

Emerging biological roles for erythropoietin in the nervous system

Michael Brines and Anthony Cerami

Abstract | Erythropoietin mediates an evolutionarily conserved, ancient immune response that limits damage to the heart, the nervous system and other tissues following injury. New evidence indicates that erythropoietin specifically prevents the destruction of viable tissue surrounding the site of an injury by signalling through a non-haematopoietic receptor. Engineered derivatives of erythropoietin that have a high affinity for this receptor have been developed, and these show robust tissue-protective effects in diverse preclinical models without stimulating erythropoiesis. A recent successful proof-of-concept clinical trial that used erythropoietin to treat human patients who had suffered a stroke encourages the evaluation of both this cytokine and non-erythropoietic derivatives as therapeutic agents to limit tissue injury.

Exactly which factors limit the spread of injury around a lesion has been a long-standing question. Surprisingly, recent studies have shown that the cytokine erythropoietin (EPO) is a crucial mediator of injury-related tissue protection in mammals. Although it was clear a century ago that the production of erythrocytes was modulated by a humoral factor, it was not possible to successfully isolate erythropoietin until 1977 (REF. 1), due to its extremely low concentrations in the serum of healthy humans (FIG. 1). Erythropoietin, which is produced by all vertebrates investigated so far, is a 165 amino acid (~30 kDa) glycoprotein that is a member of the type I cytokine superfamily. Initially, it was believed that the only function of erythropoietin was to maintain optimal tissue oxygenation by regulating the number of erythrocytes in a negative-feedback control system that operates between the kidney and the bone marrow (FIG. 2, red-shaded region). Tissue hypoxia might cause the level of erythropoietin in the serum to increase 50-fold or even more.

Erythropoietin is required for erythroid development, allowing maturation of erythroid precursors by inhibiting programmed

cell death (apoptosis). The signalling pathway involves activation of Janus tyrosine kinase 2 (JAK2), which further propagates the signal by engaging secondary signalling molecules, including signal transducer and activator of transcription (STAT), Ras-mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K). In erythroid progenitor cells this results in the upregulation of anti-apoptotic proteins of the B-cell leukaemia/lymphoma 2 (BCL2) family, such as BCL-X_L (REF. 2). Hormonal clearance of erythropoietin from the serum occurs mainly through receptor-mediated endocytosis in the bone marrow, although pharmacological doses of this cytokine are also eliminated by the liver and kidneys³. During the last 20 years, recombinant human erythropoietin (rhEPO) has improved the quality of life of more than a million patients with anaemia.

The discovery that erythropoietin has biological functions aside from regulating erythropoiesis was unexpected. Tissue expression studies carried out in 1992 identified erythropoietin mRNA in the brain, and showed that it increased following hypoxia⁴. Later investigation led to the idea that astrocytes and neurons might express both erythropoietin and its receptor (EPOR)⁵, and showed that erythropoietin has a trophic effect on central cholinergic neurons *in vitro* and *in vivo*⁶. The observation that cells in the nervous system express erythropoietin and/or EPOR⁷ indicated that erythropoietin could function as an autocrine–paracrine factor outside the bone marrow. Follow-up studies confirmed erythropoietin to be a neuroprotective factor in hypoxic–ischaemic, traumatic, excitotoxic and inflammatory injuries^{8–10}. A fundamental mechanism of erythropoietin-mediated neuroprotection is the inhibition of apoptosis in the tissue adjacent to a lesion¹¹. Other studies have shown that erythropoietin also modulates nitric oxide synthesis (especially within the vasculature)¹² and neurotransmitter release^{13,14}, and antagonizes leakiness of the blood–brain barrier (BBB) that is induced by vascular endothelial growth factor (VEGF)¹⁵ or inflammation¹⁶.

A conceptual framework for understanding these diverse actions of erythropoietin can be constructed through the realization that erythropoietin functions to protect and repair tissue damage initiated by an innate inflammatory response. This biological program first evolved as a primitive defence mechanism to destroy invading pathogens (BOX 1), but is also triggered by other damaging stimuli such as ischaemia. In vertebrates, the primary triggers of the various molecular pathways of the innate inflammatory response are the pro-inflammatory cytokines and hypoxia-inducible factor (HIF), a protein that activates the synthesis of genes such as erythropoietin, VEGF, glucose transporters and glycolytic enzymes that are adaptive for survival under reduced oxygen tension¹⁷. However, metabolic stress also promotes HIF production, which triggers a similar — but inappropriate — response to infection, as well as the production of pro-inflammatory cytokines that upregulate EPOR but inhibit the transcription of erythropoietin (FIG. 2, green shaded region; see also BOX 1). The ultimate extent of tissue injury results from the balance of a complex network of molecular signals that arise from injured cells, adjacent healthy cells and infiltrating immune cells. In primitive organisms, cytokine-like molecules of the innate inflammatory response also have crucial roles in embryonic development¹⁸. It is not surprising, therefore, that erythropoietin is important in the development of the brain and the vascular system¹⁹, and that it might function in neurogenesis and in guiding stem cells into damaged tissues in adults²⁰.

The tissue-protective molecular pathways that are triggered by erythropoietin have similarities to, as well as differences from, those activated during erythropoiesis. JAK2, MAPK and PI3K all seem to be important². Recently, we postulated that a receptor distinct from those expressed by erythroid precursors specifically mediates tissue protection²¹. This receptor probably consists of the EPOR monomer and a dimer of the β common receptor (β CR) — a shared receptor subunit of interleukin 3 (IL-3), IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF) (FIG. 3). Based on this information, engineered molecules have been developed that mediate tissue protection but do not bind to erythroid progenitors, thereby dissociating the biology of erythropoietin-mediated cytoprotection from that of erythropoiesis. Here, we briefly review recent progress in understanding the roles of erythropoietin in the nervous system

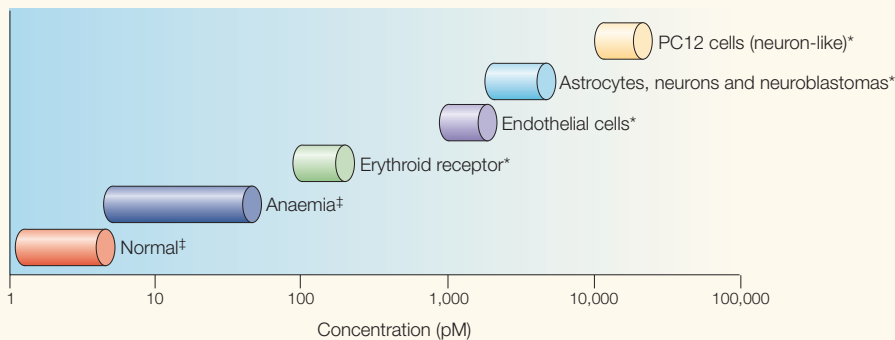


Figure 1 | Range of erythropoietin concentrations in the serum of healthy individuals and those with moderate anaemia, and the affinities of erythropoietin receptors in different cell types.

The binding affinities* of the erythropoietin receptors expressed by non-erythroid cells that have been examined so far are much lower than the concentration[†] of circulating erythropoietin in the serum, even in patients with anaemia. This discordance indicates that erythropoietin might be produced in an autocrine–paracrine manner to maintain the local concentrations of erythropoietin within the range required to activate the non-erythroid erythropoietin receptors.

and evaluate the therapeutic potential of this multifunctional molecule for the treatment of nerve injuries and neurological disorders.

Expression of erythropoietin and EPOR

Erythropoietin and EPOR are both single gene products. Although there might be differentially-edited transcripts of EPOR, they are not thought to be translated²². Erythropoietin is mainly regulated in the kidney by HIF in response to hypoxia, but other factors are also involved in specialized tissues²³. In contrast to erythropoietin, the expression of EPOR is not appreciably sensitive to hypoxia¹², rather, it is regulated by pro-inflammatory cytokines²⁴, such as tumour necrosis factor- α (TNF α) and IL-1 β , erythropoietin itself²² and probably other factors that have not yet been identified. Both the erythropoietin and EPOR proteins that are expressed in the brain are smaller than their counterparts in the periphery, which is due to differences in sialic acid content^{5,25}. In addition to some astrocytes and neurons, endothelial cells in the brain and other organs can also synthesize erythropoietin in response to hypoxia–ischaemia⁷, thereby potentially providing an available source of local erythropoietin.

The erythroid receptor exists as a pre-formed EPOR homodimer²⁶ (FIG. 3B), which was thought (we believe incorrectly) to transduce signals for tissue protection (see below). Although some cells express high concentrations of EPOR at baseline (for example, the astrocytes that surround capillaries in white matter, Purkinje neurons, the choroid plexus and ependymal cells), most regions of the healthy adult brain express only minimal levels of erythropoietin and EPOR. The precise cell-type specific expression

patterns remain controversial, as, in general, rather poorly-defined polyclonal antibodies have been used for immunohistochemical studies. However, it is clear that the expression of both proteins increases markedly following tissue injury^{7,27}.

By contrast, the fetal CNS expresses high levels of erythropoietin and EPOR, which indicates that erythropoietin might be important in neural development²⁸. The EPOR-deficient mouse is an embryonic lethal as a result of severe defects in erythropoiesis and angiogenesis¹⁹. However, mice that have a targeted knock-in of EPOR for the bone marrow (which allows survival) and are fertile²⁹, which indicates that erythropoietin is not actually required for organ development.

In the brain, erythropoietin synthesis and signalling are regulated by several mechanisms. In both the nervous system and the kidney, increased expression of erythropoietin occurs through hypoxia-induced activation of HIF in response to reduced oxygen concentrations. Moreover, insulin³⁰, hypoglycaemia³¹ and intense neuronal activity³² also activate erythropoietin synthesis in neurons and astrocytes. Furthermore, although the kidney — the main source of circulating erythropoietin — synthesizes erythropoietin mRNA only transiently in response to hypoxia, peaking within 6 h (REF. 33), the production of erythropoietin by astrocytes is maintained during hypoxia²³. Finally, the expression of renal erythropoietin and EPOR protein in the bone marrow occurs simultaneously, whereas synthesis in the brain is temporally staggered, with EPOR preceding erythropoietin³⁴ — a pattern that is also observed during normal embryogenesis¹⁹.

