

Discovering erythropoietin's extra-hematopoietic functions: Biology and clinical promise

M Brines¹ and A Cerami¹

¹The Kenneth S Warren Institute and Warren Pharmaceuticals, Ossining, New York, USA

A greatly expanded understanding of the biology of endogenous erythropoietin (EPO) has emerged since the early 1990s. Originally viewed as the renal hormone dedicated to erythrocyte production, it is now clear that EPO is produced locally by many other tissues in response to physical or metabolic stress. In its autocrine–paracrine roles, EPO mediates preconditioning (ischemic tolerance) and specifically limits the destructive potential of tumor necrosis factor α and other proinflammatory cytokines in the brain, heart, kidney, and other tissues. As local production of EPO is generally suppressed following injury, administration of exogenous EPO has been a successful therapeutic approach in preclinical and clinical studies, for example, following ischemia–reperfusion and toxin-induced renal injuries, and in human stroke. The therapeutic time window of tissue protection by EPO is typically very wide in experimental models, showing effectiveness when administered before, during, or after an insult and raising optimism for a high clinical potential. Although there is progress in understanding the signaling pathways responsible for EPO's tissue-protective actions that are similar to, but not as redundant as, those employed for erythrocyte maturation, much work remains to be carried out. Experimental observations also suggest the existence of EPO receptor (EPOR) isoforms mediating EPO's diverse biological activities and have identified a tissue-protective receptor complex consisting of the EPOR and the beta common receptor (CD131) subunit that is also employed by granulocyte-macrophage colony-stimulating factor, interleukin-3 and interleukin-5. Successfully engineered analogues of EPO that selectively activate tissue protection without stimulating hematopoiesis confirm the concept of a tissue-protective receptor and have significant potential utility in the investigational and therapeutic arenas.

Kidney International advance online publication, 31 May 2006;
doi:10.1038/sj.ki.5001546

KEYWORDS: apoptosis; erythropoietin; ischemia-reperfusion; renal injury; stem cell

Correspondence: M Brines, The Kenneth S Warren Institute and Warren Pharmaceuticals, 712 Kitchawan Road, Ossining, New York 10562, USA.
E-mail: mbrines@kswi.org

Received 21 March 2006; accepted 28 March 2006

ENDOCRINE VERSUS PARACRINE

Classic studies established long ago that the adult kidney is the source of the circulating erythropoietin (EPO) that maintains erythrocyte mass (reviewed by Fisher¹). In this role, EPO prevents programmed cell death (apoptosis) of erythrocyte precursors in a negative feedback control fashion, typical of an endocrine hormone. However, beginning with the unexpected observations of EPO receptor (EPOR) expression by the endothelial cell² and within the central nervous system,³ as well as the capacity for EPO production by astrocytes,⁴ a broader concept of EPO as a paracrine–autocrine tissue-protective molecule has emerged over the last 15 years. Simply put, metabolic stress triggers apoptosis in tissues as an adaptive response to limit the spread of infection by destroying surrounding tissue and EPO is an antagonist of this biological process (reviewed by Brines and Cerami⁵). This innate immune response works well with true infection, but results in more damage in the setting of trauma, ischemia, or toxins through excessive apoptosis of viable tissue within the region immediately surrounding an injury. Further, EPO also antagonizes the activities of proinflammatory cytokines and promotes healing following injury (e.g., by stimulating capillary growth). However, because of a significant temporal delay in EPO gene expression after an injury (hours) and the direct suppression of EPO production by inflammatory cytokines, a maximum protective benefit is only achieved by administration of exogenous EPO.

An initial understanding of the biology of EPO-mediated tissue protection largely developed from study of the nervous system, a tissue of a high basal metabolic rate and therefore an enhanced susceptibility to ischemic injury. The findings derived from the nervous system, however, also broadly apply to other tissues and organs. Like the nervous system, the normal kidney is characterized by regions in which energy substrates are limited. Commonly, chronic renal hypoxia with subsequent tubulointerstitial injury establishes a vicious cycle leading if not prevented to end-stage renal failure. Principal mediators of injury are highly reactive free radicals produced as by-products of cellular metabolism under relative hypoxic condition. Endogenous, locally produced EPO is active in preventing injury under these circumstances. Mechanistically, hypoxia activates the hypoxia inducible factor (HIF) family of proteins that turn on a number of protective genes, including EPO (reviewed by Maxwell⁶).

