Erythropoietin in Experimental Acute Renal Failure

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Key Words

Erythropoietin · Apoptosis · Acute renal failure · Animal model

Abstract

The haematopoietic factor erythropoietin (EPO) has recently been recognized to play a physiological role in the brain and other tissues. The EPO receptor is present in the glomerulus, mesangial and tubular epithelial cells in the kidney. We have reviewed the experimental use of EPO in animal models of acute renal failure. EPO attenuates the dysfunction and histological changes associated with ischaemia-reperfusion injury, with a reduction in apoptotic cell death. EPO has also shown benefit in animal models of systemic shock and cisplatin-induced nephrotoxicity. In vitro studies have shown that EPO has direct effects on proliferation and cell death in proximal tubular epithelial cells. There is increasingly strong experimental evidence that EPO may be of therapeutic use in acute renal failure, and clinical trials should be undertaken to determine its clinical applications in this field.

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Introduction

The haematopoietic growth factor erythropoietin (EPO), driven by oxygen-dependent regulation of HIF1- α stability, promotes the survival, proliferation and differ-

entiation of erythroid progenitor cells in the bone marrow. Recombinant human EPO has been established for nearly 20 years in the management of anaemia associated with end-stage renal disease. Until recently, this was thought to be the sole physiological role for EPO. This view was challenged by the discovery that EPO and its receptor were expressed in multiple tissues, including the vasculature, brain, uterus, heart and skeletal muscle. In the brain, EPO is produced by astrocytes in response to hypoxia and mediates paracrine and autocrine survival pathways, reducing cell death in the ischaemic penumbra. Exogenous administration of EPO attenuates cell death in ischaemic brain and spinal cord injury, associated with functional protection [reviewed in 1]. In this minireview, we will examine the current experimental evidence for the potential use of EPO as a therapeutic agent in acute renal injury.

In vivo Evidence for Protective Role for Erythropoietin

The discovery that the EPO receptor is expressed in glomerular, mesangial and tubular epithelial cells in human, rat and mouse kidney [2], led several groups to study the effects of EPO in small rodent models of acute kidney injury (table 1). These models are highly reproducible in the degree of renal dysfunction initially induced, and therefore are a good screening tool for novel therapeutic agents, but may not be directly comparable to human acute renal failure.

Table 1. In vivo studies of the effects of EPO on experimental injury in the kidney

Author, ref.	Model	EPO protocol		Outcome
Yang [3]	45 min bilat I/R	3,000 U/kg i.p.	-24 h	functional protection apoptosis↓
Vesey [5]	45 min unilat I/R and bilat I/R	500–3,000 U/kg i.p. -30 min		reduced necrosis and apoptosis
Sharples [4]	45 min bilat I/R	300 U/kg i.v.	-30 min At reperfusion +30 min after reperfusion	↓apoptosis, functional protection at 6 h
Abdelrahman [14]	haemorrhagic shock	300 U/kg i.v.		functional protection ↓caspase-3
Patel [13]	45 min bilateral I/R, mouse	1,000 U/kg s.c.		3 days preconditioning greater protection
Vaziri [6]	cisplatin	100 U/kg		improved C _{cr} at day 9 increased tubular proliferation
Bahlmann [12]	5/6th nephrectomy	darbopoietin 0.1 µg/kg/weekly		reduced early apoptosis maintenance of peritu- bular capillary networks
Lee [7]	cyclosporine A toxicity	100 U/kg weekly i.p.		reduced systemic inflammation

In the first study to show that EPO may protect the kidney from ischaemic injury, Yang et al. [3] administered high-dose EPO (3,000 U/kg) intra-peritoneally (i.p.) 24 h before bilateral renal artery clamping. At 24 h post-reperfusion, there was functional protection in EPO-treated animals, with significant attenuation in the rise of creatinine induced by ischaemia. EPO reduced proximal tubular epithelial cell death by TUNEL staining. EPO caused increased expression of Bcl-2, an anti-apoptotic protein that prevents mitochondrial depolarisation, and the molecular chaperone heat shock protein-70 (HSP-70) in both sham operated and animals subjected to I/R. The increase in HSP-70 expression was time- and dose-dependent, via activation of the JAK2/STAT transcription factor pathway.