Erythropoietin signalling

Erythropoietin promotes cell survival by acting through many signalling pathways. Until recently, it was thought that the erythroid receptor (which consists of an EPOR homodimer) mediated neuroprotection. The identification of a potentially different receptor isoform (see below) necessitates re-evaluation of this idea. Prior study indicates that, in the bone marrow, ligand binding of erythropoietin results in phosphorylation of JAK2. Following phosphorylation, JAK2 is activated and propagates signalling through the MAPK or the protein kinase B (PKB/Akt)–nuclear factor- κ B (NF- κ B) pathways (FIG. 4). In erythroid cells, the activation of MAPK results in accumulation of the anti-apoptotic protein BCL-X_L by the inhibition of caspases — key mediators of apoptosis that are responsible for the degradation of BCL-X_L (REF. 35). By contrast, a study of the neuroprotective effects of erythropoietin indicates that BCL-X_L might not be required³⁶. Furthermore, although it has been reported that STAT is part of erythropoietin's neuroprotective signalling cascade *in vitro*, *in vivo* studies largely have not supported the involvement of STATs in erythropoietin cytoprotection^{36–38}. Additional work has also shown that erythropoietin modulates intracellular calcium concentrations in excitable cells (for example, vascular smooth muscle cells) by activating phospholipase C- γ (PLC γ) (REF. 39), as well as in neurons. In this manner, erythropoietin directly influences neurotransmitter release as well as neuronal activity^{13,14}. *In vivo* microdialysis of hippocampal neurons reveals that nitric oxide production is elevated after erythropoietin stimulation — a process that requires the activation of voltage-gated calcium channels⁴⁰ and that could be responsible for maintaining neuronal integrity, for example, through the regulation of neurotransmitter release, gene expression and synaptic activity⁴¹.

Erythropoietin in injured tissues

The complex interactions of cellular signals that surround injured tissue result in gradients of pro-inflammatory cytokine expression that define a 'region at risk' or penumbra (FIG. 5). Similar to its action on erythroid progenitors, erythropoietin prevents apoptosis of EPOR-expressing cells within the penumbra. Although there is no compelling evidence to indicate that erythropoietin directly inhibits necrosis, it might do so indirectly by preserving vascular integrity (see below). Erythropoietin can also attenuate reactive inflammatory responses, although, in some cases, this might be a secondary effect due to reduced apoptosis, and, therefore, diminished

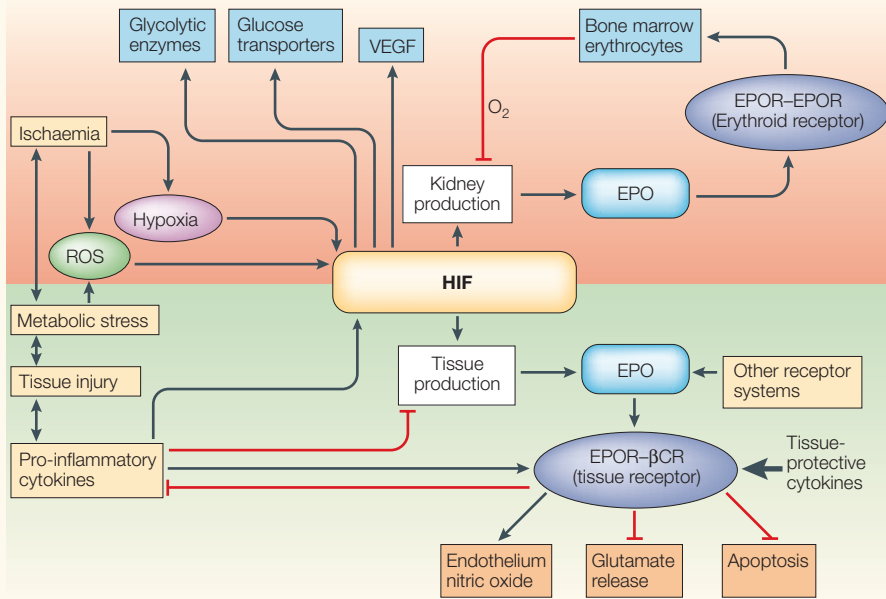


Figure 2 | The central role of hypoxia-inducible factor in erythropoiesis and tissue protection. In mammals, endogenous erythropoietin (EPO) can function either in the kidney–bone marrow system (shaded red) or in a local autocrine–paracrine system (shaded green). In both systems, hypoxia-inducible factor (HIF) has a crucial role in regulating erythropoietin expression. In addition, erythropoietin expression in the autocrine–paracrine system can be activated through other receptor systems (for example, insulin-like growth factor). In the kidney–bone marrow system, renal hypoxia induces the synthesis of HIF by the kidney, which, in turn, increases renal erythropoietin production. Newly synthesized erythropoietin enters the circulation and travels to the bone marrow, stimulating erythroid precursor survival and maturation. Increased oxygen delivery to tissues as a result of increased erythrocyte production attenuates the action of HIF in a negative feedback manner. By contrast, tissue injury induces metabolic stress and the release of pro-inflammatory cytokines, which activate HIF and increase local erythropoietin production. Pro-inflammatory cytokines can also directly inhibit the production of erythropoietin, but greatly upregulate the expression of erythropoietin receptors (EPORs). Signalling through the postulated tissue-protective EPOR-β common receptor (βCR) (see text) reduces tissue damage by inhibiting apoptosis, downregulating glutamate release and reducing the production of pro-inflammatory cytokines. Activation of the tissue-protective EPOR-βCR can also lead to increased nitric oxide synthesis (especially under conditions of hypoxia), which is important for the maintenance of vascular autoregulation. Although exogenous erythropoietin will be active in both systems, its non-erythropoietic, tissue-protective derivatives signal only through the autocrine–paracrine system, thereby mediating tissue protection without increasing the number of erythroid cells or producing other hormonal effects that are associated with erythropoietin (for example, platelet production). Tissue protection by endogenously-produced erythropoietin acting through the EPOR homodimer can not be excluded, but is not supported by current data (not shown in this figure). ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

activation of the immune-competent cells that initiate phagocytosis. Notably, erythropoietin suppresses the inflammatory mononuclear cell infiltration and the burst of pro-inflammatory cytokines in an animal model of stroke with reperfusion⁴². However, in animal models that do not involve extensive apoptosis — for example, those of experimental autoimmune encephalitis and peripheral nerve injuries — erythropoietin and its analogues also have potent anti-inflammatory effects^{16,43}. Erythropoietin has also recently been reported to be active in a model of rheumatoid arthritis⁴⁴, which is a primarily inflammatory disease. In this context, it is interesting to note that erythropoietin can apparently directly affect EPOR-expressing immune-competent cells⁴⁵.

Substantial evidence also indicates that erythropoietin mediates protective effects by maintaining normal vascular autoregulation and thereby preventing expansion of an injury through secondary microinfarction (that is, ischaemia resulting from injury-related vasospasm). A key mechanism in this process seems to be augmented nitric oxide signalling through increased endothelial nitric oxide synthase¹², which mediates vascular relaxation and maintains blood flow. However, erythropoietin can also cause vascular constriction through endothelin 1; see ‘Issues in clinical development’ (below). This observation could explain the remarkable efficacy of erythropoietin in reversing the vascular spasm that accompanies subarachnoid haemorrhage⁴⁶ and spinal cord

compression⁴⁷, which, in turn, reduces tissue damage. Moreover, erythropoietin can also act synergistically with VEGF to stimulate angiogenesis after injury⁴⁸ while maintaining vascular integrity by antagonizing inflammation¹⁶ and the leakiness of the endothelial barrier that is promoted by VEGF¹⁵. It is intriguing that the tissue distribution of EPOR is similar to that of aquaporin 4, the brain isoform of the water-transporting proteins in the astrocytic end-feet that surround capillaries, the choroid plexus and ependymal cells⁴⁹. However, whether erythropoietin and EPOR interact directly with aquaporins has not yet been determined.

Induction of erythropoietin expression by HIF is also crucial for preconditioning — a form of molecular memory characterized by resistance to injury that is activated by prior exposure to non-toxic metabolic stress. For example, ischaemic preconditioning, which occurs in the heart and the brain, is a powerful protective mechanism whereby exposure to mild hypoxia markedly increases cellular resistance to subsequent severe hypoxia. Other causes of metabolic stress, such as intense neuronal depolarization³² or hypoglycaemia³¹, also trigger preconditioning responses.

Preconditioning can markedly limit the extent of tissue damage by preventing primary injury and decreasing the secondary damage that is caused by inflammation. However, ischaemic preconditioning in the heart and the brain occurs in two phases: an early phase, mediated by potassium channel activation, that lasts only a few hours, and a delayed phase, requiring gene expression, that develops slowly over 12–24 h and persists for several days (for reviews, see REFS 50,51). Several studies have shown that endogenous erythropoietin has a crucial role in both phases of preconditioning within the brain^{36,52}. The expression of EPOR is necessary for the onset of the immediate phase of preconditioning, and the reaction is limited to only EPOR-positive cells at the time of exposure. Levels of both erythropoietin and EPOR increase significantly in the delayed phase of preconditioning, with EPOR expression upregulated first. Knowledge of the time course is crucial for testing erythropoietin in proof-of-concept clinical trials.

Finally, erythropoietin seems to modulate the migration and differentiation of stem cells and might, therefore, be important in tissue repair and functional recovery after injury^{20,53}. For example, experiments have shown that forebrain neuronal stem cells in the subventricular zone of the adult mouse differentiate into neurons after exposure to erythropoietin²⁰. Furthermore, injection of erythropoietin into the lateral ventricle

Box 1 | Tissue protection and the innate immune response

All multicellular organisms use programmed cell death (apoptosis) to eliminate superfluous or damaged cells, as well as to sculpt tissues during development. Apoptosis is also used by the innate inflammatory response to produce an acellular zone surrounding the site of an injury. This is an ancient, evolutionarily conserved defence mechanism that is triggered by the body's recognition of non-self or injured self. The innate inflammatory response in primitive organisms is the primary response



to the invasion of pathogens. In mammals, this response uses pro-inflammatory cytokines (such as interleukin-1 β and tumour necrosis factor- α) as molecular signals to limit the spread of infection. In this positive-feedback system, a counter-acting tissue-protective component is required to arrest the spread of injury, which would otherwise move like wildfire outward into healthy tissue.