Direct activation of HIF-1, for example by mild ischemia in advance of potential injury, provides considerable protection from subsequent actual injury. However, HIF-1-activated gene expression is actually relatively sluggish considering the pathological processes that injury triggers. Additionally, HIF-1 activation also includes molecules that can produce maladaptive effects, such as vascular endothelial growth factor-induced vasogenic edema. Many studies have shown that exogenous EPO administered before or after injury is highly effective for the prevention of permanent or transient ischemic injury in multiple organs and tissues. The findings from studies of ischemic injury appear to apply generally to other forms of injury, including trauma, toxins, and infection (Figure 1; reviewed by Brines and Cerami⁵).

EPO AND THE KIDNEY

A potential role for the non-hematopoietic activities of EPO in the kidney was first implied by the identification of EPOR protein expressed throughout the kidney, including both proximal and distal tubular cells.⁷ However, the affinity of these receptors (~ 1 nM) is well below the normal plasma EPO concentration (~ 1 – 10 pM), consistent with observations in other tissues responding to EPO in a paracrine fashion. These receptors have such a low probability of occupancy by circulating EPO that they are functionally isolated from the hematopoietic system. However, it is currently unclear what contribution (if any) to the local intra-renal EPO content is provided by the fibroblast-like interstitial cells that produce circulating EPO. It will be interesting to examine the pharmacological characteristics of the EPOR within this microdomain.

EPO administration elicits physiological effects within the normal kidney not primarily associated with tissue protection and these could have important implications in certain clinical situations. One prominent EPO target is the vascular smooth muscle cell (VSMC) in which EPO increases

intracellular calcium and causes VSMC contraction *in vitro* and constriction *in vivo*, significantly increasing peripheral vascular resistance.⁸ Clinically, EPO has variable effects on systemic blood pressure and vascular resistance.⁹ A substantial proportion ($\sim 35\%$) of patients with end-stage renal disease will exhibit a dose-dependent increase in blood pressure following EPO administration. Observations using preclinical models have been variable, with some groups finding large increases in blood pressure and others only moderate or no effect.⁹ Some blood pressure effects may arise from modulation of nitric oxide production, as EPO has been reported to induce changes in nitric oxide synthase directly or indirectly by stimulating the production of asymmetrical dimethyl arginine, an endogenous inhibitor of nitric oxide synthase. Further, acute EPO administration reduces cortical renal blood flow and urinary solute excretion.⁹ However, potential effects on renal nerves apparently have not been assessed. Any or all of these effects could be important in the setting of injury, but have not been extensively studied.

THE CAPILLARY ENDOTHELIUM

Capillary endothelial cells obtained from a variety of tissues express EPO and EPOR and were the first cells implicated experimentally as targets of the extra-hematopoietic activities of EPO.² Endothelial cells respond to local ischemia by producing EPO. Therefore, these cells distributed universally throughout tissues could potentially provide EPO-mediated protective function globally. The endothelial cell expresses a receptor with an affinity for EPO (~ 1 nM) intermediate between the erythrocyte precursor (~ 200 pM) and neuronal cells (~ 10 – 20 nM). Regulation of the endothelial cell EPO/EPOR has been studied, and under hypoxia, EPO strongly stimulates the expression of EPOR.¹⁰ For tissues characterized by a relatively impermeant endothelial barrier, such as the blood-brain barrier, EPO has been shown to increase cell surface proteins maintaining tight junctions *in vitro*.¹¹ A large

