Erythropoietin-mediated tissue protection: reducing collateral damage from the primary injury response

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In its classic hormonal role, erythropoietin (EPO) is produced by the kidney and regulates the number of erythrocytes within the circulation to provide adequate tissue oxygenation. EPO also mediates other effects directed towards optimizing oxygen delivery to tissues, e.g. modulating regional blood flow and reducing blood loss by promoting thrombosis within damaged vessels. Over the past 15 years, many unexpected nonhaematopoietic functions of EPO have been identified. In these more recently appreciated nonhormonal roles, locally-produced EPO signals through a different receptor isoform and is a major molecular component of the injury response, in which it counteracts the effects of pro-inflammatory cytokines. Acutely, EPO prevents programmed cell death and reduces the development of secondary, pro-inflammatory cytokine-induced injury. Within a longer time frame, EPO provides trophic support to enable regeneration and healing. As the region immediately surrounding damage is typically relatively deficient in endogenous EPO, administration of recombinant EPO can provide increased tissue protection. However, effective use of EPO as therapy for tissue injury requires higher doses than for haematopoiesis, potentially triggering serious adverse effects. The identification of a tissue-protective receptor isoform has facilitated the engineering of nonhaematopoietic, tissue-protective EPO derivatives, e.g. carbamyl EPO, that avoid these complications. Recently, regions within the EPO molecule mediating tissue protection have been identified and this has enabled the development of potent tissue-protective peptides, including some mimicking EPO’s tertiary structure but unrelated in primary sequence.

Keywords: apoptosis, cytokines, cytoprotection, healing, inflammation, regeneration.

Introduction

For many years, erythropoietin (EPO) has been viewed solely as a renal hormone with a specialized role in maintaining adequate numbers of erythrocytes. However, recent studies have revealed that EPO is a multifunctional molecule produced and utilized by many tissues. In addition to erythropoiesis, EPO’s other key roles involve the acute and subacute biological responses to tissue damage. In these roles, EPO attenuates both primary and secondary injury, as well as facilitates healing and restoration of function. This new appreciation assigns EPO as a central player in the local down-regulation of inflammatory processes triggered by injury.

Molecular versatility, the ability of one compound to elicit multiple effects, is a general characteristic of biological systems. Whilst these actions can be complementary, the physiological roles are often qualitatively different. Typically, multifunctional molecules that trigger widely different biological responses do so by utilizing different receptor isoforms with markedly different binding affinities for the cognate ligand.
Additionally, ‘cross-talk’ between biological systems utilizing a common signalling molecule is often reduced by restricting operation to within a localized environment, i.e. in a paracrine-autocrine manner.

As one of many examples, the peptide vasopressin is normally responsible for maintaining plasma osmolality via its antidiuretic action, circulating on a sustained basis in the 5–20 pmol L\(^{-1}\) range and interacting with the V2 receptor (affinity of \(\sim 0.4 \text{ nmol L}^{-1}\)) in the kidney [1]. However, in its other role as a stress hormone, vasopressin exhibits strong vasomotor, metabolic and prothrombotic activities triggered when circulating levels up to 200 nmol L\(^{-1}\) interact with its V1 receptor (affinity of \(\sim 12 \text{ nmol L}^{-1}\)) expressed by many tissues (reviewed in refs [2, 3]). Thus, differentiation of biological roles of the same molecule is achieved by differences in receptor affinity and distribution.

As a hormone, EPO circulates at concentrations in the low picomolar range (1–7 pmol L\(^{-1}\); reviewed in Jelkmann [2]). Plasma concentrations are maintained by a classic negative feedback loop in which hypoxia detected by the kidney stimulates the production and release of EPO into circulation. This renal EPO elicits an increase in erythrocyte number and therefore, improved oxygen delivery to the kidney, resulting in a compensatory dampening of EPO production. In the setting of severe, chronic hypoxia, plasma EPO levels can increase more than 100-fold above baseline.