The efficacy of the inflammatory response can be easily observed in plant leaves, as is illustrated by the response of a tobacco leaf to mosaic virus inoculation (above). Cells immediately surrounding the inoculation site are specifically removed by apoptosis (arrows) and the spread of the virus into viable tissue is prevented. The innate immune response is also highly developed in vertebrates, despite the presence of acquired immunity. (A second, more generalized level of protection is provided by the systemic stress response, for example, that mediated by the hypothalamic–pituitary–adrenal axis in mammals, which is also of crucial importance in the nervous system¹⁰⁴.) Pro-inflammatory cytokines are also induced by other injuries, which effectively identify an ‘area at risk’, known as the penumbra. In mammals, our view is that there are normally several cell types in an injured region, and the cells at risk typically include parenchymal cells and those that form capillaries. In the penumbra, cells that are destined for apoptosis express the tissue protective erythropoietin receptor within a few hours of injury, and can be rescued by erythropoietin or its analogues within an appropriate time window (FIG. 5). Over the past decade, many studies have shown that, in mammals, erythropoietin is a protective cytokine that markedly limits the extent of injury through several pathways, including inhibiting apoptosis, reducing influx of inflammatory cells into the penumbra and restoring vascular autoregulation.

be biologically active, could travel from the capillary lumen into the brain parenchyma¹⁰. Moreover, in an *in vitro* model of the BBB, erythropoietin was transported vectorially from the luminal surface to the basolateral surface in a specific and saturable manner, which is fully consistent with a mechanism of receptor-mediated transcytosis¹⁵. In addition, after exposure to erythropoietin, expression of EPOR by this endothelial cell system increased with similar kinetics to those observed for the transport of peripheral erythropoietin into the CSF¹⁵.

In contrast to this work, researchers using *in vivo* models have questioned the idea of transcytosis and instead suggest that the penetration of erythropoietin into the brain is nonspecific^{54,59}. The most comprehensive study so far used both radiolabelled erythropoietin and albumin to assess movement of these molecules from the circulation to the brain parenchyma (in contrast to the CSF as previously reported)⁵⁹. These investigators concluded that erythropoietin transport, although present and appreciable, was not specific. The issue of erythropoietin transport across the BBB *in vivo* is complicated by the fact that endothelial cells synthesize a soluble EPOR⁶⁰ (which is readily detected in humans⁶¹) that would sequester erythropoietin, neutralizing its biological activity. Further work will be necessary to allow us to understand the exact mechanism by which erythropoietin traverses the endothelium. From a therapeutic perspective, however, it is a non-controversial point that, in appropriate doses, systemically-administered erythropoietin clearly crosses the BBB to a degree that is sufficient to enable neuroprotection.

Animal models for erythropoietin

Comprehensive recent reviews have provided a good summary of studies that used erythropoietin as a tissue-protective agent^{55,62–64}. Therefore, we will only outline these investigations and will focus more specifically on new, tissue-protective erythropoietin derivatives that have no erythropoietic activity, as well as our views on the promise and challenges that exist within this area of research. However, several issues that concern the appropriateness of specific models with respect to tissue protection do merit some comments.

First, relevant models obviously require an at-risk component that depends on erythropoietin's mechanism of action. For example, when cerebral ischaemia is induced in young animals by external vascular occlusion with reperfusion, this produces a large penumbra

increased the number of olfactory bulb neurons, an effect that could be mimicked by subjecting the animal to hypoxia²⁰. Additional studies will be required to allow us to understand the potential roles that erythropoietin and its derivatives might have in the plasticity of the nervous system.

Crossing the blood–brain barrier

One conceptual therapeutic breakthrough occurred with the unexpected discovery that peripherally-administered erythropoietin crosses the intact BBB in neuroprotective amounts¹⁰. The appearance of exogenous erythropoietin in the cerebrospinal fluid (CSF) of rodents, sheep, rabbits and primates occurs with a temporal profile similar to that of erythropoietin in serum, but with a delay of 1–2 h and a peak concentration of ~1% of the plasma^{54–56} (which for large doses is within the neuroprotective range). Follow-up nuclear-imaging studies using

indium-labelled erythropoietin have shown that systemically administered erythropoietin penetrates the BBB as an intact molecule in both healthy individuals and patients with schizophrenia⁵⁶. Systemically administered erythropoietin can also cross other tissues with tight endothelial barriers, including the retina⁵⁷ and testis⁵⁸, which both express high levels of EPOR under basal conditions.

At the electron microscope level, abundant immunoreactive EPOR is localized at the surface of the endothelial cells and within caveolae — vesicles that fuse to both the luminal and abluminal membrane surfaces. This observation indicates that erythropoietin might reach the brain through receptor-mediated transcytosis^{10,27} in which erythropoietin binds to EPOR at the endothelial cell luminal surface, traverses the cytoplasm and is released at the abluminal surface. Brines *et al.*¹⁰ showed that biotinylated rhEPO, which was proved to

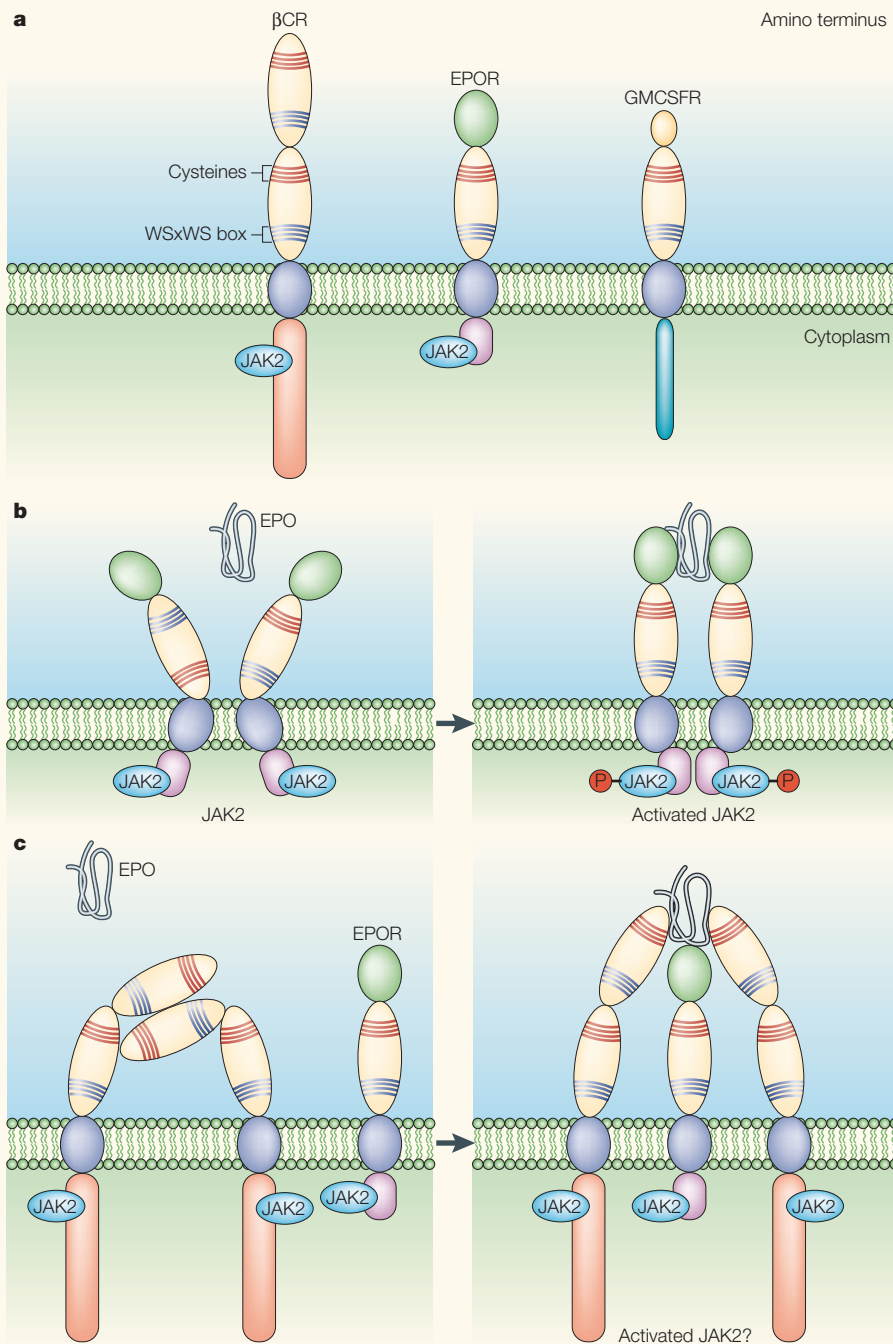


Figure 3 | Structure of cytokine receptor subunits that mediate erythropoiesis and cytoprotection. **a** | The erythroid receptor subunit (EPOR) belongs to a subfamily of the type I cytokine receptor superfamily, which also includes the β common receptor (β CR) and the granulocyte-macrophage colony-stimulating factor receptor (GMCSFR). These share several structural features. Their extracellular domain includes the 'tryptophan-serine-any amino acid-Trp-Ser' (WSxWS) motif near the plasma membrane and 4 cysteine residues that are more distally located. The large intracellular domain of the β CR subunit is also the signalling component of the receptor complex of GMCSF, interleukin-3 and interleukin-5. **b** | The erythroid erythropoietin (EPO) receptor consists of a preformed EPOR homodimer with a conformation such that in the absence of erythropoietin the intracellular domains do not interact with each other. Binding of erythropoietin to the homodimer induces a conformational change that brings the intracellular domains together, which results in phosphorylation and activation of Janus tyrosine kinase 2 (JAK2) and activation of the downstream signalling cascade. **c** | The proposed structure of the tissue-protective EPOR based on the stoichiometry of GMCSFR¹⁰⁵. β CR is unusual in that it exists as a preformed homodimer that consists of intertwined extracellular domains. We have proposed that erythropoietin induces the formation of a heterotrimer that consists of an EPOR and a β CR homodimer, which might result in interaction of the intracellular domains of the receptor subunits and activation of the signalling cascades that mediate tissue protection.

that undergoes apoptosis. By contrast, the penumbra is usually much smaller when the ischaemia is caused by intraluminal filament occlusion. In this situation, treatment with erythropoietin will be less effective⁶⁵.

Second, assessment of the degree of protection that is afforded by erythropoietin can be problematic, particularly when estimates are based on cell losses measured only during brief follow-up periods rather than on long-term changes in function.

Third, the therapeutic window of opportunity and relevant dose-response relationships need to be understood, particularly in tissues like the brain that have tight endothelial barriers. It is of note that researchers have consistently reported the dose-response curve of erythropoietin cytoprotection to follow the shape of an inverted 'U', both *in vitro* and *in vivo*^{9,12,16}, a common observation for cytokines in general, which indicates that more is not always better.