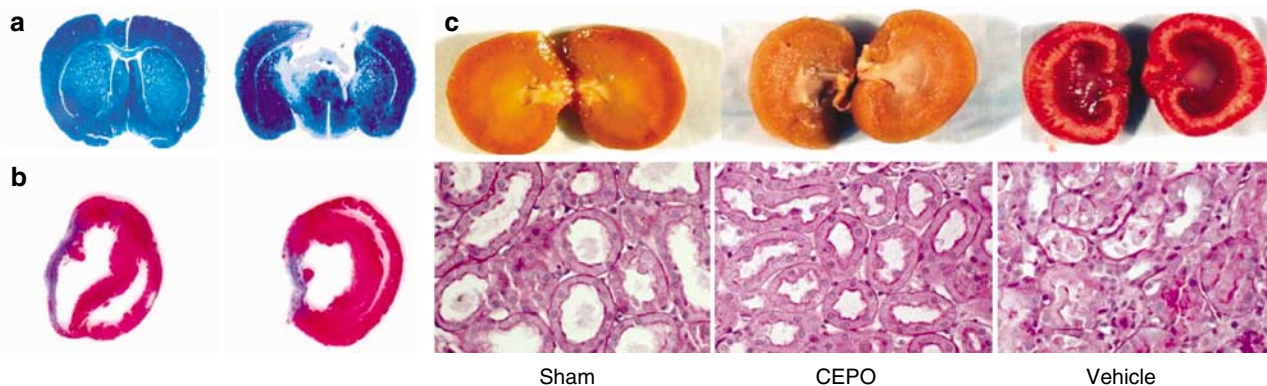


Figure 1 | Examples of gross pathology exhibiting beneficial effects of EPO or the non-erythropoietic molecule carbamylated EPO (CEPO) for tissue injury models in rodents. (a) EPO administered as a single dose (5000 U/kg-bw intraperitoneally) immediately following traumatic injury to mouse brain markedly reduces tissue loss (left) when evaluated 10 days following injury (reproduced with permission from *Proc Natl Acad Sci USA*). **(b)** Cross-sections of the heart obtained 24 h following injury from a rat model of myocardial ischemia (30 min) with reperfusion administered carbamylated EPO (10 μ g/kg-bw at reperfusion). Masson trichrome staining shows intact muscle (red) and scar (blue). **(c)** Carbamylated EPO (10 μ g/kg-bw intravenously at reperfusion) protects rat kidney from 60 min of ischemia (renal artery occlusion) followed by reperfusion for 48 h. The vehicle-treated kidney is severely necrotic, exhibiting widespread tubular destruction (original magnification $\times 100$).

contribution to tissue protection following injury is likely attributable to EPO-mediated survival of the capillary endothelium itself from destruction by microinfarction.

EPO also strongly mobilizes and regulates endothelial progenitor cells^{9,12} and recent interest has focused on a potential principal role of EPO in endothelial progenitor cell recruitment, enhancement of adhesiveness, proliferation, and promoting repair of the endothelium as well as angiogenesis in the setting of tissue injury.

EPO AND RENAL INJURY

A number of groups have shown that EPO or darbepoietin administered in preclinical models at high dose (300–5000 U/kg-bw intraperitoneally) is substantially protective of ischemia–reperfusion injury as assessed by residual renal function and histological markers of injury (reviewed by Chatterjee¹³). Similar to its effects in nervous tissue, EPO strongly suppresses the inflammatory response to ischemia–reperfusion in the kidney, reducing proinflammatory cytokine production, subsequent leukocyte recruitment, and amplification of damage. EPO is also renoprotective against toxins such as cyclosporine and cisplatin (reviewed by Chatterjee¹³). In these acute experiments, there were no increases in hemoglobin concentration so that the action of EPO was a direct one on the kidney.

A number of clinical trials have concluded that early EPO administration to predialysis patients slows the progression of kidney disease. However, the chronic administration of EPO also increases hemoglobin levels and complicates assignment of cause and effect. For example, one clinical trial in which the efficacy of EPO was evaluated in a predialysis population observed that patients in the EPO arm had a 60% reduction of the risk of initiating renal replacement or death.¹⁴ However, as the EPO group also had a higher hemoglobin concentration (12.9 versus 10.3), the beneficial effect of receiving EPO was ascribed to the increased erythrocyte number. In contrast to studies where patients received EPO chronically, virtually no clinical studies concerning tissue protection following acute EPO administration exist. One exception is a successful proof of concept trial of EPO in human stroke. In this study, EPO (100 000 U administered intravenously, divided over 3 days) has been shown to be effective for improving the short-term clinical outcome of patients with middle cerebral arterial infarction, without any acute changes in hemoglobin concentrations.¹⁵ Further clinical trials will need to be designed with the potential direct or indirect effects in mind.

PHARMACOLOGICAL APPROACHES TO TISSUE PROTECTION

Although EPO presumably is the only endogenous ligand mediating tissue protection through the EPOR, its therapeutic utility could be complicated by increases in red cell mass and development of a prothrombotic state. One approach to avoid this problem arose from an appreciation that effective production of erythrocytes requires a continuous presence of EPO in the circulation.¹⁶ The EPO molecule is highly

glycosylated and the sialic acid moieties terminating the oligosaccharide chains prevent rapid clearance from the plasma. Selective tissue protection without activating erythropoiesis can be accomplished using desialated EPO which has a very short half-life. In multiple preclinical models, this analogue demonstrates potent tissue protection without any detectable stimulation of the bone marrow.¹⁶ AsialoEPO is also a useful reagent to assess whether components of tissue protection depend upon triggering gene expression, as this compound disappears from the circulation after several minutes.