Within the bone marrow, EPO binds to a preformed homodimeric EPO receptor (EPOR)\(_2\) present on the cell membrane of the erythroid colony-forming units (Fig. 1a). This in turn initiates a signalling cascade that allows erythroid maturation by inhibiting an ongoing apoptotic programme. Although EPO is absolutely required for erythroid development, other cytokines act in a synergistic fashion, e.g. stem cell factor [3]. The affinity of the homodimeric receptor for EPO is \(\sim 100–200 \text{ pmol L}^{-1}\) (Fig. 2). To maintain a normal pool of erythrocytes in humans, about 0.8–1% of the red cell mass must be replaced daily. However, because only a small fraction of potential new erythrocytes express (EPOR)\(_2\) at any time, EPO concentrations must be continuously sustained within the circulation. (EPOR)\(_2\) occupancy under normal EPO plasma concentrations is, however, low (\(\sim 3\%\)), which is achieved when the EPO concentration reaches \(\sim 15 \text{ pmol L}^{-1}\) [4].

In contradistinction to the low picomolar endocrine system, EPO is also produced and released into the surrounding environment by virtually every tissue studied under conditions of hypoxia or metabolic stress, e.g. glucopenia, intense cellular metabolic work, infection or trauma. This locally-produced EPO interacts with a receptor that is typically not highly expressed, but instead is up-regulated following injury or stress. Signalling through this receptor initiates diverse but co-ordinated functions that generally counteract the potential collateral damage triggered by injury. In contrast to the hormonal activities mediated by EPO, the local concentrations of EPO in these tissue-protective roles are higher and the affinity of the receptor lower (1–20 nmol L\(^{-1}\)). Notably, these pharmacological characteristics also reduce the probability that circulating EPO of renal origin can interact with this local, tissue-protective system.

In this review, we will compare and contrast EPO’s activities in the erythropoietic versus tissue-protective arenas. Notably, evidence supports the existence of a receptor isoform that mediates tissue protection composed of EPOR and the beta common receptor (\(\beta cR\)). Based on this knowledge, EPO derivatives have been engineered that interact with this receptor but possess no affinity for (EPOR)\(_2\) and thus are not erythropoietic. Finally, specific regions of the EPO molecule that do not interact with the binding sites of (EPOR)\(_2\) have been identified that are tissue protective but are not erythropoietic. These have enabled the synthesis of small peptides that possess the full range of tissue protection exhibited by the parent EPO molecule.

**Biology of the tissue-protective system: the stereotypic injury response**

All multicellular organisms are challenged with the need to localize pathogen invasion and prevent a lethal, generalized spread of infection. Evolutionarily,
this problem has been solved by the employment of a group of molecules: pro-inflammatory cytokines of the type I superfamily – whose role is to orchestrate a sequence of biological activities summarized as the innate immune response [5]. These actions evolved to isolate and destroy pathogens, but normal cells within the proximate environment are also unavoidably damaged. Thus, a prominent characteristic of the stereotypic response to infection (i.e. independent of pathogen type) is that the adjacent surrounding, noninfected tissue is at risk of destruction. Collateral damage inflicted outside the pathogen environment ultimately results in lesions that are larger than the infected volume of tissue. Moreover, this innate immune response is also triggered by many noninfectious stimuli such as ischaemia, immune precipitates, toxins, chemical agents or infarction, amongst others. These sterile stimuli also precipitate collateral damage that significantly amplifies the initial insult.

In mammals, the injury response has been studied in great detail and is a highly orchestrated process with distinct stages (reviewed by Lotze et al. [6]). The first component of this cascade is initiated by immune-competent cells recognizing characteristic molecular signatures of the pathogen. In response, a defensive programme is activated by the production of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α). These cytokines kill cells by causing the production of a variety of highly toxic molecules, e.g. free radicals as well as recruiting immune-competent cells into the injury site. Pro-inflammatory cytokines also activate physiological processes that serve to isolate the damage, such as vascular thrombosis and oedema.

The pro-inflammatory arm of the injury response is inherently highly self-amplifying. Unchecked, tissue injury in response to pathogen invasion will spread far beyond the nidus of infection, potentially even overwhelming the host (Fig. 3a). Therefore, a crucial phase of the normal injury response is an active attenuation of inflammation occurring after a critical time delay that enables an initial phase of sterilization. If successful, the end result is a necrotic region that isolates and contains the invading pathogen.