Fourth, rhEPO, rather than species-specific erythropoietin, has been used in most preclinical *in vivo* studies and, therefore, eventually elicits immune reactions, including neutralizing antibodies against both exogenous and endogenous erythropoietin. The consequent anaemia (as well as potential neutralization of locally-produced erythropoietin) can obviously limit the duration and interpretation of these experiments.

Hypoxic-ischaemic injuries in the CNS. In pioneering *in vivo* studies that were elegantly performed by Sasaki and colleagues, erythropoietin was delivered intrathecally and its neuroprotective effects directly assessed^{9,66}. In rat, gerbil and mouse models of global and focal ischaemia induced by vascular compression, these investigators proved that erythropoietin dramatically reduced tissue damage and improved recovery of cognitive function as assessed by a water maze task⁶⁶. The important role of endogenous erythropoietin was confirmed by administering soluble EPOR, which increased the degree of injury by neutralizing endogenous erythropoietin⁹. Our experiments, which were carried out with peripherally-administered erythropoietin, showed that there is a threshold dose of ~500 U/kg-body weight (bw) intraperitoneally (i.p.) (~0.5 μ g/kg-bw) in rats, with a therapeutic time window of ~6 h when evaluated by tetrazolium staining 24 h after injury¹⁰. Studies using other injury models, such as ischaemia of the spinal cord⁶⁷ and retina⁶⁸, and neonatal hypoxia⁶⁹, have yielded similar robust findings. Systemic administration of erythropoietin can also

protect the retina from oxidative stress that results from light-induced injury⁵⁷ and mild-to-moderate photoreceptor mutations⁷⁰. As in all models, injury that is very severe (that is, causing necrosis) is not benefitted by erythropoietin.

Inflammation. In rodent models of auto-immune diseases, injection of myelin basic protein or myelin oligodendrocyte glycoprotein (MOG) provokes BBB leakage and axonal damage in the cortex and the spinal cord, and infiltration of inflammatory cells. Studies have shown that erythropoietin is highly effective in preventing and reversing these effects^{10,16,43}. Systemic administration of erythropoietin has also been shown to markedly increase the survival of retinal ganglion cells in rats with optic neuritis produced by the injection of MOG⁷¹.

Trauma- and toxin-induced injuries. Similar to the effects of erythropoietin on cerebral ischaemia in rats, erythropoietin has been shown to markedly reduce contusive cortical injury in mice¹⁰ when the equivalent of 5,000U/kg-bw i.p. (~50 µg/kg-bw) erythropoietin was administered for 24 h before and up to 6 h after injury, and then daily for 4 further days. Five days after treatment, the volume of injury, which was estimated by quantifying serial sections of the cerebral cortex, was markedly reduced when erythropoietin had been administered as late as 6 h following trauma. A recent *in vitro* study using organotypic hippocampal slices has largely confirmed this observation, as well as showing that erythropoietin has a greater neuroprotective effect than MK-801 (dizocilpine) — an antagonist of the cytotoxic NMDA (*N*-methyl-D-aspartate) receptor⁷² that is activated by trauma. Similarly, studies using contusion or compression models of spinal cord injury have also validated the idea that even single doses of erythropoietin administered immediately following injury provide robust protection⁴⁷. In these experiments, animals that received erythropoietin recovered almost completely within 3 weeks, whereas their control counterparts remained profoundly paraplegic.

The neuroprotective effects of erythropoietin have also been confirmed using traumatic or metabolic-toxic (for example, cisplatin⁷³ or acrylamide⁷⁴) injury models in the peripheral nerve^{75–78}. Finally, erythropoietin has been shown to have beneficial effects in subarachnoid haemorrhage^{46,79} and glutamate-induced excitotoxicity, as in models of Parkinson's disease⁸⁰.

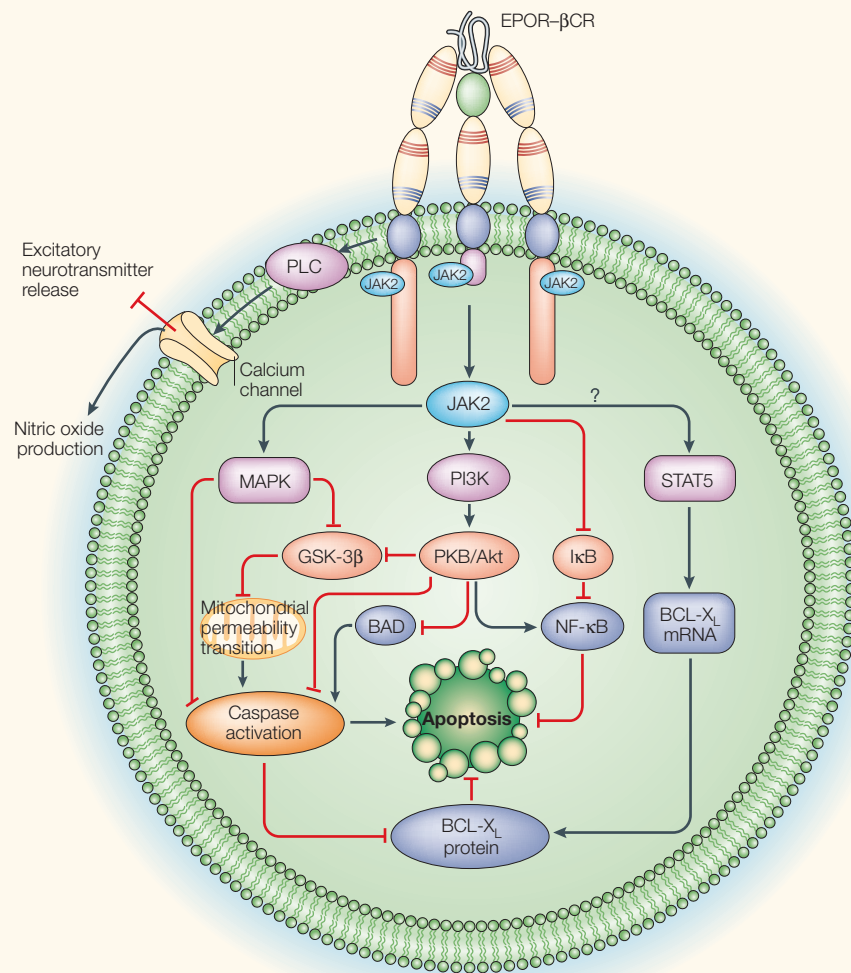


Figure 4 | Intracellular erythropoietin signalling pathways implicated in tissue protection. Some of the signalling pathways that are used by erythropoietin for tissue protection are similar to those of erythropoiesis. Recent evidence indicates that the proposed tissue-protective erythropoietin receptor (EPOR- β common receptor (β CR)) first activates Janus tyrosine kinase 2 (JAK2), which then engages secondary signalling pathways that involve mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and nuclear factor- κ B (NF- κ B). Some *in vitro* studies show that signal transduction and activator of transcription 5 (STAT5) can also be activated by the tissue-protective erythropoietin receptor, but *in vivo* evidence is lacking. Ultimately, these secondary signalling events lead to an increase in anti-apoptotic proteins of the B-cell leukaemia/lymphoma (BCL) family. Some of the pathways act directly, whereas others act indirectly by inhibiting the activity of a group of enzymes called caspases. These are key mediators of apoptosis and are responsible for the degradation of anti-apoptotic proteins. The tissue-protective erythropoietin receptor also inhibits glycogen synthase kinase 3 β (GSK3 β), which, in turn, inhibits the mitochondrial permeability transition pore¹⁰⁶, a major determinant of cell death, through caspase activation. Finally, erythropoietin modulates the activity of calcium channels through phospholipase C (PLC), thereby reducing the release of excitatory neurotransmitters and augmenting nitric oxide production. Erythropoietin has also been implicated in other anti-apoptotic pathways that are not shown in this diagram (for an example, see REF. 107). BAD, BCL2-associated death protein; I κ B, inhibitor of κ B; PKB (Akt), protein kinase B.

Neurotrophic effects. The administration of erythropoietin provides significant effects on axonal regrowth of peripheral nerve fibres, both after transection^{75,76} and in animals with established diabetic neuropathy⁸¹. In fact, an early study that indicated a role for erythropoietin in the nervous system showed that the administration of erythropoietin had a neurotrophic effect on transected nerve tracts and on the growth

of neuronal processes in culture⁶. Although insufficient work has been done to assess a role for erythropoietin in learning and memory, we have shown that, in normal mice, administration of erythropoietin markedly increases conditioned taste aversion, a particularly robust type of learning⁵⁶. We believe that the effects of erythropoietin on learning and cognition are likely to be an especially fertile area for future investigation.

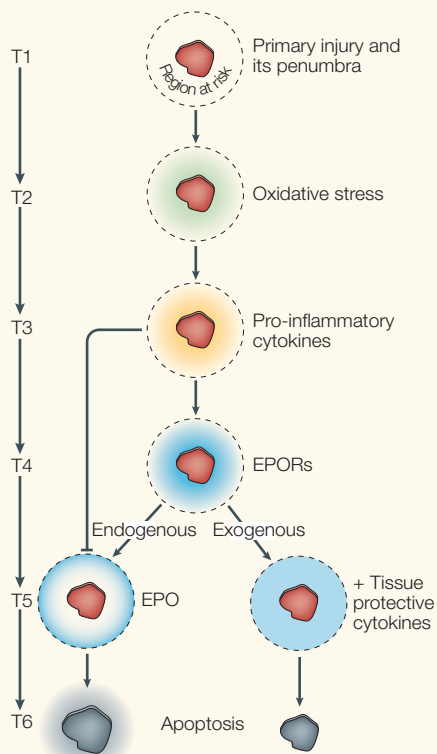


Figure 5 | Evolution of tissue injury and its modulation by endogenous erythropoietin or exogenous non-erythropoietic tissue-protective cytokines. After a primary insult (for example, a vascular infarct) a penumbra develops around necrotic cells in a region where the oxygen tension falls below a critical limit (time point 1; T1). Cells around the core undergo oxidative and nitrosative stress as a result of metabolism in the absence of sufficient oxygen, are at risk of undergoing apoptosis and form the penumbra (T2). Cells within the penumbra and immune cells recruited from a distance by the products of necrosis begin to secrete pro-inflammatory cytokines. These cytokines induce the expression of erythropoietin receptors (EPORs) by cells in the penumbra, with the highest activity at the lesion epicentre where the cytokines are most concentrated (T3) and expression of EPOR is highest (T4). The hypoxic conditions in the penumbra also induce the expression of hypoxia-inducible factor (HIF) in some cell types (such as astrocytes), which initiates increased erythropoietin (EPO) production. However, pro-inflammatory cytokines can also directly inhibit erythropoietin synthesis, thereby producing a gradient that is the inverse (T5, left) of that of EPOR (T4). This results in diminished salvage of the penumbra. The apoptotic programme in EPOR-expressing cells can be terminated by the binding of erythropoietin within a few hours of injury. Application of exogenous erythropoietin or other tissue-protective cytokines to the injury site can substitute for endogenous erythropoietin (if it is suppressed) and protect EPOR-expressing cells from apoptosis (T5, right), so reducing the degree of tissue loss by terminating the apoptotic programme (T6).

