Erythropoietin Protects Against Acute Kidney Injury and Failure

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Abstract: Erythropoietin (EPO) has traditionally been viewed as a hormone dedicated to the regulation of erythropoiesis. More recently, the EPO receptor (EPOR) has been found to be expressed in a large variety of non-haematopoietic tissues suggesting that EPO might have important actions beyond erythrocyte production. Over the last five years, short-term, high dose administration of EPO has been shown to ameliorate acute kidney injury (AKI), as evidenced by suppressed tubular epithelial apoptosis, enhanced tubular epithelial proliferation, and hastened functional recovery. This renoprotective effect is still apparent when administration is delayed up to 6 hours after the onset of injury and has been demonstrated in a variety of animals (mouse, rat, dog, pig) in response to a variety of renal insults (ischaemia-reperfusion injury, cisplatin, ureteral obstruction, cyclosporine, radiographic contrast, endotoxaemia, heat-shock, and aristolochic acid). Based on these highly encouraging results, several large randomised controlled trials of EPO therapy in ischaemic or toxic AKI are currently underway. The purpose of this article is to review the experimental and clinical evidence for a renoprotective benefit of EPO in acute kidney injury (AKI), the potential mechanisms underpinning these renoprotective actions and possible future directions for research in this important area.

Keywords: Apoptosis, cytoprotection, darbepoetin, erythropoietin, functional recovery, kidney failure, acute, regeneration, renoprotection, signalling pathways.

INTRODUCTION

Erythropoietin (EPO) is a 30.4 kDa, 165 amino acid, glycoprotein hormone member of the type 1 cytokine superfamily that is produced primarily by renal cortical and outer medullary type 1 fibroblasts [1] in response to tissue hypoxia, *via* stabilisation of the transcriptional regulator, hypoxia-inducible factor-1-alpha (HIF-1α) [2]. Its erythropoietic function occurs by mediation of anti-apoptotic pathways, principally involving Akt and the Bcl-2 gene family, in bone marrow, thereby, facilitating the maturation and differentiation of erythroid progenitor cells [3]. The net effect is a compensatory adaptation to renal tissue hypoxia by augmenting the oxygen-carrying capacity of the blood.

EPO was first detected as a haemopoietic factor in the blood of exsanguinated rabbits [4], but the protein was not isolated until 1977 [5]. Following the cloning of the EPO gene in 1985 [6, 7], commercially available recombinant human EPO (rhEPO) forms, (EPOα, EPOβ, EPOδ, EPOθ, darbepoetin α, and continuous erythropoietin receptor activator [CERA]), have been widely used for the treatment of anaemia of chronic kidney disease (CKD) and cancer chemotherapy-associated anaemia [8]. Over the last 10 years, there has also been a growing body of evidence that rhEPO may have important therapeutic roles beyond anaemia correction in preventing or ameliorating ischaemic or toxic damage to critical organs, such as the kidney, brain and heart [1, 9-12]. The purpose of this article is to review the experimental and clinical evidence for a renoprotective benefit of rhEPO in acute kidney injury (AKI), the potential mechanisms underpinning these renoprotective actions and possible future directions for research in this important area.

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EPO-MEDIATED CYTOPROTECTION

The early unexpected observations of EPO receptor (EPOR) expression by endothelial [13] and neuronal cells [14] in the 1990s prompted speculation that EPO might have important tissue-specific actions beyond its traditional endocrine role of stimulating erythropoiesis. Since this time, EPO receptors have been identified in a large range of cell types, including proximal tubule epithelial cells, mesangial cells, renal cell carcinomas, neurons, microglia, astrocytes, myelin sheaths, endothelial cells, vascular smooth muscle cells, cardiomyocytes, myoblasts, enterocytes, mast cells, pancreatic islet cells, myeloid cells, lymphocytes, megakaryocytes, prostatic cells, breast cancer cells, chorioallantoic membrane, uterine adenocarcinomas and ovarian carcinomas [14-17]. Hypoxia-inducible EPO production regulated by hypoxia-inducible factor-1 (HIF-1) has also been observed in astrocytes in the brain and endometrial cells [18], suggesting that EPO may mediate a number of organ responses to low tissue oxygen tension, beyond simple erythropoiesis. The actions of EPO in these cases may be autocrine-paracrine, as well as endocrine.

In 1993, Konishi *et al.* [19] demonstrated that EPO exerted important neurotrophic effects on mouse embryonic primary septal neurons and a cholinergic hybridoma cell line, SN6.10.2.2, influencing their differentiation, maintenance, regeneration, and survival after serum withdrawal. EPO produced by astroglia was subsequently shown to protect cultured cortical neurons from the effects of hypoxia and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)