Recent investigations have shown that EPO, another member of the type I cytokine superfamily, is the
principal molecular mediator charged with attenuating the immune response triggered by injury. Results of studies have shown that in many respects, the biological effects of TNF-α and EPO are antagonistic. Each is capable of suppressing the synthesis and, to a variable, tissue-dependent extent, the biological activity of the other (Fig. 3b). In general, tissues and organs possess the capacity to synthesize pro-inflammatory cytokines or functionally can do so because of cytokine production by immune-competent cells recruited to the injury site. In general, the ambient tissue concentrations of these opposing molecules determine whether inflammation or tissue protection dominates. Within the core region of an innate immune response (Fig. 3a), the TNF-α concentration is high and EPO is low. Towards the periphery of the lesion, TNF-α levels decrease due to the effects of diffusion away from the production source, allowing EPO synthesis.

An understanding of the actions of the innate immune response is also relevant to noninfectious tissue injury. Cell damage, irrespective of the cause, activates an ‘injury identification’ stage via production of molecular markers – alarmins – that indicate tissue damage has occurred [7]. The alarmins are a diverse group of molecules that include the leakage of intracellular contents, generation of highly reactive free radicals and the result of activation of cellular death signals. As one example, infectious agents cause nearby cells to release nuclear protein high-mobility group box 1 protein (HMGB1), which in turn activates an inflammatory response [8, 9]. Thus, it is critically important to appreciate that tissue injury in the absence of pathogens also produces molecular signatures that trigger a full injury response. However, as pathogens are not present, the sterilized volume of injury is always inappropriately large. It is in this setting, locally-produced EPO acts to limit the collateral damage initiated by the generalized injury response.

In addition to the production or inhibition of these functionally opposing type I cytokines, another control in the spread of injury relies upon the expression of receptors for the molecular mediators of the injury response. Similar to the opposing biological effects of the ligands, a similar relationship exists in the expression of their cognate receptors. Within the tissue-protective arm, EPO receptor expression is activated in advance of EPO production [10]. Critically for the regulation of the extent of the injury response, EPO receptor is induced by TNF-α [11], opposite to EPO which is suppressed. Thus, a double safety system exists wherein both the ligands and their respective receptors are actively regulated to ultimately provide a protective tissue response. For example, in mice with TNF receptor 1 knocked-out, EPO receptor fails to up-regulate and therefore EPO is not protective of kainate-induced seizures and hippocampal neuronal losses [12]. In general,
EPO is a primary component of the innate immune response to injury in which it rescues cells from apoptosis and opposes the actions of pro-inflammatory cytokines. (a) Diverse initiators of injury, e.g. infection, ischaemia-reperfusion, or trauma, each activate a stereotypic response characterized by the production of pro-inflammatory cytokines. For example, TNF-α released by cells within the central region diffuses out into adjacent healthy tissue, inducing widespread apoptosis and further amplification of inflammation. The region surrounding the central core of injury (grey; the penumbra) is initially viable, but if not rescued, eventually dies and enlarges the lesion volume. (b) Pro-inflammatory cytokines within the penumbra stimulate the expression of receptors for EPO by penumbral cells (grey). Although metabolic stress within the penumbra would ordinarily initiate EPO production via the stabilization of hypoxia inducible factor, high levels of pro-inflammatory cytokines present there suppress EPO. At the border of the penumbra the lower levels of pro-inflammatory cytokines do not effectively inhibit EPO production, which diffuses out (blue arrows) and interacts with the tissue-protective receptor, halting the spread of injury. The rationale for use of exogenous EPO or other tissue-protective compounds is to flood the penumbral region expressing receptors for EPO, salvaging those cells from pro-inflammatory cytokine-induced death. EPO also antagonizes other deleterious actions of pro-inflammatory cytokines, e.g. decreases vasospasm, and within a later time frame initiates healing and regeneration by recruitment of stem cells and neoangiogenesis. (c) A delicate balance in tissue injury exists between EPO and pro-inflammatory cytokines such as TNF-α. A sufficient amount of tissue destruction is required to localize infection and to mobilize a debris-clearing response. This critical response is orchestrated by inflammation. Compensatory EPO production by nearby tissue balances the effects of inflammatory mediators and prevents the further spread of damage.
EPO does not increase the expression of its receptor, but has been observed to do so under conditions of hypoxia in vascular endothelial cells [13].