Human clinical studies of rhEPO

Stroke. Despite the widespread use of erythropoietin for the treatment of anaemia, there are few clinical observations that postulate any direct effect of erythropoietin (that is, separate from the effects that are mediated by changes in haemoglobin concentration) in neurological diseases. This is probably because the doses of erythropoietin that are used to stimulate erythropoiesis are typically much lower than the minimum dose required for neuroprotection. By using high doses and rigorous selection criteria that enrolled only patients with documented middle cerebral artery (MCA) non-haemorrhagic infarcts (and, therefore, ensuring a homogeneous patient group), a successful proof-of-concept clinical trial using rhEPO has been performed. In this study, 40 patients who had suffered a stroke less than 8 h before trial entry (mean 5.5 h; range 2.9–8.0 h) were randomized into rhEPO or saline treatment groups. A total of 100,000 U of rhEPO was delivered intravenously as equally-divided doses over the first 3 days. After 30 days both neurological scores and functionality improved significantly in the active rhEPO treatment group. A larger study is in progress to test this conclusion. It is especially notable that, in the past, the translation of several promising results from preclinical animal models of stroke into therapeutic applications in humans has failed almost universally. The positive result of the erythropoietin clinical trial raises the hope that the efficacy of erythropoietin in wide-ranging preclinical models might translate into similar positive results for other diseases.

Schizophrenia. The brain pathology of patients with schizophrenia indicates that there is a neurotoxic component in this disease⁸². Paradoxically, clinically effective antipsychotic drugs are neurotoxic *in vitro*, but are effectively antagonized by erythropoietin⁵⁶. Interestingly, a recent study that used nuclear imaging technology showed that more rhEPO penetrates the CSF in patients with schizophrenia than in healthy controls⁵⁶. In addition, postmortem examination of the brain indicates that EPOR is upregulated in the cerebral cortex and hippocampus in patients with schizophrenia compared with healthy controls. These observations indicate that erythropoietin therapy could have a role in schizophrenia, and demand a proof-of-concept clinical trial.

An alternative erythropoietin receptor

Until recently, erythropoietin was thought to be an atypical cytokine that possessed only a single function (erythropoiesis). The first

suggestion that erythropoietin might have an additional biological activity resulted from a chance observation by Anagnostou and co-workers⁸³ that erythropoietin induced chemotaxis and mitosis of cultured endothelial cells. Characterization of the endothelial cell EPOR was consistent with a functionally different receptor, as it was present at a much higher density but had a much lower affinity for erythropoietin when compared with its counterpart expressed by erythroid cells (~5 nM versus ~200 pM respectively; see also FIG. 1). Receptor-protein crosslinking studies also showed differences, with only a single associated protein of ~45 kDa in endothelial cells, rather than the two larger ones of ~110 and ~95 kDa that are found in erythroid cells⁸⁴. Later work showed that neuronal-like PC12 cells express an erythropoietin receptor with even lower erythropoietin affinity (~20 nM), which also produces a single crosslinked product⁶⁰ but differs from the endothelial cell. These erythropoietin receptors with only nanomolar erythropoietin affinities indicated that erythropoietin could act locally only in an autocrine or paracrine manner, as the level of circulating erythropoietin is normally too low (1–5 pM) to signal through the endothelial EPOR (affinity ~2 nM; FIG. 1).

These findings clearly indicated the existence of a different type of erythropoietin receptor. However, the results of previous biochemical studies demanded that this alternative receptor also contained the erythroid receptor subunit EPOR, because tissue expression following injury or ischaemia is characterized by increased EPOR mRNA and protein. In addition, EPOR antagonism using neutralizing antibodies abolished neuroprotection⁸⁵.

One potential solution to this puzzle was indicated by previous observations of an EPOR-expressing, IL-3-dependent murine cell line, Baf3. In the Baf3 cells, EPOR was found to associate with the β CR subunit (FIG. 3C), which is shared by the IL-3, GM-CSF and IL-5 receptors⁸⁶. Although studies of β CR-knockout mice showed unambiguously that this subunit is not a component of the erythroid receptor that is involved in erythropoiesis⁸⁷, this mutant confirmed that the β CR subunit is required for erythropoietin's tissue-protective effects²¹. The proposed structure would also explain the changes that occur in expression and the requirement for EPOR, as it is a component of this heteroreceptor. However, there could also be a role for homodimeric EPOR in tissue protection, for example, in vascular responses. This

is unlikely, however, as erythropoietin is not tissue protective in the β CR-knockout mouse that expresses EPOR normally²¹.

In summary, the evidence supports different erythropoietin receptor complexes with non-overlapping functions. Biological cross-talk between the bone marrow and other tissues would effectively be prevented by the low level of circulating erythropoietin for erythropoiesis and the focal production of higher concentrations of erythropoietin within tissues for cytoprotection. Further work is needed, particularly to investigate the biology of β CR.

Novel tissue-protective molecules

The identification of a putative EPOR heteroreceptor that has a lower affinity for erythropoietin than the bone marrow and mediates tissue protection indicates that molecules might be engineered that are tissue-protective, but not for erythroid precursors. These non-erythropoietic, tissue-protective molecules could have significant clinical applications. So far, three approaches have successfully produced engineered molecules. It should also be noted that a 17-amino acid erythropoietin peptide⁸⁵ shows neuroprotective effects *in vitro*, although these are not apparent *in vivo*.

Increased plasma erythropoietin clearance.

As discussed above, tissue damage can be ameliorated by erythropoietin when apoptosis has a role in determining the extent of secondary injury. Notably, apoptosis can be aborted by only brief exposure (~5 min) to erythropoietin⁸. By contrast, continuous production of newly erythropoietin-responsive erythroid precursor cells in the bone marrow requires the sustained presence of erythropoietin. Therefore, a very short-acting erythropoietin molecule should be effective. To this end, the removal of sialic acid, which terminates the oligosaccharide chains on erythropoietin, produces such a molecule (asialo-erythropoietin). This has a half-life of only several minutes *in vivo*. The conversion can be achieved by sialidases *in vitro*, and it has been reported that systemically administered asialo-erythropoietin rapidly disappears from the circulation. Several studies show that asialo-erythropoietin has tissue-protective effects in rodent ischaemia and spinal cord injury models that are comparable to those of erythropoietin, but that it does not stimulate the production of erythrocytes^{77,88}. However, since asialo-erythropoietin is neither metabolized nor

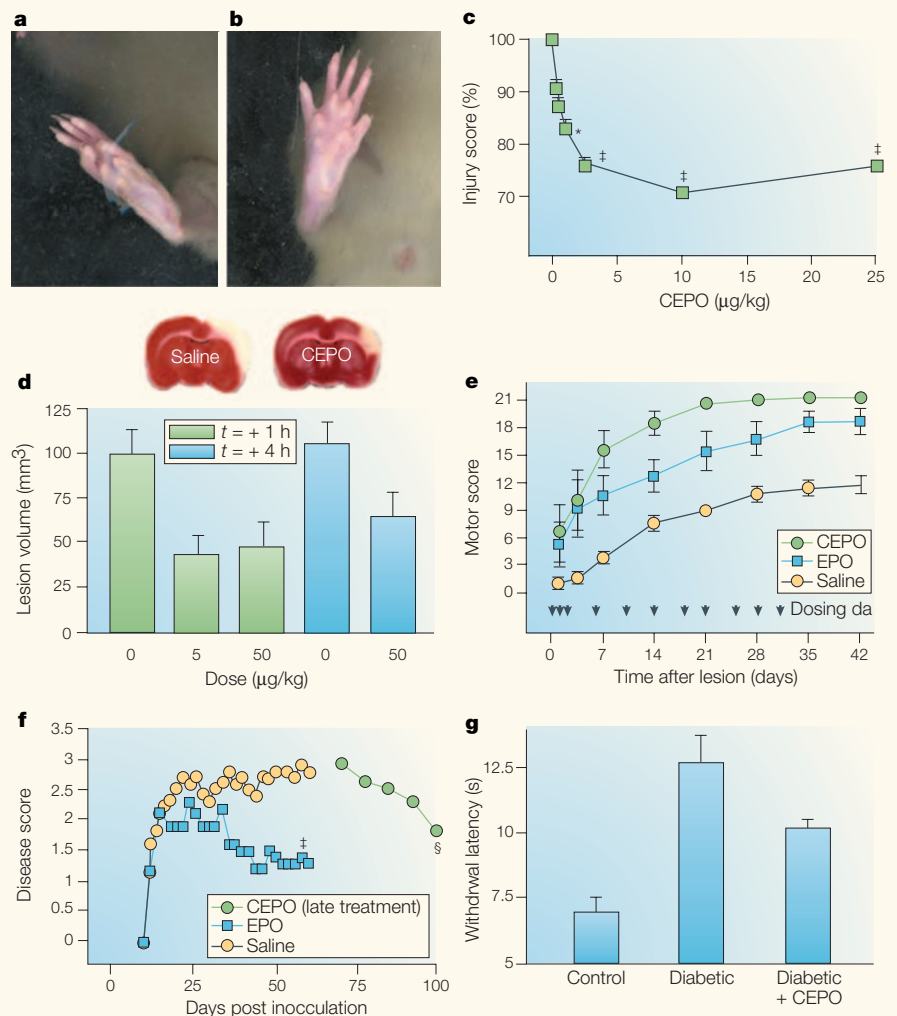


Figure 6 | *In vivo* efficacy of non-erythropoietic, tissue-protective erythropoietin derivatives.