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toxicity [20]. Moreover, neutralisation of endogenous EPO with soluble EPOR augmented ischaemic brain damage in an *in vivo* model [21, 22]. However, the relatively low and/or late endogenous production of EPO within the brain may only provide limited protection against hypoxic or toxic brain injury since several studies have demonstrated that systemic administration of a large dose (generally 5000 IU/kg body weight) of rhEPO to rats prior to middle cerebral artery occlusion, common carotid artery occlusion, blunt head trauma, autoimmune encephalomyelitis or neurotoxin exposure substantially reduced, by up to 75%, the extent of subsequent experimental brain injury [14, 22, 23]. Systemic administration of EPO in a rabbit model of subarachnoid haemorrhage also produced significant increases in cerebrospinal fluid EPO concentrations and reduced vasoconstriction of the basilar artery, ischaemic neuronal damage, and subsequent neurological functional deficit [24]. Moreover, the agent was still able to exert significant neuroprotective effects even if administration was delayed up to 6 hours after the onset of cerebral ischaemic injury [23].

rhEPO administration has also been documented to ameliorate ischaemia-reperfusion and mechanical injuries of the spinal cord, retinal neurons, skin, peripheral nerves, gut, lung, bowel, liver, pancreas and heart [16, 17, 25-29]. In all models studied to date, cytoprotection appeared to result from a combination of suppressed apoptosis and stimulated mitogenesis and cellular differentiation. EPO also appeared to mediate a number of additional beneficial effects including stimulation of nitric oxide produced by the action of endothelial nitric oxide synthase (eNOS) [12, 30], stimulation of cytotrophic factors (such as brain-derived neurotrophic factor) [31] and mobilization of endothelial progenitor cells (EPCs) from the bone marrow and subsequent promotion of neovascularisation [12, 32, 33]. EPO may also exert anti-inflammatory actions, either directly by antagonism of pro-inflammatory cytokines (such as tumour necrosis factorα) [9] or indirectly by mitigation of tissue injury [34].

EPO CYTOPROTECTION IN ACUTE KIDNEY INJURY

Endogenous EPO is known to be primarily produced by renal cortical fibroblasts in response to hypoxia [35]. A potential role for a renal paracrine EPO axis with cytoprotective actions was first suggested by the identification of functional EPO receptors on renal tubular epithelial, mesangial and endothelial cells [15]. Specific binding of ^{125}I ligand revealed that the affinity of these receptors (96 pM to 1.4 nM) [15] was well below the normal plasma EPO concentration (~1-10 pmol/L) consistent with observations in other tissues demonstrating paracrine EPO responses [9].

Our group was one of the first to demonstrate that administration of exogenous rhEPO in both *in vitro* and *in vivo* models of ischaemic acute kidney injury (AKI) significantly hastened renal structural and functional recovery [36]. In an *in vitro* model of ischaemia-reperfusion injury, treatment of human proximal tubule cells with 200 IU/mL rhEPO inhibited apoptosis in cells exposed to hypoxia with or without subsequent anoxia. Dosage at 400 U/mL also stimulated cell proliferation in the same model. In an *in vivo* rat model of ischaemia-reperfusion injury, EPOR expression was well maintained during the early phase, whereas endogenous EPO was unchanged or minimal. When rats were treated with rhEPO (5000 U/kg) at the time of ischaemia-reperfusion injury, tubular cell apoptosis was significantly decreased compared with vehicle-treated controls, particularly in the region of the hypoxia-sensitive proximal straight tubule in the outer stripe of the outer medulla [36]. The kidneys of rhEPO-treated animals also demonstrated enhanced tubular epithelial regeneration (mitosis) and decreased tubular luminal cast formation. Peak serum creatinine concentrations were significantly lower in rhEPO-treated rats (21±8 *vs* controls 40±10 μmol/L, $p<0.05$). Although haematocrit readings rose in the rhEPO-treated animals compared with vehicle-treated controls, this increase was not statistically or clinically significant and, therefore, could not have accounted for the renoprotective actions of rhEPO. In a subsequent paper [37], our group demonstrated that the EPO analogue, darbepoetin α, exerted comparable renoprotection to EPOα in bilateral renal ischaemia-reperfusion injury and that the benefits of either agent were still apparent even when their administration was delayed by up to 6 hours after the onset of reperfusion. These findings raised the possibility that both darbepoetin α and EPO may have potential clinical applications as novel renoprotective agents for not only those patients at risk of ischaemic ARF, but also those within a certain time period after having sustained an ischaemic renal insult. These results have also been confirmed by our group in canine [38] and porcine [39] models of ischaemic AKI.

Other investigators have subsequently reported similar findings of augmented renal histologic and functional recovery in animal models of bilateral renal ischaemia-reperfusion injury, as well as in toxin-induced acute renal failure (cisplatin nephrotoxicity, cyclosporine nephrotoxicity), haemorrhagic shock-associated kidney failure, radiographic contrast-induced nephropathy, endotoxaemia, aristolochic acid-induced AKI, and obstructive uropathy (Table **1**). Most of the studies to date have employed EPOα, but comparable renoprotective actions have been demonstrated for EPOβ [40].

