclusion that EPO directly protects proximal tubule epithelial cells by (1) activating EPO receptor/JAK-2 kinase, (2) activating PI3K leading to activation of Akt, (3) upregulation of Bcl-X<sub>L</sub> and XIAP, and (4) preventing the activation of caspase-3 and ultimately apoptosis. We confirm that EPO reduced the activation of caspase-3 caused by I/R of the kidney by inhibiting the activation of the caspase cascade both through the mito-



**Figure 7.** EPO prevents apoptosis caused by serum starvation. (A) Serum starvation of HK-2 proximal tubule epithelial cells is associated with significant apoptosis assessed by DNA fragmentation by 24 h. EPO treatment attenuated the increase in DNA fragmentation ( $P < 0.05$ ). (B) Serum starvation reduced cell viability [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS assay) when compared with control 10% FCS. Incubation with EPO (1 to 100 U/ml) reversed the reduction in cell viability and caused a dose-dependent cell proliferation ( $P < 0.001$ ) above that seen in control cells. (C) Lysates of HK-2 cells were separated with SDS-PAGE and immunoblotted with antibodies for Bcl-X<sub>L</sub>, caspase-3, and XIAP. Serum starvation was associated with a reduction of Bcl-X<sub>L</sub> and XIAP after 24 h. EPO prevented the loss of Bcl-X<sub>L</sub> and XIAP, which was accompanied by the inhibition of caspase-3 activation. Immunoblots for caspase-3, Bcl-X<sub>L</sub>, and XIAP shown are representative of three independent experiments.

chondrial caspase-9 pathway and possibly to a lesser degree the death receptor-mediated activation of caspase-8. The death receptors Fas (CD95), TNF-R1, and CD27 have been implicated in the pathogenesis of renal I/R injury (31), and TNF- $\alpha$  is induced after reperfusion by the upregulation of NF- $\kappa\text{B}$  via p38. We saw significant activation in mean caspase-8 activity after I/R. The increase in caspase-8 activity was less than the increase in caspase-9 activity. This difference may be due to





**Figure 8.** EPO-induced AKT phosphorylation is PI3K dependent. Immunoblot analysis using specific antibody to phospho-AKT (serine 473) in control and EPO-treated HK-2 cells confirms activation of AKT by EPO. The activation of AKT was prevented by co-incubation with LY294002 (10  $\mu$ M). The membrane was re-probed for total AKT to confirm equal protein loading. EPO also activated extracellular signal-regulated kinases 1 and 2, which was not dependent on PI3K.

differences in the relative sensitivity of the assay but may also be attributed to changes in mitochondrial membrane potential leading to caspase-9 activation beginning during ischemia, whereas the death receptor pathway may be activated only at a later time point during reperfusion (30).

In summary, we demonstrate here that a single intravenous bolus injection of recombinant human EPO, either preischemia or just before the onset of reperfusion, attenuates renal I/R injury in rats, *via* the inhibition of proapoptotic caspase activation. We propose that the nonhemopoietic pleiotropic effects of EPO have major implications in the treatment of I/R injury to the kidney, in particular, and possibly other organs.

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## References

1. Lacombe C, and Mayeux P: Biology of erythropoietin. *Haematologica* 83: 724–732, 1998
2. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim AV, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ: Targeting of HIF- $\alpha$  to the von Hippel-Lindau ubiquitylation complex by O<sub>2</sub>-regulated prolyl hydroxylation. *Science* 292: 468–472, 2001
3. Kaelin WG Jr: How oxygen makes its presence felt. *Genes Dev* 16: 1441–1445, 2002
4. Matsumoto M, Makino Y, Tanaka T, Tanaka H, Ishizaka N, Noiri E, Fujita T, Nangaku M: Induction of renoprotective gene expression by cobalt ameliorates ischemic injury of the kidney in rats. *J Am Soc Nephrol* 14: 1825–1832, 2003
5. Brines ML, Ghezzi P, Keenan S, Agnello D, de Lanerolle NC, Cerami C, Itri LM, Cerami A: Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci U S A* 97: 10526–10531, 2000
6. Siren AL, Fratelli M, Brines M, Goemans C, Casagrande S, Lewczuk P, Keenan S, Gleiter C, Pasquali C, Capobianco A, Mennini T, Heumann R, Cerami A, Ehrenreich H, Ghezzi P:

Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc Natl Acad Sci U S A* 98: 4044–4049, 2001