Examples of tissue protection by carbamylated erythropoietin (CEPO) in rodent models. **a–c** | Sciatic nerve compression. Rats were subjected to a 50-g compression of the right sciatic nerve for 1 min at a level that predominantly affects innervation of the intrinsic muscles of the toes. A single dose of carbamylated erythropoietin (10 μ g/kg-bw i.v.) or saline was then administered. Four days after compression, the animals receiving saline could not spread the digits of their right feet while standing (**a**) whereas those that had been treated with carbamylated erythropoietin showed almost normal function (**b**). Panel **c** shows the clear dose-response curve derived from this experiment (injury of 100% is complete paralysis). **d** | Cerebral ischaemia. Rats received a 1-h arterial occlusion that produced a large penumbra within the parietal cortex. They then received a single dose of carbamylated erythropoietin (10 μ g/kg-bw i.v.) either 1 h or 4 h after the restoration of blood flow. Compared with saline (dose of 0) carbamylated erythropoietin significantly decreased the volume of non-viable tissue, which was measured 24 h later using tetrazolium staining (red indicates viable tissue, by more than 50% (white areas on the right superior aspect of the coronal brain sections above the graph). **e** | Spinal cord compression. Rats were subjected to a 58-g compression for 20 s at the spinal level T3 using an aneurysm clip, dosed with either carbamylated erythropoietin, erythropoietin or saline (10 μ g/kg-bw) at the times indicated by arrow heads and evaluated for motor function in open-field testing (score of 21 is normal and 0 is complete paraplegia). Animals that received carbamylated erythropoietin or erythropoietin performed markedly better than saline-treated animals. Whereas carbamylated erythropoietin-treated animals fully recovered, erythropoietin-treated animals retained mild disability 6 weeks after the injury. **f** | Experimental autoimmune encephalitis. Mice were inoculated with myelin oligodendrocyte glycoprotein and, if untreated, developed moderately severe disease within several weeks (maximum score of 5; 0 is normal). Treatment with carbamylated erythropoietin (50 μ g/kg sc three times weekly) could both prevent the development of symptoms as well as reverse already established disease. **g** | Diabetic neuropathy. Rats made diabetic by the administration of streptozotocin developed a peripheral neuropathy by 4 weeks, which was characterized by hypoaesthesia such that the time for foot withdrawal from a hot plate was significantly delayed. Administration of carbamylated erythropoietin (50 μ g/kg-bw sc three times weekly) for 4 weeks significantly improved temperature sensitivity. * p <0.05; † p <0.001; ‡ p <0.01. **a–g** reproduced, with permission, from REF. 78 © (2004) American Association for the Advancement of Science.

cleared under tissue culture conditions, asialo-erythropoietin stimulates erythroid differentiation as potently as erythropoietin *in vitro*⁸⁹.

Mutations in erythropoietin. On the basis of structure–function studies and crystallographic analysis of ligand–receptor binding, it is clear that two regions within the erythropoietin molecule are crucial for enabling binding to the receptor homodimer and activation of downstream signalling cascades⁹⁰. Mutations at either of these sites produce molecules that lack affinity for the EPOR homodimer, but these molecules retain their tissue-protective effects⁷⁸. This observation also confirms that erythropoietin's actions in tissue protection and erythropoiesis are mediated by separate regions of the molecule.

Chemical modifications of erythropoietin. Specific chemical modifications in the erythropoietin domains that are not involved in binding to EPOR can also produce molecules with only tissue-protective effects⁷⁸. For example, carbamylation of erythropoietin, which modifies lysine to homocitrulline, produces a carbamylated molecule, which is non-erythropoietic but fully tissue-protective. The active site(s) of these modified tissue-protective cytokines, with respect to receptor binding, have not yet been identified.

Studies have shown that erythropoietin and carbamylated erythropoietin might have a similar efficacy for tissue protection (FIG. 6). When delivered in a single dose (10 µg/kg-bw), the potencies of carbamylated erythropoietin, asialo-erythropoietin and erythropoietin seem to be comparable in rat models of stroke and spinal cord compression⁷⁸. However, when multiple doses are administered, carbamylated erythropoietin is more potent than erythropoietin and asialo-erythropoietin in the spinal cord compression model⁷⁸ (FIG. 6). Detailed dose–response studies have yet to be carried out to compare the performance of other erythropoietin analogues in additional preclinical models.

Issues in clinical development

A number of important factors must be considered when contemplating the clinical use of rhEPO for cytoprotection. These arise from the fact that treatment with rhEPO will activate both the hormonal (for example, bone marrow stimulation) as well as the local autocrine–paracrine erythropoietin systems.

First, erythropoietin induces vascular smooth muscle contraction, which produces dose-dependent increases in blood pressure in experimental animals and humans^{91,92}. Hypertension, a known adverse effect of rhEPO therapy, seems to be due, in part, to excessive endothelin production^{93,94}. Importantly, marked overexpression of erythropoietin in transgenic mice is associated with decreased survival because of cardiovascular abnormalities when nitric oxide synthesis is inhibited. These animals have greatly increased endothelin expression in many tissues and endothelin receptor antagonism markedly reduces the pathology shown by this model⁹⁵.

Second, erythropoietin significantly increases the risk of thrombosis, especially in certain patient groups. It is well known that erythropoietin is pro-thrombotic in a dose-dependent manner, which is partly mediated by augmented expression of P- and E-selectins. However, erythropoietin also induces the production of young, hyperreactive platelets, which is especially problematic for chronic administration of erythropoietin, and for the high doses that would be required for neuroprotection. Notably, dogs receiving several high doses of erythropoietin readily develop thromboses⁹⁶. Clinical studies indicate that patients undergoing haemodialysis and patients with cancer seem to be at a higher risk of the adverse effects of erythropoietin. For example, the use of erythropoietin to keep the haematocrit (the ratio of the volume of erythrocytes to the total volume of blood) within the normal range has shown that these prophetic adverse effects are real and problematic^{97,98}. Furthermore, the frequency and severity of the adverse effects increased when larger doses of erythropoietin were used to target a higher haematocrit level that was still within the normal range⁹⁹.

A recent prospective study of patients receiving haemodialysis reported a strong correlation between erythropoietin dosing and patient mortality¹⁰⁰ and a clinical trial of erythropoietin therapy in patients with breast cancer was halted because of increased mortality in the active erythropoietin-treatment group⁹⁷.

Third, many malignant tumours express both erythropoietin and EPOR, and tumour growth is supported by erythropoietin both *in vitro* and *in vivo*¹⁰¹. It is worrying that some cancer patients treated with rhEPO have also shown tumour expansion^{102,103}. Therefore, chronic high dose treatment using rhEPO raises clear concerns of risk versus benefit. Because of the factors described

above, it would be prudent to resist the use of erythropoietin in any 'open-label' fashion for the treatment of tissue injury until the appropriate formal clinical trials have been conducted and confirmed to have reached positive endpoints.

Finally, how the tissue-protective erythropoietin receptor is regulated and whether the receptor is even involved in the adverse effects of erythropoietin remain to be determined. Many studies have shown that the biological activity of EPOR is proportional to the level of protein expression. The tissue-protective erythropoietin receptor is presumably similar, but there could be surprises. This basic information will be critical for planning clinical studies. For example, it will be important to determine the temporal expression pattern of erythropoietin and its receptors in relation to the onset of pathological conditions, as this will dictate the therapeutic window for effective treatment.

Future perspectives

Erythropoietin has proved to be a remarkably neuroprotective molecule that limits the extent of injury produced by diverse pathologies. Its non-erythropoietic tissue-protective derivatives possess at least comparable efficacy and potency and could offer important therapeutic advantages by avoiding adverse effects activated by the homodimeric EPOR. Erythropoietin analogues with short half-lives (such as asialo-erythropoietin) could be part of an 'on-off' approach to tissue protection. These molecules, together with other erythropoietin derivatives with longer half-lives that do not interact with the homodimeric EPOR, constitute a potentially diverse therapeutic armamentarium. Considerable further work is required, but the future looks bright for designing molecules that selectively target the tissue protection pathways of an innate inflammatory response that is activated following tissue injury.

Michael Brines and Anthony Cerami are at The Kenneth S. Warren Institute and Warren Pharmaceuticals, Inc., 712 Kitchawan Road, Ossining, New York 10562, USA. Correspondence to M.B. e-mail: mbrines@warrenpharma.com

doi:1038/nrn1687

- Miyake, T., Kung, C. K. & Goldwasser, E. Purification of human erythropoietin. *J. Biol. Chem.* **252**, 5558–5564 (1977).
- Fisher, J. W. Erythropoietin: physiology and pharmacology update. *Exp. Biol. Med. (Maywood)* **228**, 1–14 (2003).
- Jelkmann, W. The enigma of the metabolic fate of circulating erythropoietin (Epo) in view of the pharmacokinetics of the recombinant drugs rhEpo and NESP. *Eur. J. Haematol.* **69**, 265–274 (2002).