In contrast to the universally observed renoprotective benefits of EPO in the aforementioned AKI models, Andratshke *et al.* [41] reported that concomitant EPO administration significantly increased the degree of radiation-induced kidney damage in a C3H mouse model of unilateral kidney irradiation. A rhEPO dose of 2,000 IU/kg body weight per injection tended to cause more damage than 500 IU/kg. The explanation for the observed deleterious effect of EPO on radiation-induced kidney injury is unclear, but may relate to the predominant injury of endothelial cells in this model. Interestingly, an EPO-mediated increase in vascular nitric oxide bioavailability has only been observed in intact vessels, whereas administration of EPO to injured arteries provoked vasoconstriction [12]. Significant endothelial damage, as occurs in radiation-induced kidney injury, may alter the risk:benefit balance of EPO therapy in this setting.

Table 1. Summary of Studies of EPO Therapy in Experimental Kidney Failure

Animal	Model	Treatment	Outcome
Rat [36]	Ischaemia-reperfusion (30 min ischaemia, ≤48h reperfusion)	5000 U/kg EPO IP 30 min before ischaemia	↓Tubular apoptosis ↑ Tubular regeneration ↓ Tubular casts ↓ Plasma creatinine
Human [36]	*In vitro* exposure of proximal tubule cells to 1% O_2 for 24h with or without subsequent normoxia	25 – 400 U/mL EPO incubation for 5h or 24h	↓ Apoptosis ↑ DNA synthesis
Rat [37]	Ischaemia-reperfusion (45 min ischaemia, 1-7d reperfusion)	5000 U/kg EPO or 25 μg/kg darbepoetin ip before ischaemia or 6h after reperfusion	↓Tubular apoptosis ↑ Tubular regeneration ↓ Plasma creatinine ↓ Renal Bax expression
Mouse [54]	Ischaemia-reperfusion (30 min ischaemia, 24h reperfusion)	1000 U/kg EPO sc daily for 3 days before ischaemia or 1000 U/kg EPO 5 min before reperfusion	↓ Plasma creatinine ↓ Histologic injury ↓ Plasma AST levels ↓ Kidney MPO levels ↓ Kidney MDA levels
Rat [67]	Ischaemia-reperfusion (40 min ischaemia, ≤96h reperfusion)	200 U/kg EPO ip 0h and 6h after ischaemia then daily for 48h or 96h	↓ Plasma creatinine ↓ Polyuria ↓ FE_{Na} ↑ AQP, NHE, TSC expression
Rat [68]	Ischaemia-reperfusion (30 or 45 min ischaemia, ≤72h reperfusion)	500 or 3000 U/kg EPO iv before ischaemia and then repeated sc at 24h and 48h post-ischaemia	No benefit on renal function ↑ Survival (severe ischaemia group)
Rat [44]	Ischaemia-reperfusion (45 min ischaemia, 6h reperfusion)	300 U/kg EPO iv 30 min before ischaemia, 5 min before reperfusion or 30 min after ischaemia	↓ Plasma creatinine ↓ Tubular apoptosis
Rat [69]	Ischaemia-reperfusion (45 min ischaemia, ≤72h reperfusion)	3000 U/kg EPO iv 24h prior to ischaemia	↓ Plasma creatinine ↓ Tubular apoptosis ↓ Tubular proliferation
Rat [40]	Ischaemia-reperfusion (45 min ischaemia, 24h reperfusion) + right nephrectomy	1000 U/kg EPO sc 2h before ischaemia	↓ Blood urea nitrogen ↓ Plasma creatinine ↓ LDH
Rat [43]	Ischaemia-reperfusion (45 min ischaemia, 24h reperfusion)	500 U/kg EPO ip 20 min before ischaemia	↓ Plasma creatinine ↓ Tubular apoptosis
Rat [70]	Ischaemia-reperfusion (30 min ischaemia, 1h reperfusion)	1000 U/kg EPO 5 min pre-ischaemia	↓ Histologic injury ↓ Interstitial inflammation ↓ MDA, SOD, catalase
Rat [10]	Ischaemia-reperfusion (30 min ischaemia, 24h reperfusion)	HBSP (0.08-8 nmol/kg) at 1 min, 6 h, and 12 h after reperfusion.	↓ Plasma creatinine
Pig [39]	Unilateral nephrectomy followed 1 week later by Ischaemia-reperfusion (60 min ischaemia, 5 days reperfusion)	5000 U/kg EPO IV at time of ischaemia followed by 1000 U/kg SC daily, or no treatment	↓ Histologic injury ↓ Plasma creatinine
Pig / Mouse [42]	*In vitro* exposure of LLC/PK1 and mouse mesangial cells to prostaglandin D_2 synthase,campothecin, hydrogen peroxide or hypoxia	50 ng/mL darbepoetin incubation for 16h	↓ Tubular apoptosis
Rat [71]	Cis-platinum toxicity	100 U/kg EPO ip daily for 9 days	↑ GFR ↑ Renal blood flow ↑ Tubular regeneration
Rat [72]	Cis-platinum toxicity	100 U/kg EPO ip daily for 9 days	↑ Functional recovery ↑ Tubular regeneration
Rat [73]	Cis-platinum toxicity	100 U/kg EPO ip daily for 9 days	↑ Functional recovery

(Table 1) contd…..