7. Digicaylioglu M, Lipton SA: Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF- $\kappa$ B signalling cascades. *Nature* 412: 641–647, 2001
8. Grimm C, Wenzel A, Groszer M, Mayser H, Seeliger M, Samardzija M, Bauer C, Gassman M, Reme CE: HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. *Nat Med* 8: 718–724, 2002
9. Westenfelder C, Biddle DL, Baranowski RL: Human, rat, and mouse kidney cells express functional erythropoietin receptors. *Kidney Int* 55: 808–820, 1999
10. Weight SC, Bell PR, Nicholson ML: Renal ischaemia-reperfusion injury. *Br J Surg* 83: 162–170, 1996
11. Yaqoob MM, Alkhunaizi AM, Edelstein CL, Conger JD, Schrier RW: Acute renal failure: Pathogenesis, diagnosis, and management. In: *Renal and electrolyte Disorders*, 5th Ed., edited by RW Schrier, Philadelphia, Lippincott-Raven, 1997
12. Lieberthal W, Koh JS, Levine JS: Necrosis and apoptosis in acute renal failure. *Semin Nephrol* 18: 505–518, 1998
13. Daemen MA, Van 't Veer C, Denecker G, Heemskerk VH, Wolfs TG, Clauss M, Vandenaabeele P, Buurman WA: Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. *J Clin Invest* 104: 541–549, 1999
14. Lieberthal W, Menza SA, Levine JS: Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells. *Am J Physiol* 274: F315–F327, 1998
15. Edelstein CL, Shi Y, Schrier RW: Role of caspases in hypoxia-induced necrosis of rat renal proximal tubules. *J Am Soc Nephrol* 10: 1940–1949, 1999
16. Kaushal GP, Singh AB, Shah SV: Identification of gene family of caspases in rat kidney and altered expression in ischemia-reperfusion injury. *Am J Physiol* 274: F587–F595, 1998
17. Chien CT, Lee PH, Chen CF, Ma MC, Lai MK, Hsu SM: De novo demonstration and co-localization of free-radical production and apoptosis formation in rat kidney subjected to ischemia/reperfusion. *J Am Soc Nephrol* 12: 973–982, 2001
18. Supavekin S, Zhang W, Kuchelapati R, Kaskel FJ, Moore LC, Devarajan P: Differential gene expression following early renal ischemia/reperfusion. *Kidney Int* 63: 1714–1724, 2003
19. Yoshida T, Kurella M, Beato F, Min H, Ingelfinger JR, Stears RL, Swinford RD, Gullans SR, Tang SS: Monitoring changes in gene expression in renal ischemia-reperfusion in the rat. *Kidney Int* 61: 1646–1654, 2002
20. Chatterjee PK, Zacharowski K, Cuzzocrea S, Otto M, Thiemeermann C: Inhibitors of poly (ADP-ribose) synthetase reduce renal ischemia-reperfusion injury in the anesthetized rat in vivo. *FASEB J* 14: 641–651, 2000
21. Cuello-Carrion FD, Ciocca DR: Improved detection of apoptotic cells using a modified in situ TUNEL technique. *J Histochem Cytochem* 47: 837–839, 1999
22. Allen DA, Harwood S, Varagunam M, Raftery MJ, Yaqoob MM: High glucose-induced oxidative stress causes apoptosis in proximal tubular epithelial cells and is mediated by multiple caspases. *FASEB J* 17: 908–910, 2003
23. Huang C, Davis G, Johns EJ: Study of the actions of human recombinant erythropoietin on rat renal haemodynamics. *Clin Sci (Lond)* 83: 453–459, 1992
24. Chong ZZ, Kang JQ, Maiese K: Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial

- modulation of cysteine proteases. *Circulation* 106: 2973–2979, 2002
25. Cantley LC: The phosphoinositide 3-kinase pathway. *Science* 296: 1655–1657, 2002.
  26. Holcik M, Gibson H, Korneluk RG: XIAP: Apoptotic brake and promising therapeutic target. *Apoptosis* 6: 253–261, 2001
  27. Levkau B, Garton KJ, Ferri N, Kloke K, Nofer JR, Baba HA, Raines EW, Breithardt G: XIAP induces cell-cycle arrest and activates NF- $\kappa$ B: New survival pathways disabled by caspase-mediated cleavage during apoptosis of human endothelial cells. *Circ Res* 88: 282–290, 2001
  28. Megyesi J, Andrade L, Vieira JM Jr, Safirstein RL, Price PM: Positive effect of the induction of p21WAF1/CIP1 on the course of ischemic acute renal failure. *Kidney Int* 60: 2164–2172, 2001
  29. Nogae S, Miyazaki M, Kobayashi N, Saito T, Abe K, Saito H, Nakune PK, Nakanishi Y, Koji T: Induction of apoptosis in ischemia-reperfusion model of mouse kidney: Possible involvement of Fas. *J Am Soc Nephrol* 9: 620–631, 1998
  30. Donnahoo K, Shames B, Harken A, Meldrum D: Review article: The role of tumour necrosis factor in renal ischaemia reperfusion. *J Urol* 162: 196–203, 1999

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