4. Tan, C. C., Eckardt, K. U., Firth, J. D. & Ratcliffe, P. J. Feedback modulation of renal and hepatic erythropoietin mRNA in response to graded anemia and hypoxia. *Am. J. Physiol.* **263**, F474–F481 (1992).
5. Masuda, S. *et al.* A novel site of erythropoietin production. Oxygen-dependent production in cultured rat astrocytes. *J. Biol. Chem.* **269**, 19488–19493 (1994).
6. Konishi, Y., Chui, D. H., Hirose, H., Kunishita, T. & Tabira, T. Trophic effect of erythropoietin and other hematopoietic factors on central cholinergic neurons *in vitro* and *in vivo*. *Brain Res.* **609**, 29–35 (1993).
7. Siren, A. L. *et al.* Erythropoietin and erythropoietin receptor in human ischemic/hypoxic brain. *Acta Neuropathol. (Berl.)* **101**, 271–276 (2001).
8. Morishita, E., Masuda, S., Nagao, M., Yasuda, Y. & Sasaki, R. Erythropoietin receptor is expressed in rat hippocampal and cerebral cortical neurons, and erythropoietin prevents *in vitro* glutamate-induced neuronal death. *Neuroscience* **76**, 105–116 (1997).
9. Sakanaka, M. *et al.* *In vivo* evidence that erythropoietin protects neurons from ischemic damage. *Proc. Natl Acad. Sci. USA* **95**, 4635–4640 (1998).
10. Brines, M. L. *et al.* Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. *Proc. Natl Acad. Sci. USA* **97**, 10526–10531 (2000).
11. Siren, A. L. *et al.* Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. *Proc. Natl Acad. Sci. USA* **98**, 4044–4049 (2001).
12. Beleslin-Kocic, B. B. *et al.* Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells. *Blood* **104**, 2073–2080 (2004).
13. Kawakami, M., Iwasaki, S., Sato, K. & Takahashi, M. Erythropoietin inhibits calcium-induced neurotransmitter release from clonal neuronal cells. *Biochem. Biophys. Res. Commun.* **279**, 293–297 (2000).
14. Koshimura, K., Murakami, Y., Sohmiya, M., Tanaka, J. & Kato, Y. Effects of erythropoietin on neuronal activity. *J. Neurochem.* **72**, 2565–2572 (1999).
15. Martinez-Estrada, O. M. *et al.* Erythropoietin protects the *in vitro* blood-brain barrier against VEGF-induced permeability. *Eur. J. Neurosci.* **18**, 2538–2544 (2003).
16. Li, W. *et al.* Beneficial effect of erythropoietin on experimental allergic encephalomyelitis. *Ann. Neurol.* **56**, 767–777 (2004).
17. Semenza, G. L. HIF-1 and mechanisms of hypoxia sensing. *Curr. Opin. Cell Biol.* **13**, 167–171 (2001).
18. Schuster, J. M. & Nelson, P. S. Toll receptors: an expanding role in our understanding of human disease. *J. Leukoc. Biol.* **67**, 767–773 (2000).
19. Kertesz, N., Wu, J., Chen, T. H., Suvoc, H. M. & Wu, H. The role of erythropoietin in regulating angiogenesis. *Dev. Biol.* **276**, 101–110 (2004).
20. Shingo, T., Sorokan, S. T., Shimazaki, T. & Weiss, S. Erythropoietin regulates the *in vitro* and *in vivo* production of neuronal progenitors by mammalian forebrain neural stem cells. *J. Neurosci.* **21**, 9733–9743 (2001).
21. Brines, M. *et al.* Erythropoietin mediates tissue protection through an erythropoietin and common β -subunit heteroreceptor. *Proc. Natl Acad. Sci. USA* **101**, 14907–14912 (2004).
22. Chin, K. *et al.* Production and processing of erythropoietin receptor transcripts in brain. *Brain Res. Mol. Brain Res.* **81**, 29–42 (2000).
23. Chikuma, M., Masuda, S., Kobayashi, T., Nagao, M. & Sasaki, R. Tissue-specific regulation of erythropoietin production in the murine kidney, brain, and uterus. *Am. J. Physiol. Endocrinol. Metab.* **279**, E1242–E1248 (2000).
24. Nagai, A. *et al.* Erythropoietin and erythropoietin receptors in human CNS neurons, astrocytes, microglia, and oligodendrocytes grown in culture. *J. Neuropathol. Exp. Neurol.* **60**, 386–392 (2001).
25. Masuda, S. *et al.* Functional erythropoietin receptor of the cells with neural characteristics. Comparison with receptor properties of erythroid cells. *J. Biol. Chem.* **268**, 11208–11216 (1993).
26. Livnah, O. *et al.* Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation. *Science* **283**, 987–990 (1999).
27. Eid, T. *et al.* Increased expression of erythropoietin receptor on blood vessels in the human epileptogenic hippocampus with sclerosis. *J. Neuropathol. Exp. Neurol.* **63**, 73–83 (2004).
28. Juul, S. E., Anderson, D. K., Li, Y. & Christensen, R. D. Erythropoietin and erythropoietin receptor in the developing human central nervous system. *Pediatr. Res.* **43**, 40–49 (1998).
29. Suzuki, N. *et al.* Erythroid-specific expression of the erythropoietin receptor rescued its null mutant mice from lethality. *Blood* **100**, 2279–2288 (2002).
30. Masuda, S., Chikuma, M. & Sasaki, R. Insulin-like growth factors and insulin stimulate erythropoietin production in primary cultured astrocytes. *Brain Res.* **746**, 63–70 (1997).
31. Johns, L., Sinclair, A. J. & Davies, J. A. Hypoxia/hypoglycemia-induced amino acid release is decreased *in vitro* by preconditioning. *Biochem. Biophys. Res. Commun.* **276**, 134–136 (2000).
32. Blondeau, N., Plamondon, H., Richelme, C., Heurteaux, C. & Lazdunski, M. K(ATP) channel openers, adenosine agonists and epileptic preconditioning are stress signals inducing hippocampal neuroprotection. *Neuroscience* **100**, 465–474 (2000).
33. Ho, V., Acquaviva, A., Duh, E. & Bunn, H. F. Use of a marked erythropoietin gene for investigation of its cis-acting elements. *J. Biol. Chem.* **270**, 10084–10090 (1995).
34. Bernaudin, M. *et al.* A potential role for erythropoietin in focal permanent cerebral ischemia in mice. *J. Cereb. Blood Flow Metab.* **19**, 643–651 (1999).
35. Mori, M. *et al.* Activation of extracellular signal-regulated kinases ERK1 and ERK2 induces Bcl-XL up-regulation via inhibition of caspase activities in erythropoietin signaling. *J. Cell Physiol.* **195**, 290–297 (2003).
36. Ruscher, K. *et al.* Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an *in vitro* model. *J. Neurosci.* **22**, 10291–10301 (2002).
37. Kilic, U. *et al.* Erythropoietin protects from axotomy-induced degeneration of retinal ganglion cells by activating ERK-1/-2. *FASEB J.* **19**, 249–251 (2004).
38. Digicaylioglu, M. & Lipton, S. A. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF- κ B signalling cascades. *Nature* **412**, 641–647 (2001).
39. Marrero, M. B., Venema, R. C., Ma, H., Ling, B. N. & Eaton, D. C. Erythropoietin receptor-operated Ca²⁺ channels: activation by phospholipase C- γ 1. *Kidney Int.* **53**, 1259–1268 (1998).
40. Yamamoto, M., Koshimura, K., Sohmiya, M., Murakami, Y. & Kato, Y. Effect of erythropoietin on nitric oxide production in the rat hippocampus using *in vivo* brain microdialysis. *Neuroscience* **128**, 163–168 (2004).
41. Yun, H. Y., Dawson, V. L. & Dawson, T. M. Neurobiology of nitric oxide. *Crit. Rev. Neurobiol.* **10**, 291–316 (1996).
42. Villa, P. *et al.* Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis. *J. Exp. Med.* **198**, 971–975 (2003).
43. Agnello, D. *et al.* Erythropoietin exerts an anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis. *Brain Res.* **952**, 128–134 (2002).
44. Cuzzocrea, S. *et al.* Erythropoietin reduces the degree of arthritis caused by type II collagen in the mouse. *Arthritis Rheum.* **52**, 940–950 (2005).
45. Sela, S. *et al.* The polymorphonuclear leukocyte – a new target for erythropoietin. *Nephron* **88**, 205–210 (2001).
46. Grasso, G. *et al.* Beneficial effects of systemic administration of recombinant human erythropoietin in rabbits subjected to subarachnoid hemorrhage. *Proc. Natl Acad. Sci. USA* **99**, 5627–5631 (2002).
47. Gorio, A. *et al.* Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma. *Proc. Natl Acad. Sci. USA* **99**, 9450–9455 (2002).
48. Wang, L., Zhang, Z., Wang, Y., Zhang, R. & Chopp, M. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. *Stroke* **35**, 1732–1737 (2004).
49. Nagelhus, E. A., Mathiesen, T. M. & Ottersen, O. P. Aquaporin-4 in the central nervous system: cellular and subcellular distribution and coexpression with KIR4.1. *Neuroscience* **129**, 905–913 (2004).
50. Dirnagl, U., Simon, R. P. & Hallenbeck, J. M. Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci.* **26**, 248–254 (2003).
51. Dawson, T. M. Preconditioning-mediated neuroprotection through erythropoietin? *Lancet* **359**, 96–97 (2002).
52. Bernaudin, M. *et al.* Normobaric hypoxia induces tolerance to focal permanent cerebral ischemia in association with an increased expression of hypoxia-inducible factor-1 and its target genes, erythropoietin and VEGF, in the adult mouse brain. *J. Cereb. Blood Flow Metab.* **22**, 393–403 (2002).
53. Lee, S. M. *et al.* EPO receptor-mediated ERK kinase and NF- κ B activation in erythropoietin-promoted differentiation of astrocytes. *Biochem. Biophys. Res. Commun.* **320**, 1087–1095 (2004).
54. Juul, S. E. *et al.* Erythropoietin concentrations in cerebrospinal fluid of nonhuman primates and fetal sheep following high-dose recombinant erythropoietin. *Biol. Neonate* **85**, 138–144 (2004).
55. Dame, C., Juul, S. E. & Christensen, R. D. The biology of erythropoietin in the central nervous system and its neurotrophic and neuroprotective potential. *Biol. Neonate* **79**, 228–235 (2001).
56. Ehrenreich, H. *et al.* Erythropoietin: a candidate compound for neuroprotection in schizophrenia. *Mol. Psychiatry* **9**, 42–54 (2004).
57. Grimm, C. *et al.* HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. *Nature Med.* **8**, 718–724 (2002).
58. Foresta, C. *et al.* Erythropoietin stimulates testosterone production in man. *J. Clin. Endocrinol. Metab.* **78**, 753–756 (1994).
59. Banks, W. A., Jumbe, N. L., Farrell, C. L., Niehoff, M. L. & Heatherington, A. C. Passage of erythropoietic agents across the blood-brain barrier: a comparison of human and murine erythropoietin and the analog darbepoetin alfa. *Eur. J. Pharmacol.* **505**, 93–101 (2004).
60. Yamaji, R. *et al.* Brain capillary endothelial cells express two forms of erythropoietin receptor mRNA. *Eur. J. Biochem.* **239**, 494–500 (1996).
61. Harris, K. W. & Winkelmann, J. C. Enzyme-linked immunosorbent assay detects a potential soluble form of the erythropoietin receptor in human plasma. *Am. J. Hematol.* **52**, 8–13 (1996).
62. Juul, S. Recombinant erythropoietin as a neuroprotective treatment: *in vitro* and *in vivo* models. *Clin. Perinatol.* **31**, 129–142 (2004).
63. Gassmann, M. *et al.* Non-erythroid functions of erythropoietin. *Adv. Exp. Med. Biol.* **543**, 323–330 (2003).
64. Ghezzi, P. & Brines, M. Erythropoietin as an antiapoptotic, tissue-protective cytokine. *Cell Death Differ.* **11** (suppl. 1), S37–S44 (2004).
65. Tsuchiya, D., Hong, S., Rajdev, S., Panter, S. & Weinstein, P. Evaluation of the neuroprotective effect of erythropoietin in cerebral ischemia produced by the intraluminal suture model. *Soc. Neurosci. Abstr.* **764**, 17 (2001).
66. Sadamoto, Y. *et al.* Erythropoietin prevents place navigation disability and cortical infarction in rats with permanent occlusion of the middle cerebral artery. *Biochem. Biophys. Res. Commun.* **253**, 26–32 (1998).
67. Celik, M. *et al.* Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. *Proc. Natl Acad. Sci. USA* **99**, 2258–2263 (2002).
68. Junk, A. K. *et al.* Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. *Proc. Natl Acad. Sci. USA* **99**, 10659–10664 (2002).
69. Kumral, A. *et al.* Neuroprotective effect of erythropoietin on hypoxic-ischemic brain injury in neonatal rats. *Biol. Neonate* **83**, 224–228 (2003).
70. Rex, T. S. *et al.* Systemic but not intraocular Epo gene transfer protects the retina from light- and genetic-induced degeneration. *Mol. Ther.* **10**, 855–861 (2004).
71. Sattler, M. B. *et al.* Neuroprotective effects and intracellular signaling pathways of erythropoietin in a rat model of multiple sclerosis. *Cell Death Differ.* **11** (suppl. 2), S181–S192 (2004).
72. Adembi, C. *et al.* Erythropoietin attenuates post-traumatic injury in organotypic hippocampal slices. *J. Neurotrauma* **21**, 1103–1112 (2004).
73. Orhan, B., Yalcin, S., Nurlu, G., Zeybek, D. & Muftuoglu, S. Erythropoietin against cisplatin-induced peripheral neurotoxicity in rats. *Med. Oncol.* **21**, 197–203 (2004).
74. Keswani, S. C. *et al.* A novel endogenous erythropoietin mediated pathway prevents axonal degeneration. *Ann. Neurol.* **56**, 815–826 (2004).
75. Campana, W. M. & Myers, R. R. Erythropoietin and erythropoietin receptors in the peripheral nervous system: changes after nerve injury. *FASEB J.* **15**, 1804–1806 (2001).
76. Campana, W. M. & Myers, R. R. Exogenous erythropoietin protects against dorsal root ganglion apoptosis and pain following peripheral nerve injury. *Eur. J. Neurosci.* **18**, 1497–1506 (2003).
77. Erbayraktar, S. *et al.* Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity *in vivo*. *Proc. Natl Acad. Sci. USA* **100**, 6741–6746 (2003).
78. Leist, M. *et al.* Derivatives of erythropoietin that are tissue protective but not erythropoietic. *Science* **305**, 239–242 (2004).
79. Springborg, J. B. *et al.* A single subcutaneous bolus of erythropoietin normalizes cerebral blood flow autoregulation after subarachnoid haemorrhage in rats. *Br. J. Pharmacol.* **135**, 823–829 (2002).
80. Genc, S. *et al.* Erythropoietin exerts neuroprotection in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated C57/BL mice via increasing nitric oxide production. *Neurosci. Lett.* **298**, 139–141 (2001).