In our study, low-dose EPO (300 U/kg) was administered as a single intravenous bolus in three treatment groups, either as a 30-min pre-treatment, at the time of reperfusion, or 30 min after the onset of reperfusion, to determine which protocol offered the best renal protection [4]. EPO significantly reduced renal dysfunction, and markers of tubular dysfunction, including fractional sodium excretion. Administration of EPO 30 min after reperfusion was associated with lesser, but still significant

protection at 6 h. The preservation of renal function was mirrored by a lack of disruption in tubular architecture on histological examination. EPO caused a significant reduction in the number of apoptotic tubules, with attenuation in the activity of cysteine proteases, or caspases, that mediate apoptosis.

These studies showed that EPO altered the initiation phase of acute renal injury. Vesey et al. [5] showed that EPO was effective when administered only 30 min before the initiation of ischaemia, in a model in which Sprague-Dawley rats underwent nephrectomy and unilateral ischaemia. High-dose EPO (5,000 U/kg i.p.) attenuated the rise in creatinine at 24 h, and the difference between the treatment groups was maintained at 72 h. Importantly, a single pre-ischaemia administration of EPO stimulated early initiation of tubular regeneration, evidenced by increased tubular mitosis observed at 24 h, confirmed by increased immunostaining for proliferating nuclear cell antigen (PCNA) within the proximal tubules. This apparent effect on the development of the tubular regenerative process is an important potential mechanism by which EPO may act in the recovery phase of acute renal failure. EPO has been shown to stimulate proliferation of proximal tubular epithelial cells in vitro in a dose-dependent

manner. Chronic administration of EPO may enhance tubular proliferation following injury. Previously, Vaziri et al. [6] had used EPO in a model of cisplatin nephrotoxicity (7 mg/kg i.p.) in Sprague-Dawley rats, and then administered EPO 100 U/kg daily for 9 days. Erythropoietin significantly increased the calculated creatinine clearance by day 9 when compared with vehicle treated animals. The enhanced functional recovery was accompanied by increased ³H-thymidine incorporation as a marker of increased tubular regeneration. The early improvement in creatinine clearance in EPO treated animals was maintained over the entire course of 6 weeks, at which point full functional recovery occurred in the vehicle-treated animals. Chronic EPO therapy has also been shown to be beneficial in a model of cyclosporine toxicity. Lee et al. [7] demonstrated reduced apoptosis and inflammation in kidneys treated with EPO (100 U/kg thrice weekly) when compared to control animals.

Endothelial Effects of EPO in Ischaemia-Reperfusion Injury

Endothelial dysfunction and injury have been recognized as playing major roles in the extension and maintenance phases of acute renal failure. Transplantation and engraftment of functionally mature endothelial cells into the circulation of post-ischaemic rats to reverse endothelial dysfunction protected the kidney from ischaemic injury [8]. Minimization of renal dysfunction was also observed following transplantation of human embryonic kidney cells (HEK293) stably transfected with human endothelial nitric oxide synthase (eNOS), demonstrating the importance of eNOS-derived nitric oxide in the preservation of endothelial integrity, and hence maintenance of normal vascular auto-regulation, preventing expansion of injury by local microinfarction and persistent hypoxia.

Capillary endothelial cells express the EPO receptor in their intraluminal surfaces. EPO antagonizes apoptosis of endothelial cells subjected to hypoxic stress *in vitro* [9], and might therefore have a role in maintaining integrity of the microvasculature. EPO upregulates expression of eNOS mRNA in endothelial cells, and might also increase eNOS activity via AKT-dependent eNOS phosphorylation (serine-1179) [10]. Through these mechanisms, EPO may maintain normal vascular autoregulation, thereby preventing amplification of tubular hypoxia during the extension phase of acute renal failure. These beneficial effects of EPO on the endothelium may be dose-depen-

dent, as high doses of EPO may reduce the activity of dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that degrades asymmetrical dimethylarginine (ADMA), which is an endogenous inhibitor of eNOS and accumulation of ADMA is associated with oxidative stress and endothelial dysfunction [11].