A study of the expression of EPO and its receptor within the area at risk surrounding the necrotic core (the penumbra) was initially performed following ischaemic injury in the central nervous system [10]. These observations revealed a stereotypic response (Fig. 3b) characterized by the appearance of EPOR at ~8–12 h following injury, and only after another ~12 h, the synthesis of EPO. Specific cell types expressed EPO with a characteristic time delay, e.g. endothelial cells (1 day), microglia/monocytes (3 days) and reactive glia (7 days). Within the injury response, EPO-producing cells were mostly observed at the periphery of the lesion. In contrast, EPOR-expressing cells were located more centrally within the lesion. The spatial mismatch between the expression of EPO and its receptor arises from the effects of pro-inflammatory cytokines that suppress EPO but stimulate EPOR expression, as mentioned above. The size of the resulting lesion therefore is a result of a balance of the effects of pro-inflammatory cytokines and EPO as mediated through their respective receptors. In confirmation of this mechanism, increasing local EPO by direct intracranial infusion decreases ischaemia-induced brain injury, whereas neutralization of EPO by infusion of a soluble EPO receptor amplifies injury [14]. Similar effects have been observed in other tissues, e.g. the retina [15]. In summary, a beneficial role for administering exogenous EPO is to rescue cells at risk of apoptosis within the penumbra by signalling though its already expressed receptor.

Erythropoietin also mediates a number of additional effects related to the resolution of damage and recovery from injury. Prominent amongst these is the recruitment of stem cells into the region of injury. The molecular mediator of EPO’s action on endothelial progenitor cells (EPCs) has been shown to be nitric oxide produced by the action of endothelial nitric oxide synthase (eNOS), as eNOS knock-out mice cannot display an EPC response to EPO [16]. Further, EPO also triggers production of tissue-specific growth factors. For example, in the central nervous system, EPO stimulates the production of brain-derived neurotrophic factor (BDNF), which subsequently exerts its own strong effects on neuronal plasticity [17]. In addition to spatially-localized effects in solid tissues, another prominent target for EPO is the capillary vascular endothelial cell. In this role, EPO protects metabolically stressed (e.g. hypoxic) endothelial cells from apoptosis [18] and in this way helps maintain an adequate blood supply into the region of injury.

From this perspective, EPO is an endogenous antagonist of pro-inflammatory cytokines (Fig. 3c). However, in many situations a penumbra exists in which receptor expression is up-regulated, but little or no endogenous EPO is present because of suppression by pro-inflammatory cytokines. This circumstance constitutes the conceptual basis of administering exogenous EPO and thereby potentially salvaging the tissue at risk. Because delivery of EPO into the lesion’s core cannot fully antagonize the strong inflammatory components present, EPO does not impair the ability of the innate immune response to neutralize pathogens unlike strong immunosuppressants, e.g. glucocorticoids. Finally, since the tissue-protective effects of EPO are triggered by injury and evolve over hours, the ‘window of opportunity’ for treatment is typically very broad. In many preclinical models EPO can be administered hours or days after injury with effects qualitatively similar to its immediate administration. For these reasons, EPO and its tissue-protective derivatives are particularly attractive pharmaceutical candidates.

Erythropoietin-mediated erythropoiesis

Erythropoietin is a 165 amino acid protein member of the type I cytokine superfamily [2]. It is a compact, globular molecule because of the hydrophobic interaction of its four alpha helices present in an antiparallel configuration. In the adult kidney, EPO is ordinarily produced by the renal tubular interstitium in response to hypoxia, via stabilization of hypoxia-inducible factor-1 alpha (HIF-1α) which activates the transcription of the EPO gene [2]. There is significant homology of the EPO molecule across all vertebrates that have been studied [19, 20]. Although mammals generally respond briskly to a cross-species EPO, chronic exposure to nonnative EPO invariably leads to the development of anti-EPO antibodies. These frequently
neutralize endogenous EPO, resulting in a profound anaemia secondary to pure red cell aplasia. This fact limits the chronic use of recombinant human EPO in animal models.