Animal	Model	Treatment	Outcome
Rat [47]	Unilateral ureteral obstruction	3000 U/kg EPO or vehicle IP per day for 3 days	↓ Tubular apoptosis ↓ Inflammatory infiltrates ↓ Inflammatory mediators
Rat [64]	Unilateral ureteral obstruction	EPO or CEPO (500 or 1000 IU/kg) every 2 days for 7 days	↓ Tubular apoptosis ↓ α-smooth muscle actin
Rats [63]	Unilateral ureteral obstruction	EPO or CEPO (5000 U/kg) 1 day prior and days 3, 7 and 10 post	↓ Tubulointerstitial injury ↓ Tubular apoptosis ↓ α-smooth muscle actin
Rat [46]	Cycosporine nephrotoxicity	100 U/kg thrice weekly for 1 or 4 weeks	↓ Tubulointerstitial fibrosis ↓ Apoptosis ↓ Bcl-2 and caspase-3 ↓ Osteopontin and CRP ↓ TGFβ and βIG-H3
Rat [25]	Haemorrhagic schock and endotoxic shock	300 U/kg EPO iv before resuscitation	↓ Renal dysfunction in haemorrhagic, but not endotoxic, shock
Rat [61]	Contrast-induced nephropathy	80 ng/g asialo-EPO (equivalent to 10,0000 U/kf EPO) 1 hour pre-contrast injection	↓ Histologic injury ↓ Apoptosis
Mice [74]	Endotoxaemia	4000 U/kg EPO 30 min pre-LPS administration	↑ GFR ↓ Renal SOD
Pig LLC-PK1 [75]	Aristolochic acid nephrotoxicity	EPO (10 or 20 U/mL) for 24 hours	↓ Apoptosis ↑ Regeneration
Mouse [41]	Radiation-induced kidney injury	EPO (500 or 2000 U/kg)	↑ Histologic injury

Abbreviations: AST, aspartate aminotransferase; AQP, aquaporin; CRP, C-reactive protein; FE_{Na} ; fractional excretion of filtered sodium; βIG-H3, transforming growth factor-β inducible gene-h3; CEPO, carbamylated EPO; GFR, glomerular filtration rate; HBSP, helix B surface peptide; ip, intraperitoneal; iv, intravenous; LDH, lactate dehydrogenase; MDA, malondialdehyde; MPO, myeloperoxidase; NHE, sodium-hydrogen exchanger; sc, subcutaneous; SOD, superoxide dismutase; TSC, thiazide-sensitive cotransporter.

MECHANISMS OF EPO RENOPROTECTION IN AKI

Activation of the EPO receptor mediates renoprotection by activation of multiple signalling pathways (Fig. **1**). As has been shown for other organs, a key cytoprotective mechanism of EPO in AKI is its anti-apoptotic action. Apoptosis of renal tubular epithelial cells plays a major role in AKI following hypoxia, ischaemia-reperfusion, nephrotoxins, ureteral obstruction and sepsis [34]. We [36-39] and others [42, 43] have observed marked inhibition of tubular epithelial cell apoptosis by rhEPO in various models of AKI. EPO exerts its anti-apoptotic effects *via* several pathways. Binding of EPO to its homodimerised receptor allows Janus kinase-2 (Jak2) (a tyrosine kinase bound to the β-subunit of the EPOR) to autophosphorylate, which in turn cascades through to tyrosine phosphorylation of multiple sites on the intracellular portion of the receptor. The phospho-tyrosine residues facilitate binding of proteins containing SH-2 domains. The p85 subunit of phosphatidylinositol-3 kinase (PI3 kinase) interacts with the receptor leading to phosphorylation of Akt (or protein kinase B). Akt in turn promotes cell survival and anti-apoptotic effects *via* inhibition of forkhead transcription factor (FOXO3a), inactivation of glycogen synthase kinase-3β (GSK-3β), reduced activity of pro-apoptotic proteins (Bad, Bax), increased activity of anti-apoptotic proteins (Bcl-2, Bcl-x_L), induced expression of X-chromosome-linked inhibitors of apoptotic protein (XIAP), prevention of activation of caspases-9 and -3, maintenance of mitochondrial membrane potential ($\Delta\Psi m$), prevention of cytochrome C release, and preservation of glycolysis and ATP synthesis [16, 17]. EPOR activation also activates members of the signal transducer and activator of transcription (STAT) family of transcription factors that enhance cell proliferation and survival. STAT5 is the member activated during stimulation of erythropoiesis, but involvement of a specific STAT family member appears to be cell type-specific [9, 34]. JAK2 activation also activates the nuclear transcription factor, NF-κB, which is known to induce a number of antiapoptotic proteins, such as X-linked inhibitor of apoptosis (XIAP), growth arrest, and DNA damage protein 45 (Gadd45β), heat shock protein 70 (HSP-70) and Bcl-X_L. Other key cell survival/anti-apoptotic pathways affected by EPO involve activation of the mitogen-activated protein kinase (MAPK) signal transduction proteins, extracellular signal-regulated survival kinases (ERK) and the receptor activator of NF-κB (RANK/RANK-L) proteins.