EPO has also been shown to stimulate endothelial cell mitogenesis and angiogenesis, which improve tissue oxygenation. Studies in mice and humans have shown that EPO is a potent stimulator of endothelial progenitor cell (EPC) mobilization from the bone marrow, increasing circulating EPCs which may contribute to the regeneration of damaged endothelium [12]. A 3-day subcutaneous pre-conditioning EPO regimen (1,000 U/kg EPO per day), based on the regimen known to stimulate production of endothelial progenitor cells, was compared with a single dose of EPO (1,000 U/kg) at the time of reperfusion in a murine model of ischaemia reperfusion injury [13]. Male C57BL/6J mice weighing 25–30 g underwent 30 min bilateral renal clamping followed by 24-hour reperfusion. Both EPO protocols conferred significant protection from ischaemia-reperfusion injury, although a greater reduction in injury marker intensity was observed in the pre-conditioned group. Further work is required to determine the role of EPCs and endothelial regeneration in the protective effects of EPO on ischaemic injury in the kidney and heart.

Does EPO Have Similar Effects in Other Models of Experimental Renal Failure?

EPO has been effective in the standard small rodent clamp models of ischaemia reperfusion. Abdelrahman et al. [14] showed that EPO had a similar degree of renal protection in a model of haemorrhagic shock, but did not alter the course of renal dysfunction induced by LPS, as had been shown in the brain.

The long-term benefit of reducing the severity of initial kidney injury has also been shown to reduce progressive dysfunction in a rat remnant kidney model. Sprague-Dawley rats underwent a single stage 5/6th nephrectomy procedure, and were followed for 6 weeks. Low-dose darbopoetin (0.1 µg/kg) subcutaneously administered once weekly significantly reduced apoptotic cell death between days 4 and 14 post-surgery, associated with persistent AKT phosphorylation, and this reduction in apoptosis led to partial preservation of renal function at 6 weeks when compared with vehicle treated animals. Importantly, this study demonstrates similar protection from injury

Fig. 1. Potential mechanism of anti-apoptotic effects of erythropoietin. EPO and the EPO receptor prevent apoptosis and cellular inflammation through a series of pathways that originate with autophosphorylation and activation of Janus kinase-2 (JAK2) following binding of EPO to its receptor. JAK2 phosphorylates several tyrosine residues on the intracellular portion of the receptor, facilitating binding of proteins containing src-homology (SH-2) domains. The p85 subunit of phosphatidylinositol-3 kinase (PI3K) interacts with the receptor, possibly via scaffolding proteins, and this leads to phosphorylation of protein kinase B (AKT). AKT has multiple effects on cell survival, by maintaining mitochondrial integrity directly and through the inhibition of several pro-apoptotic media-

tors, including Bad, caspase-9, GSK-3 and inhibits the activation of JNK by stabilization of ASK-1. Via JAK2, EPO activates members of the STAT family of transcription factors that enhance cell proliferation and survival. Activation of the transcription factor NF-κB is dependent on JAK2 activity, and might be amplified by phosphorylation of IκB kinase by AKT. NF-κB induces expression of endogenous inhibitors of apoptosis, including X-linked inhibitor of apoptosis, which inhibits caspase-3, caspase-7 and caspase-9. EPO maintains mitochondrial membrane integrity and prevents apoptosis by enhancing expression of Bcl-X_L, which interacts with the pro-apoptotic BH3 protein Bax. This interaction prevents release of cytochrome c, and activation of caspase-9 and caspase-3.

using a low dose of darbopoietin, which was insufficient to stimulate significant erythropoiesis [15].

Another potential avenue for a therapeutic role of EPO in acute kidney injury has been opened by the discovery that podocytes express the EPO receptor, and EPO reduces glomerular injury in Thy1.1 model of glomerulonephritis [16].