Likewise, EPOR is a member of the type I cytokine receptor family that exhibits high homology within regions of the extracellular domain. These are characterized by a cytokine binding homology region, formed by tandem fibronectin repeats, four conserved cysteine residues within the amino terminal domain and a tryptophan-serine motif (Trp-Ser-X-Trp-Ser) near the membrane insertion site [reviewed in Boulangger and Garcia [21]]. Monomeric EPOR subunits expressed on the surface of red cell progenitors spontaneously associate as a function of their concentration within the membrane [22], via formation of a leucine zipper within the transmembrane domain [23]. Whilst the monomers lack enzymatic activity and therefore do not autophosphorylate, the cytoplasmic portion of each of the receptor subunits is physically associated with janus tyrosine kinase-2 (JAK-2). Binding of EPO to the haematopoietic receptor (EPOR)2 has been extensively studied and is well understood [24]. EPO interacts with specific sites in the extracellular portion of the receptor within each subunit, eliciting a conformational change which subsequently activates JAK-2 phosphorylation. This biochemical signal in turn initiates a signalling cascade that utilizes a number of downstream molecular pathways, including the STAT5-Bcl-2 pathway, ultimately inhibiting apoptosis of the erythrocyte precursor.

In addition to EPO itself, several erythropoietic EPO-mimetic peptides have been reported to date. One class consists of short peptides that interact with (EPOR)2 by interdigitating with the receptor dimer at a location close to the membrane insertion site, causing a conformational change, and initiating signalling [25]. This peptide is composed of amino acids 194–216 of the EPOR and binds to the same sequence within the intact EPOR molecule, a region that is involved in the dimerization of the receptor monomers. Another class of EPO-mimetic peptides was discovered using phage display technology. These are small, cyclized peptides unrelated by sequence or structure to EPO [26]. The EPO-mimetic peptides also interact exclusively with (EPOR)2 by binding to the extracellular portion in a manner similar to that of the EPO protein. These peptides are much less potent than EPO, but their activity can be significantly improved by dimerization.

It is important to appreciate that in addition to erythropoiesis, EPO also performs other haematopoietic functions related to minimizing acute and subacute perturbations in haemoglobin levels, as, for example, caused by haemorrhage. Specifically, EPO in synergy with thrombopoietin induces an accelerated maturation of megakaryocytes [27], producing young, reticulated, ‘stress’ platelets that are highly reactive [28, 29]. EPO also activates endothelial cells to express adhesion molecules that attract platelets and leukocytes to plug vessels by thrombus formation. Increased EPO levels also stimulate vascular smooth muscle contraction that reduces regional blood flow, potentially limiting blood loss, as well as altering systemic vascular resistance in a manner to improve blood flow, and therefore O2 delivery, to exercising muscle [30]. Within a longer time frame, EPO also initiates angiogenesis which can improve tissue oxygenation. It is important to appreciate that in addition to erythropoiesis, these other hormonal activities of EPO can be particularly problematic when therapy is directed to the tissue-protective realm, as higher doses of EPO are required (see below).

**Erythropoietin-mediated tissue protection**

In 1993, Konishi et al. [31] reported that EPO exhibited trophic effects on cultured embryonic central cholinergic neurones, including the promotion of cell survival following serum withdrawal and the growth of neuronal processes. Simultaneously, Masuda et al. [32] described the existence of a functional EPO receptor on the neural-like clonal cell lines PC12 and SN6. The in vivo relevance of these findings was confirmed by the demonstration of the specific binding of radiolabelled EPO in a number of brain regions of the mouse, in which EPOR mRNA was detected [33]. Further, EPO mRNA was present in the normal brain and was up-regulated in mice subjected to hypoxic conditions. The existence of EPO and EPOR was also
confirmed in human and monkey brain, with astrocytes as the EPO source, and EPOR-positive neurones the target [34]. These seminal discoveries were expanded by an elegant series of experiments performed by the Sasaki group [14, 35] which established that exogenous recombinant human EPO infused directly into the brain was strongly protective of ischaemic injury. In further confirmation of a neuroprotective role for EPO, neutralization of endogenous EPO by infusion of soluble EPOR markedly worsened injury [14]. In sum, the results of these investigations clearly demonstrated that EPO is made locally in the brain and serves to limit the extent of brain injury.