81. Bianchi, R. *et al.* Erythropoietin both protects from and reverses experimental diabetic neuropathy. *Proc. Natl Acad. Sci. USA* **101**, 823–828 (2004).
82. Lieberman, J. *et al.* Longitudinal study of brain morphology in first episode schizophrenia. *Biol. Psychiatry* **49**, 487–499 (2001).
83. Anagnostou, A., Lee, E. S., Kessimian, N., Levinson, R. & Steiner, M. Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. *Proc. Natl Acad. Sci. USA* **87**, 5978–5982 (1990).
84. Tojo, A., Fukamachi, H., Kasuga, M., Urabe, A. & Takaku, F. Identification of erythropoietin receptors on fetal liver erythroid cells. *Biochem. Biophys. Res. Commun.* **148**, 443–448 (1987).
85. Campana, W. M., Misasi, R. & O'Brien, J. S. Identification of a neurotrophic sequence in erythropoietin. *Int. J. Mol. Med.* **1**, 235–241 (1998).
86. Jubinsky, P. T., Krijanovski, O. I., Nathan, D. G., Tavernier, J. & Sieff, C. A. The beta chain of the interleukin-3 receptor functionally associates with the erythropoietin receptor. *Blood* **90**, 1867–1873 (1997).
87. Scott, C. L. *et al.* Reassessment of interactions between hematopoietic receptors using common beta-chain and interleukin-3-specific receptor beta-chain-null cells: no evidence of functional interactions with receptors for erythropoietin, granulocyte colony-stimulating factor, or stem cell factor. *Blood* **96**, 1588–1590 (2000).
88. Wang, X. *et al.* The nonerythropoietic asialoerythropoietin protects against neonatal hypoxia-ischemia as potently as erythropoietin. *J. Neurochem.* **91**, 900–910 (2004).
89. Dong, Y. J., Kung, C. & Goldwasser, E. Receptor binding of asialoerythropoietin. *J. Cell Biochem.* **48**, 269–276 (1992).
90. Elliott, S., Lorenzini, T., Chang, D., Barzilay, J. & Delorme, E. Mapping of the active site of recombinant human erythropoietin. *Blood* **89**, 493–502 (1997).
91. Miyashita, K. *et al.* Blood pressure response to erythropoietin injection in hemodialysis and predialysis patients. *Hypertens. Res.* **27**, 79–84 (2004).
92. Vaziri, N. D. *et al.* *In vivo* and *in vitro* pressor effects of erythropoietin in rats. *Am. J. Physiol.* **269**, F838–F845 (1995).
93. Carlini, R., Obialo, C. I. & Rothstein, M. Intravenous erythropoietin (rHuEPO) administration increases plasma endothelin and blood pressure in hemodialysis patients. *Am. J. Hypertens.* **6**, 103–107 (1993).
94. Carlini, R. G., Dusso, A. S., Obialo, C. I., Alvarez, U. M. & Rothstein, M. Recombinant human erythropoietin (rHuEPO) increases endothelin-1 release by endothelial cells. *Kidney Int.* **43**, 1010–1014 (1993).
95. Quaschnig, T. *et al.* Erythropoietin-induced excessive erythrocytosis activates the tissue endothelin system in mice. *FASEB J.* **17**, 259–261 (2003).
96. Wolf, R. F. *et al.* Erythropoietin potentiates thrombus development in a canine arterio-venous shunt model. *Thromb. Haemost.* **77**, 1020–1024 (1997).
97. Leyland-Jones, B. Breast cancer trial with erythropoietin terminated unexpectedly. *Lancet Oncol.* **4**, 459–460 (2003).
98. Rosenzweig, M. Q., Bender, C. M., Lucke, J. P., Yasko, J. M. & Brufsky, A. M. The decision to prematurely terminate a trial of r-HuEPO due to thrombotic events. *J. Pain Symptom Manage.* **27**, 185–190 (2004).
99. Peterson, L. FDA oncologic drugs advisory committee (ODAC) meeting on the safety of erythropoietin in oncology. *Trends Med.* 1–4 (2004).
100. Cotter, D. *et al.* Hematocrit was not validated as a surrogate end point for survival among epoetin-treated hemodialysis patients. *J. Clin. Epidemiol.* **57**, 1086–1095 (2004).
101. Yasuda, Y. *et al.* Erythropoietin regulates tumour growth of human malignancies. *Carcinogenesis* **24**, 1021–1029 (2003).
102. Pajonk, F., Weil, A., Sommer, A., Suwinski, R. & Henke, M. The erythropoietin-receptor pathway modulates survival of cancer cells. *Oncogene* **23**, 8987–8991 (2004).
103. Henke, M. *et al.* Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised, double-blind, placebo-controlled trial. *Lancet* **362**, 1255–1260 (2003).
104. Glezer, I. & Rivest, S. Glucocorticoids: protectors of the brain during innate immune responses. *Neuroscientist* **10**, 538–552 (2004).
105. McClure, B. J. *et al.* Molecular assembly of the ternary granulocyte-macrophage colony-stimulating factor receptor complex. *Blood* **101**, 1308–1315 (2003).
106. Juhaszova, M. *et al.* Glycogen synthase kinase-3 β mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J. Clin. Invest.* **113**, 1535–1549 (2004).
107. Digicaylioglu, M., Garden, G., Timberlake, S., Fletcher, L. & Lipton, S. A. Acute neuroprotective synergy of erythropoietin and insulin-like growth factor I. *Proc. Natl Acad. Sci. USA* **101**, 9855–9860 (2004).

Acknowledgements

Recently, the number and diversity of publications concerning non-erythroid actions of erythropoietin has grown in an exponential manner. We apologize to our many colleagues for omission of their important work due to stringent space limitations in this brief perspective. We especially thank our principal colleagues, T. Coleman, Q.-w. Xie and M. Yamin at Warren Pharmaceuticals; P. Ghezzi, R. Latini and colleagues at the Mario Negri Pharmacological Institute in Milan, Italy; G. Grasso and A. Sfacteria, University of Messina, Sicily, Italy; M. Leist, T. Sager, L. Torup and co-workers at H. Lundbeck A/S, Copenhagen, Denmark; O. Yilmaz, S. Erbayraktar, Z. Erbayraktar and N. Gokmen at Dokuz Eylul University, Izmir, Turkey; and H. Ehrenreich and A. Siren, Max Planck Institute for Experimental Medicine, Göttingen, Germany. M.B. and A.C. are supported by the Kenneth S. Warren Institute and Warren Pharmaceuticals. We are employed by Warren Pharmaceuticals, which is engaged in developing non-erythropoietic tissue protective cytokines for clinical use.

Competing interests statement

The authors declare **competing financial interests**: see Web version for details.

Online links

DATABASES

The following terms in this article are linked online to:

Entrez Gene: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>
 BCL2 | BCL-X_L | EPO | EPOR | GMCSF | IL-3 | IL-5 | JAK2 | PKB | PI3K | PLC γ | STAT | TNF α | VEGF

FURTHER INFORMATION

Warren Pharmaceuticals: <http://www.warrenpharmaceuticals.com/pages/home.html>

Access to this interactive links box is free online.