Ates and coworkers [40] recently demonstrated that the protective effect of EPO in a rat model of renal ischaemia-reperfusion injury was abrogated by the tyrosine kinase inhibitor, genistein. Sharples *et al.* [44] similarly observed that the anti-apoptotic effects of EPO on human HK-2

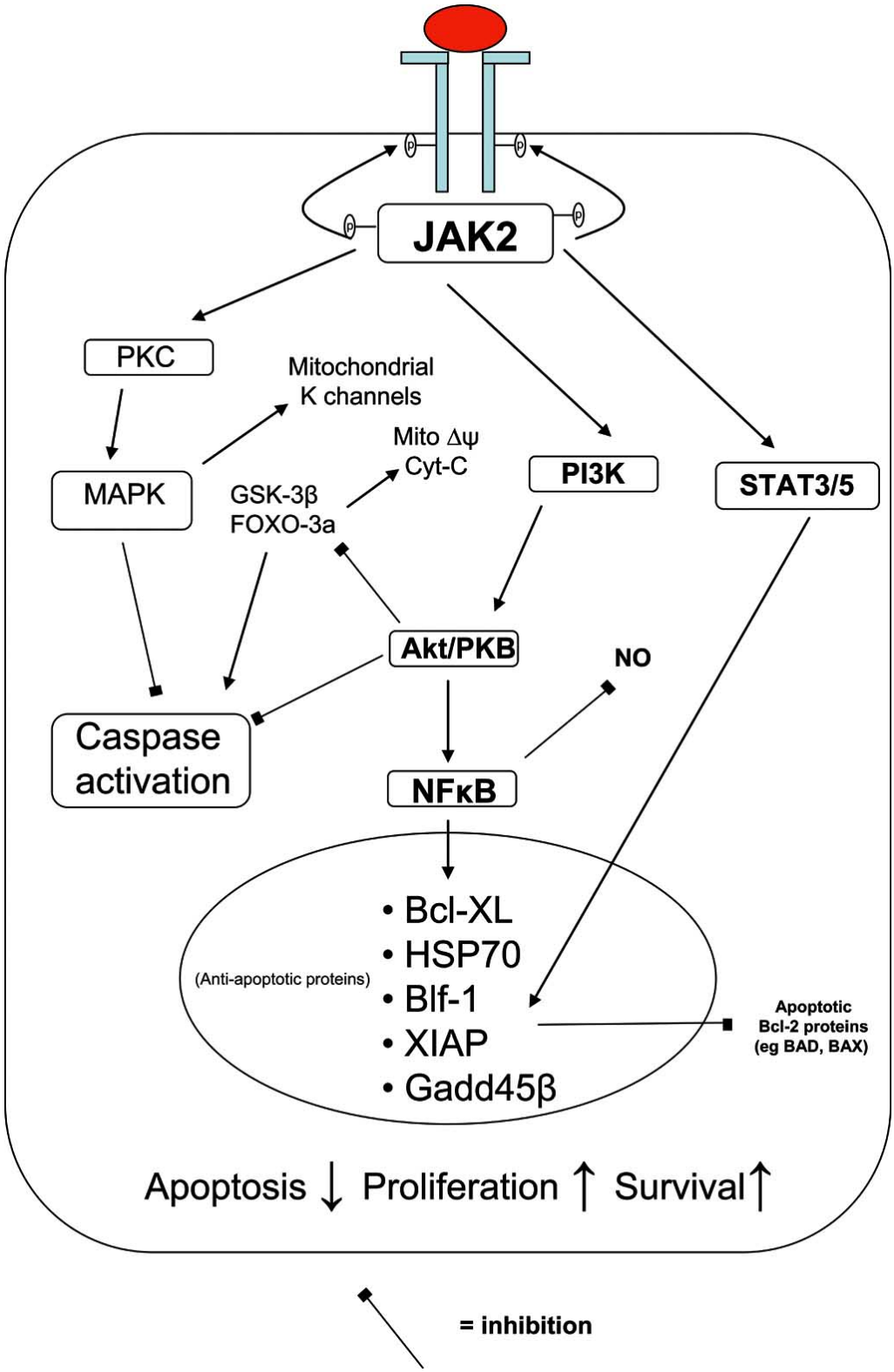

Fig. (1). Activation of the EPO receptor mediates renoprotection by activation of multiple signalling pathways. JAK2 (Janus activated kinase 2) represents the central signalling protein activated by the EPO receptor. The key downstream signalling pathways include STAT5/Bcl-2/Bcl-XL, PI3K/Akt/NFkB and the MAPK family. These pathways lead to an increased level of anti-apoptotic and proliferative proteins that lead to increased cell survival and maintained cell numbers. Abbreviations: Cyt-C, cytochrome C; EPO-R, EPO receptor; FOXO3a, forkhead transcription factor 3a; Gadd45β,growth arrest and DNA damage protein 45; GSK-3β; glycogen synthase kinase-3β; HIF-1, hypoxia-inducible factor-1; HSP-70, heat-shock protein 70; IKK, inhibitor of nuclear factor kappaB kinase; Jak2, Janus activated kinase 2; MAPK, mitogen activated protein kinase; Mito ΔΨm, mitochondrial membrane permeability; PI3K, phosphoinositol 3 kinase; PKB, protein kinase B; PKC, protein kinase C; STAT5, signal transducer and activator of transcription 5; XIAP, X-chromosome-linked inhibitor of apoptosis.