Another approach for selectively targeting tissue protection was predicted from the mismatch between the affinity of the non-hematopoietic, tissue EPO receptor and the much lower levels of circulating EPO. EPO is a member of the type 1 cytokine superfamily that is characterized by multifunctionality mediated by different assemblages of receptor subunits. Our group has identified signaling via a putative EPO receptor isoform different from the homodimer mediating erythropoiesis, consisting of a heteromeric complex containing EPOR and the beta receptor subunit (CD131) common to granulocyte–macrophage colony-stimulating factor, interleukin-3, and interleukin-5.¹⁷ This was substantiated by demonstrating that knockout of the beta common receptor gene fully abolishes tissue protective properties of EPO in the nervous system and heart.

The presence of another receptor isoform clearly raises the possibility of developing ligands with differential affinity to receptor subtypes. The EPO receptor isoform that transduces erythropoiesis has been extensively studied and consists of a preformed homodimer to which EPO binds via two well-defined sites.¹⁸ The interaction between EPO and its homodimeric receptor isoform can be abolished by chemical modification of lysine, or mutation of critical amino-acid residues within the binding sites. Engineered molecules so produced exhibit no affinity for the homodimeric EPO receptor isoform, as expected, but are equipotent to EPO for protection of tissues from injury.¹⁶

MECHANISMS OF TISSUE PROTECTION

Abundant evidence has accumulated in studies of injury in the nervous system and heart that EPO is antiapoptotic, similar to its endocrine actions within the bone marrow. Additionally, EPO has a marked effect on inflammatory processes stimulated by injury (reviewed by Brines and Cerami⁵). In some cases, for example, cerebral cortical ischemia and reperfusion,¹⁹ anti-inflammatory effects arise secondarily via a reduction of primary injury that in turn reduces the production of proinflammatory cytokines such as tumor necrosis factor α . In other models, EPO has a direct effect on proinflammatory cytokine production.²⁰ The precise interaction and relative importance of each pathway likely differs for each specific tissue. The implication of locally produced tumor necrosis factor as a major factor in the pathophysiology of renal ischemia–reperfusion injury (reviewed in Donnahoo *et al.*²¹) suggests that EPO is likely to

reduce renal injury partly by inhibiting proinflammatory cytokines. It is notable that recent work also shows that in some tissues suppression of proinflammatory cytokine expression alone (e.g., by glucocorticoids in the spinal cord²²) is not sufficient to prevent tissue injury. Thus, the contribution of anti-inflammatory effects to injury reduction may be less than that initially thought. EPO may also directly stimulate other protective molecules, for example, BDNF, which explains some of the beneficial effects of EPO within the nervous system.²³

CELLULAR MEDIATORS OF PROTECTION

Multiple, complicated, and often conflicting observations have been made concerning signaling pathways activated by EPO in mediating tissue protection. Much of the early investigation was driven by knowledge of pathways activated in the prevention of apoptosis of erythrocyte precursors and not surprisingly, similarities were identified. Subsequent study has identified certain pathways not shared. Undoubtedly, some observed differences derive from the tissue or cell studied and the particular methodology employed. Unlike erythropoiesis, survival pathways do not appear to be redundant, as inhibition of any single pathway generally blocks tissue protection.

Three main signaling pathways are involved in mediating tissue protection (reviewed by Brines and Cerami⁵). (1) The Jak2-STAT-Bcl-2 pathway, a dominant EPO-dependent anti-apoptotic mechanism for hematopoietic cells, also has been implicated in tissue protection. A number of investigators have identified regulation of this pathway, either by induction of antiapoptotic molecules (Bcl-2 and Bcl-X_L) or decrease in proapoptotic molecules (bax, bak). (2) EPO-mediated activation of the survival kinases extracellular signal-regulated kinase and Akt are critical in the nervous system. The neuroprotective effect of EPO involves the Ras/mitogen-activated protein kinase or the phosphatidylinositol 3'-kinase pathway and selective inhibitors of mitogen-activated protein kinase and phosphatidylinositol 3'-kinase block the phosphorylation of extracellular signal-regulated kinases and Akt/Protein kinase B and prevent the effect of EPO, as has been shown by multiple workers using different model systems *in vitro* and *in vivo*. (3) The nuclear factor- κ B system: Jak2-dependent activation of nuclear factor- κ B has been proposed to mediate neuroprotection of cortical neurons *in vitro* as inhibition of these messengers blocked protection. Activation of nuclear factor- κ B has also been implicated by others, although some studies suggest that nuclear factor- κ B does not play a role in the antiapoptotic effect of EPO for certain neuronal cell lines.