In vitro Studies of the Mechanism of Cellular Protection with EPO

The EPO receptor is expressed and functional in proximal tubular epithelial cells, and initial experiments had shown evidence of a proliferative response to EPO in cul-

ture [2]. Fishbane et al. [17] exposed porcine kidney epithelial cells (LLC-PK₁) to several insults with and without darbopoietin (50 ng/ml). Darbopoietin significantly reduced apoptotic cell death in response to 16 h of hypoxia (1% oxygen). Similar experiments performed with an inactive recombinant erythropoietin molecule did not offer protection. Vesey et al. [5] demonstrated that high doses of EPO reduced hypoxia induced cell death in primary human proximal tubular epithelial cells. These studies did not address the mechanism by which EPO exerts these effects.

We have examined the signaling pathways triggered by EPO in an immortalized human proximal tubular epithelial cell line (HK-2) in a variety of experimental settings. EPO caused a dose-dependent (10–50 U/ml) in-

crease in cell viability in serum-deprived cells, associated with a reduction in DNA fragmentation and caspase-3 activation, although higher doses (70–100 U/ml) were associated with reduced cell survival. EPO signaling is JAK2 dependent, and inhibition of JAK2 prevents the anti-apoptotic effect in vitro. There is strong evidence that phosphatidylinositol 3-kinase (PI3-K)/AKT pathway is involved in EPO-dependent cell survival with the demonstration that the PI3-K inhibitor LY294002 inhibits the protective effects of EPO in these models. AKT phosphorylates multiple targets that influence apoptotic signalling [4]. EPO receptor signaling leads to the phosphorylation of the mitogen-activated protein kinases ERK1/2, although the role in prevented cell death is not clearly understood.

EPO activates gene transcription via the activation and nuclear translocation of the transcription factors NF- κ B, including expression of several anti-apoptotic proteins, including Bcl-X_L and X-linked inhibitor of apoptosis (XIAP), and activates members of the STAT family of transcription factors. The exact pattern of STAT activation appears to be cell-type specific, with STAT3 and STAT5A predominately induced in the kidney and heart. The role of STAT-dependent proteins in the anti-apoptotic effects of EPO in the kidney and the proliferative response require further investigation. The differentiation of EPO signaling into genomic and non-genomic begins to distinguish which pathways may be essential to the anti-apoptotic effects of EPO over the time-course of renal injury (fig. 1). The differences in renal protection seen with a pre-conditioning regime in the study by Patel et al. [13], when compared to immediate treatment at reperfusion may be due to the relative pre-dominance of the genomic factors over the immediate effects such as AKT phosphorylation. Further work, particularly utilizing EPO receptor mimetics that may differentially activate these two types of signaling should lead to better understanding of the mechanism of action of EPO.

Translation to a Therapeutic Role?

There is now clear experimental evidence from animal models that EPO can reduce the severity of acute renal injury induced by ischaemia reperfusion, systemic shock, and nephrotoxic insults, and may also contribute to the process of tubular regeneration through direct effects on tubular epithelial cells. The mechanism of this protection is being elucidated, but it is established that EPO exerts direct effects on both endothelial and tubular

epithelial cells that contribute to the reduction in organ damage.

Further studies are required to determine the effectiveness of different treatment strategies and particularly the timing of treatment. The doses used in several early trials (3,000–5,000 U/kg) would equate to massive doses in human subjects, although recent studies have demonstrated beneficial effects at much lesser doses, more consistent with that utilized in the management of the anaemia of chronic kidney disease. The results of a ‘proof of concept’ trial in stroke should encourage clinical trials in acute renal failure [18]. The obstacles to successful clinical trials have been suggested by a lack of effect on clinical outcomes in a recent trial of EPO administration in patients with multi-organ failure in an intensive care setting. Patients received 40,000 U weekly, and although there was a reduction in transfusion requirements, and a reduced mortality (19% EPO, 29.5% placebo) in the EPO group, this did not reach significance [19].

The introduction of new derivatives of erythropoietin that do not affect haemopoiesis may make these agents attractive for use in clinical trials, but standard erythropoietin formulations have the advantage of long-term patient safety data, familiarity and availability [20]. Recent discoveries that both EPO and the EPO receptor are widely expressed, and appear to be functional in cancer cell lines required further study, but is more likely to impact on chronic use of erythropoietin rather than single-dose or short-term strategies in organ protection protocols. In the light of persistent high mortality rates in acute renal failure, there is an urgent need for clinical trials to determine the therapeutic effects of EPO in these patients.

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