proximal tubule cells were blocked by an inhibitor of Jak2 phosphorylation (tyrphostin or AG490) and by specific inhibitors of PI3K (LY294002 and wortmannin). In an *in vitro* model of contrast-induced nephropathy, inhibition of renal tubular epithelial apoptosis by asialo-EPO was blocked by a JAK2 inhibitor. Our group has also demonstrated that the protection against ischaemic AKI afforded by either EPO or darbepoetin α was associated with decreased expression of the pro-apoptotic protein Bax, although no changes were observed in the expression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L [37]. Spandou *et al.* [43] similarly observed diminished expression of Bax in experimental ischaemic ARF following EPO therapy, whilst Sharples and colleagues [44] reported upregulation of Bcl-X_L and XIAP in serum-starved HK-2 cells incubated with EPO. In an *in vitro* model of aristolochic acid-induced kidney injury, the anti-apoptotic effects of EPO were shown to be mediated by inhibition of caspase-3 activation and upregulation of the expression of the anti-apoptotic gene, Bcl-X_L. EPO and darbepoetin renoprotection has also been associated with prevention of caspase-3, -8 and -9 activation *in vivo* [42, 44].

In addition to its anti-apoptotic actions, EPO exerts important anti-inflammatory actions either *via* a direct effect on pro-inflammatory cytokine production (eg mouse arthritis models) or an indirect effect following reduction of primary injury (eg cerebral cortical ischaemia and reperfusion) (reviewed by Brines and Cerami [45]). The relative importance of each pathway appears to be tissue-dependent [9]. Lee *et al.* [46] demonstrated significant reduction in proinflammatory mediators (osteopontin and C-reactive protein) and profibrotic mediators (transforming growth factor-β1 and transforming growth factor-β1-inducible gene-h3) in cyclosporin-induced kidney injury in rats treated with rhEPO (100 IU/kg thrice weekly). This anti-inflammatory effect was related to rhEPO-induced reduction of apoptosis in the tubular epithelium. Similar findings have been reported in an experimental model of rat unilateral ureteral obstruction [47].

Mobilisation of EPCs by EPO may also protect against AKI. In the vascular endothelium, the reparative response is promoted by rhEPO stimulation of EPCs [33]. Patients with anaemia of CKD receiving standard pharmacological doses of darbepoetin α experienced enhanced EPC mobilisation, proliferation, and differentiation [12]. There is also now considerable interest in mesenchymal stem cells and their potential role in promoting renal regeneration. If the adult kidney contains interstitial mesenchymal cell progenitors with embryonic stromal cell characteristics that are able to provide paracrine support for surrounding vessels and tubular epithelial cells, they may also be stimulated to differentiate into EPO-producing fibroblasts [48]. Bi and coworkers [49] recently demonstrated that administration of rhEPO to mice resulted in the expansion of CD45-Flk1-CD105+ marrow stromal cells in the bone marrow and in the spleen and mobilised these cells, as well as CD45-Flk1+ endothelial progenitor cells into the peripheral circulation. The subsequent amelioration of cisplatin nephrotoxicity by rhEPO was partially reproduced by intraperitoneal injection of cultured EPO-mobilised cells in cisplatin-treated mice. The authors speculated that the *in vivo* expansion and/or activation of stromal cells by rhEPO may have contributed to its renoprotective actions.

Whilst EPO renoprotection might potentially be mediated by stimulation of erythropoiesis and augmented blood O_2-carrying capacity, we [36, 37] and others [42, 44] have observed that the renoprotective benefits of EPO occur prior to, or in the absence of, significant changes in haematocrit. Moreover, other studies have demonstrated that the cytoprotective actions of EPO can be dissociated from its erythropoietic effects since EPO analogues, which do not interact with the homodimerised EPOR, continue to be cytoprotective [9, 50, 51]. It is also unlikely that EPO engenders any favourable haemodynamic changes in the kidney 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 [52].

WHAT IS THE OPTIMAL DOSE OF EPO IN AKI?

Compared with the usual doses of EPO and darbepoetin α employed for correction of anaemia in CKD (50-100 IU/kg and 0.45 μg/kg, respectively), considerably higher doses have generally been employed for cytoprotection in experimental models of organ injury (up to 5000 IU/kg and 25 μg/kg). The standard use of higher doses partly arose because the central nervous system was the first organ investigated in EPO cytoprotection studies and higher systemic concentrations were required to overcome the blood-brain barrier. These doses have carried over into studies of AKI, where most groups have used 1000 IU/kg or more. Despite this, one group [53] reported effective amelioration of progressive renal failure by 0.1 μg/kg darbepoetin α in a subtotal nephrectomy rat model, whilst Sharples *et al.* [44] demonstrated that 300 U/kg EPO significantly reduced kidney dysfunction and histologic damage in ischaemia-reperfusion injury in the rat. Although there have been no *in vivo* studies to determine the optimal renoprotective dose of erythropoetic agents in ischaemic ARF, a previous *in vitro* study by our group demonstrated that the doses of EPO needed to promote maximal anti-apoptotic and mitogenic effects in human proximal tubule cells were quite high (200-400 IU/ml) [36].