An additional comment concerning tissue protection pathways is warranted. For some of these pathways, EPO was shown to reverse the decrease in these pathways induced by the noxious stimulus, rather than directly activating the pathway. This was shown, for instance, in hippocampal neurons *in vitro* where hypoxia decreased the expression of the phosphorylated forms of Stat5, Akt, and extracellular

signal-regulated kinase, and EPO restored their levels.²⁴ Further examination of signaling mechanisms is warranted.

TIMING AND DOSAGE CONSIDERATIONS FOR TISSUE PROTECTION

Optimum timing and size of dose required for tissue protection has been incompletely explored and likely is significantly tissue-specific. Clearly, the presence or absence of receptors for EPO within the target tissue at baseline is a critical issue. In general, EPO and non-erythropoietic tissue-protective cytokines do not appear to directly modulate EPOR levels in normal tissues. However, in the setting of hypoxia, EPO has been observed to increase message for its own receptor in endothelial cells.¹⁰ Some tissues have only low levels of EPOR in the absence of ischemia (e.g., cerebral cortex). Pretreatment by EPO or its tissue-protective analogues provides significant protection in some tissues, for example, the heart, in which exposure either immediately (<1 h; activating acute preconditioning) or 24 h before (triggering delayed preconditioning) reduced subsequent ischemic-reperfusion injury (reviewed by Baker²⁵). The possibility for successful prevention of tissue damage by pretreatment with EPO is obviously attractive for addressing iatrogenic injury, such as that produced by surgical procedures.

With respect to dosing, generally most preclinical models have used very high doses compared to that used for treatment of anemia (but there are exceptions, e.g. Bahlmann *et al.*²⁶ used 0.1 μ g/kg of darbepoietin to attenuate progressive renal failure in a 5/6 rat nephrectomy model). The use of high doses became standard presumably arose because the central nervous system was the first organ for which peripheral administration of EPO was shown to protect from injury.²⁷ For tissues characterized by tight endothelial barriers, like the central nervous system, minimum effective doses of EPO are very high \sim 500 U/kg-bw administered intraperitoneally or intravenously. Other tissues, for example, the heart and kidney, have much lower minimum effective doses falling within the dosing range typically employed clinically for the treatment of anemia (10–50 U/kg-bw; reviewed by Chatterjee¹³). Much more work needs to be carried out concerning dose-ranging and pharmacodynamics using preclinical models appropriate for supporting potential clinical studies.

FUTURE PROSPECTS

Abundant preclinical data are supportive of a potential role for EPO in treatment of clinical diseases of diverse etiology. Although attempted translation of these findings into the clinic has only just started, a successful proof of concept trial in stroke caused by middle cerebral artery occlusion is reassuring. Many investigator-initiated trials for a number of indications (e.g., multiple sclerosis, stroke, and acute myocardial infarction) are currently underway and will provide additional information with which to conduct further clinical evaluation. The identification of functionally

distinct receptor isoforms of EPO mediating independent hematopoietic and tissue-protective effects has enabled the development of compounds specifically tailored for tissue-protective use. The likely requirement of high doses EPO needed to achieve adequate drug concentration in some tissues, coupled with the well-known dose-dependent potential adverse effects of EPO, especially thrombosis, suggest that the specifically engineered non-erythropoietic tissue-protective agents could offer significant advantages over EPO itself.