WHAT IS THE OPTIMAL TIMING OF EPO DOSING FOR AKI?

As with EPO dosing, the optimal timing of EPO for promoting maximal tissue protection has not been fully elucidated and is likely to be tissue-specific. Typically, the therapeutic window for EPO administration in relation to tissue injury is broad, with many experimental models suggesting that EPO can be administered hours or days after injury to achieve cytoprotective effects that are qualitatively similar to those with immediate administration [9]. Although our previous studies did not find a reduction in renoprotective effect with delayed EPO administration up to 6 hours. Patel *et al.* [54] reported that daily pre-treatment with EPO (1000 IU/kg/day subcutaneously for 3 days) afforded greater renoprotection in a mouse model of renal ischaemia-reperfusion injury than a single bolus (1000 IU/kg subcutaneously) delivered at the time of reperfusion. However, the differences observed between the 2 groups may have been primarily explained by the effects of 3 repeated doses administered to the pre-treatment group rather

than the timing of administration. More work is required to determine the optimal timing of EPO dosing and the size of the therapeutic window for AKI.

WHAT ARE THE POTENTIAL RISKS OF EPO THERAPY?

The beneficial effects of EPO in acute and chronic kidney injury must be balanced against potential adverse effects, particularly as relatively high doses of EPO employed in animal studies translate into boluses of up to 350,000 U and 1750 μg, respectively, for a standard 70 kg man. Such doses have the potential for serious adverse clinical sequelae, such as hypertension, retinopathy, seizures, and thrombosis related to haematocrit increases. Several controlled trials of EPO therapy for anaemia correction in CKD have raised the possibility that achieving higher haemoglobin levels in the context of EPO therapy may be associated with increased cardiovascular events and arteriovenous access thrombosis [9]. Thrombotic events have also been reported to complicate EPO therapy in cancer patients and trauma patients [11] and concerns have been raised about the potential for EPO to stimulate the growth of various tumours [55], including renal cell carcinomas [56]. Recent clinical trials in cancer patients have found that EPO therapy was associated with reduced tumour-free survival [11].

In spite of these concerns regarding the safety of administration of large EPO boluses for cytoprotection, it is reassuring to note that a recent pilot safety study for a multi-centre, randomised controlled trial of EPO in acute stroke demonstrated no adverse effects (including no significant change in haematocrit over 30 days) from a single EPO dose of 100,000 U (approximately 1500 U/kg), despite a transient 500-fold increase in serum EPO concentrations and a 100-fold increase in cerebrospinal fluid EPO concentrations [57]. Moreover, several large randomised controlled trials of EPO administration (40,000 U weekly for up to 3 doses) in critically ill patients have demonstrated improved survival in those receiving EPO [58-60], although one study observed a significant 41% increase in the incidence of thrombotic events [58]. These trials suggest that a similar study of renoprotection in humans is feasible. Alternative strategies for reducing systemic exposure might also be possible, for example by direct perfusion of donor kidneys in renal transplantation.

CAN THE CYTOPROTECTIVE ACTIONS OF EPO BE DISSOCIATED FROM ITS ERYTHROPOIETIC EFFECTS?

Several engineered EPO variants have been developed that are cytoprotective, but do not significantly stimulate erythropoiesis. Asialo-EPO binds to the EPOR, but has an extremely short half-life. It remains in the circulation for only a few minutes, which is sufficient to trigger tissue-protective responses, but does not provide the requisite sustained levels to promote erythropoiesis or other EPO-dependent effects, such as thrombopoiesis and thrombosis [9]. Yokomaku and associates [61] reported that a single dose of asialo-EPO prevented contrast-induced renal dysfunction and renal tubular injury without stimulating haematocrit. Asialo-EPO has also been shown to be as effective as rhEPO (500 IU/kg) in preventing bilateral ischaemia-reperfusion AKI in mice [62].

The cytoprotective and erythropoietic effects of EPO can also be dissociated by carbamylating lysine residues within the binding sites of EPO (CEPO). CEPO binds with low affinity to the homodimerised EPOR thereby reducing its erythropoietic and procoagulant effects [9]. However, CEPO is still able to stimulate cytoprotective pathways *via* its interaction with the cell membrane heterodimer complex of EPOR and the common cytokine β-receptor subunit (CD131) [9]. Several investigators have demonstrated that CEPO was able to inhibit tubular epithelial apoptosis and subsequent tubulointerstitial injury without promoting erythropoiesis in animal models of ureteral obstruction [63, 64] and ischaemia-reperfusion AKI [65]. CEPO has also been shown to lack the procoagulant, proangiogenic, and vasoactive properties of EPO and to have no significant adverse effects on systemic blood pressure or renal blood flow [66].

Recently, an 11-amino acid peptide composed of adjacent amino acids forming the aqueous face of helix B of the EPO molecule (HBSP) has been found to be tissue-protective without stimulating erythropoiesis [9]. In a mouse model of ischaemia-reperfusion AKI, intraperitoneal administration of pyroglutamate HBSP (pHBSP) at 1 minute, 6 hours and 12 hours produced dose-dependent renoprotection, with the highest dose (8 nmol/kg) exhibiting nearly normal serum creatinine levels 24 hours post-injury [10].

CLINICAL TRIALS OF EPO IN AKI

Based on these encouraging findings to date in animal models, a randomised, placebo-controlled trial of EPO therapy in AKI in patients at high risk of ischaemic acute renal failure is currently underway in New Zealand (http://www.anzctr.org.au/trial_view.aspx?ID=1009). The aim of this trial is to randomise 130 general and cardiac ICU patients with urinary gammaglutamyltranspeptidase x alkaline phosphatase indices > 46.3 to either rhEPO (EPOβ two 500 IU/kg IV 24 hours apart; maximum dose 100,000 IU) or placebo. The primary outcome measure will be serum creatinine concentration after 7 days, although patients will be followed for up to 12 months following enrolment. The secondary outcome measures include dialysis-free survival, dialysis-free interval, duration of ICU stay, mortality rate, and serum creatinine/eGFR at 30 days.

Several other clinical trials of EPO cytoprotection are also underway in acute kidney injury (ERIN NCT00476619, ICU patients NCT00676234, post-cardiac surgery NCT00654992, renal transplantation NCT00425698), acute stroke (BETAS trial NCT00362414, EPOα in acute stroke NCT00604630, CEPO in acute stroke NCT00756249, REGENESIS NCT00663416) and acute mycocardial infarction (erythropoietin in acute myocardial infarction [EAMI] NCT00149058, Erythro Poetin in Myocardial Infarction [EPOMI] NCT00648089, HEBEIII NCT00449488, MAGIC Cell-5-Combicytokine Trial NCT00501917, NCT00378352).

CONCLUSIONS

There is now abundant evidence that administration of rhEPO at the time of, or up to 6 hours after, experimental AKI significantly augments histologic and functional

recovery. These renoprotective effects appear to be primarily mediated by targets of EPOR signalling pathways including Akt, STAT5 and MAPK leading to inhibition of tubular epithelial cell apoptosis. Other beneficial cytoprotective effects exerted by EPO may also include stimulation of mitogenesis, mobilisation and differentiation of EPCs and suppression of proinflammatory cytokine mediators. As a result of concerns regarding the possible procoagulant, proangiogenic and hypertensive effects of short-term, high-dose EPO administration, variants have been engineered which appear to provide comparable levels of renoprotection without stimulating erythropoiesis. Several large randomised, controlled trials of EPO administration in AKI are underway and the results of such studies are eagerly awaited.

CONFLICT OF INTEREST

Prof Johnson has received consultancy fees, research grants and speakers' honoraria from Amgen, Janssen-Cilag and Roche.

ABBREVIATIONS

AKI	=	Acute kidney injury
AMPA	=	α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AQP	=	Aquaporin
ARF	=	Acute renal failure
AST	=	Aspartate aminotransferase
ATP	=	Adenosine triphosphate
βIG-H3	=	Transforming growth factor-β inducible gene-h3
CD131	=	Common cytokine β-receptor subunit
CEPO	=	Carbamylated erythropoietin
CKD	=	Chronic kidney disease
CRP	=	C-reactive protein
cyt-C	=	Cytochrome C
eNOS	=	Endothelial nitric oxide synthase
ERK	=	Extracellular signal-regulated survival kinases
EPC	=	Endothelial progenitor cell
EPO	=	Erythropoietin
EPOR	=	Erythropoietin receptor
FE_{Na}	=	Fractional excretion of filtered sodium
FOXO3a	=	Forkhead transcription factor 3a
Gadd45β	=	Growth arrest and DNA damage protein 45
GFR	=	Glomerular filtration rate
GSK-3β	=	Glycogen synthase kinase-3β
HBSP	=	Helix B surface peptide
HIF-1	=	Hypoxia-inducible factor-1
HSP-70	=	Heat-shock protein 70
IKK	=	Inhibitor of nuclear factor kappaB kinase
ip	=	Intraperitoneal
iv	=	Intravenous
Jak2	=	Janus activated kinase 2
LDH	=	Lactate dehydrogenase
MAPK	=	Mitogen activated protein kinase
MDA	=	Malondialdehyde
MPO	=	Myeloperoxidase
Mito ΔΨm	=	Mitochondrial membrane permeability
NCT	=	National clinical trials registration number
NHE	=	Sodium-hydrogen exchanger
pHBSP	=	Pyroglutamate helix B surface peptide
PI3K	=	Phosphoinositol 3 kinase
PKB	=	Protein kinase B
PKC	=	Protein kinase C
rhEPO	=	Recombinant human erythropoietin
sc	=	Subcutaneous
SOD	=	Superoxide dismutase
STAT5	=	Signal transducer and activator of transcription 5
TSC	=	Thiazide-sensitive cotransporter
XIAP	=	X-chromosome-linked inhibitor of apoptosis.

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