REFERENCES

1. Fisher JW. A quest for erythropoietin over nine decades. *Annu Rev Pharmacol Toxicol* 1998; **38**: 1–20.
2. Anagnostou A, Lee ES, Kessimian N *et al*. Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. *Proc Natl Acad Sci USA* 1990; **87**: 5978–5982.
3. Digicaylioglu M, Bichet S, Marti HH *et al*. Localization of specific erythropoietin binding sites in defined areas of the mouse brain. *Proc Natl Acad Sci USA* 1995; **92**: 3717–3720.
4. Marti HH, Wenger RH, Rivas LA *et al*. Erythropoietin gene expression in human, monkey and murine brain. *Eur J Neurosci* 1996; **8**: 666–676.
5. Brines M, Cerami A. Emerging biological roles for erythropoietin in the nervous system. *Nat Rev Neurosci* 2005; **6**: 484–494.
6. Maxwell P. HIF-1: an oxygen response system with special relevance to the kidney. *J Am Soc Nephrol* 2003; **14**: 2712–2722.
7. Westenfelder C, Biddle DL, Baranowski RL. Human, rat, and mouse kidney cells express functional erythropoietin receptors. *Kidney Int* 1999; **55**: 808–820.
8. Schiff H, Lang SM. Hypertension induced by recombinant human erythropoietin (rHU-EPO) can be prevented by indomethacin. Pathogenic role of cytosolic calcium. *Eur J Med Res* 1997; **2**: 97–100.
9. Coleman T, Westenfelder C, Toegel F *et al*. Cytoprotective doses of erythropoietin or carbamylated erythropoietin possess markedly different procoagulant and vasoactive activities. *Proc Natl Acad Sci USA* 2006; **103**: 5965–5970.
10. Beleslin-Cokic BB, Cokic VP, Yu X *et al*. Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells. *Blood* 2004; **104**: 2073–2080.
11. Martinez-Estrada OM, Rodriguez-Millan E, Gonzalez-De Vicente E *et al*. Erythropoietin protects the *in vitro* blood–brain barrier against VEGF-induced permeability. *Eur J Neurosci* 2003; **18**: 2538–2544.
12. Bahlmann FH, De Groot K, Spandau JM *et al*. Erythropoietin regulates endothelial progenitor cells. *Blood* 2004; **103**: 921–926.
13. Chatterjee PK. Pleiotropic renal actions of erythropoietin. *Lancet* 2005; **365**: 1890–1892.
14. Gouva C, Nikolopoulos P, Ioannidis JP *et al*. Treating anemia early in renal failure patients slows the decline of renal function: a randomized controlled trial. *Kidney Int* 2004; **66**: 753–760.
15. Ehrenreich H, Hasselblatt M, Dembowski C *et al*. Erythropoietin therapy for acute stroke is both safe and beneficial. *Mol Med* 2002; **8**: 495–505.
16. Erbayraktar S, Grasso G, Sfacteria A *et al*. Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity *in vivo*. *Proc Natl Acad Sci USA* 2003; **100**: 6741–6746.
17. Brines M, Grasso G, Fiordaliso F *et al*. Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. *Proc Natl Acad Sci USA* 2004; **101**: 14907–14912.
18. Cheetham JC, Smith DM, Aoki KH *et al*. NMR structure of human erythropoietin and a comparison with its receptor bound conformation. *Nat Struct Biol* 1998; **5**: 861–866.
19. Villa P, Bigini P, Mennini T *et al*. Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis. *J Exp Med* 2003; **198**: 971–975.
20. Cuzzocrea S, Mazzon E, di Paola R *et al*. Erythropoietin reduces the degree of arthritis caused by type II collagen in the mouse. *Arthritis Rheum* 2005; **52**: 940–950.
21. Donnahoo KK, Shames BD, Harken AH *et al*. Review article: the role of tumor necrosis factor in renal ischemia–reperfusion injury. *J Urol* 1999; **162**: 196–203.
22. Gorio A, Madaschi L, Di Stefano B *et al*. Methylprednisolone neutralizes the beneficial effects of erythropoietin in experimental spinal cord injury. *Proc Natl Acad Sci USA* 2005; **102**: 16379–16384.
23. Viviani B, Bartesaghi S, Corsini E *et al*. Erythropoietin protects primary hippocampal neurons increasing the expression of brain-derived neurotrophic factor. *J Neurochem* 2005; **93**: 412–421.
24. Siren AL, Fratelli M, Brines M *et al*. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc Natl Acad Sci USA* 2001; **98**: 4044–4049.
25. Baker JE. Erythropoietin mimics ischemic preconditioning. *Vascul Pharmacol* 2005; **42**: 233–241.
26. Bahlmann FH, Song R, Boehm SM *et al*. Low-dose therapy with the long-acting erythropoietin analogue darbepoetin alpha persistently activates endothelial Akt and attenuates progressive organ failure. *Circulation* 2004; **110**: 1006–1012.
27. Brines ML, Ghezzi P, Keenan S *et al*. Erythropoietin crosses the blood–brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci USA* 2000; **97**: 10526–10531.