The question naturally arose as to whether the EPO and EPOR proteins expressed in the brain were the same as those of the erythropoietic system. The EPO produced by hypoxic-cultured primary astrocytes was found to be smaller in size and more potent in a receptor-binding assay, findings consistent with a lower degree of sialylation [36]. Notably, the EPOR expressed by PC12 cells displayed a lower affinity for EPO when compared with erythroid cells (a binding constant of 16 nmol L\(^{-1}\) vs. 95 pmol L\(^{-1}\) respectively), but the EPOR cDNA sequence was identical to that of erythroid cells [32]. Cross linking experiments also showed that EPOR was bound to different sized proteins in neuronal-like versus erythroid cells.

These observations raise the possibility of the existence of a receptor isoform for EPO that specifically mediates tissue protection. As will be described below, we discovered that carbamyl EPO (CEPO) prepared by reacting EPO with cyanate no longer binds (EPOR), and therefore is not haematopoietic in vivo or in vitro. CEPO is, nevertheless, as active as EPO in tissue protection. To further understand the nature of the tissue-protective receptor, membrane proteins prepared from various tissues were passed over a column of immobilized CEPO. The bound fraction contained EPOR and the beta common receptor (βcR), linked by a disulphide bond [37]. βcR is so called because it is the signallng part of the receptor complex of interleukin (IL)-3, granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-5. For these receptors, it has been shown that ligand specificity is conferred by the associated unique alpha chain (IL-3Ra, GM-CSFRα and IL-5Ra respectively). A review of the literature revealed that this heteroreceptor had been previously observed by several groups [38, 39] and that EPO could phosphorylate βcR [40]. However, the earlier investigations were not extended when it was observed that βcR knock-out mice were haematopoietically normal. Only later was it appreciated that these mice are unable to respond to EPO’s tissue-protective activities following injury in vitro (cardiomyocyte protection) or in vivo (spinal cord compression) [37].

Available evidence shows that βcR exists as a preformed, intertwined, dimer on the cell surface [41]. Following ligand binding, the alpha receptor subunit is covalently attached via a cystine linkage to βcR [42]. Based on this model, the tissue-protective receptor is thought to consist of two EPOR bound to 2 βcR (Fig. 1b). In the βcR family, it is the βcR subunit that undergoes phosphorylation at a number of cytoplasmic sites following ligand binding [43]. Therefore, the biological effects of receptor signalling in cells that express multiple βcR-containing receptors are similar. For example, GM-CSF, which signals through βcR, has been reported to be neuroprotective in a manner similar to EPO [44]. Because the type I cytokine receptors are often formed by the spontaneous self-association of subunits present in the cell membrane (which is markedly accelerated in the presence of some ligands), the abundance of each subunit determines the probability of self-assembly. For example, as EPOR concentration in the membrane increases, so does the probability of EPOR homodimerization [22]. Conversely, it is also likely that as the ratio of βcR to EPOR increases, the probability of a self-assembly of EPOR and βcR into a heteromer increases. Details of the regulation of receptor subunits present in the cell membrane in cells that express multiple receptor subtypes are not currently well understood and require further study. Finally, although the general form of the tissue-protective receptor in vivo appears to be that of a heteromer, conditions in which the concentration of βcR is very low or absent would then favour EPOR dimerization, which
could trigger tissue protection. For example, using a cultured neuronal-like cell lines that express only EPOR, Um *et al.* [45] reported that cytoprotection by EPO is mediated by the EPOR homodimer. It is expected that nonerythropoietic EPO analogues and mimetics that are specific for the tissue-protective receptor (see below) would not be cytoprotective under these experimental conditions.

**Does the blood–brain barrier exclude EPO?**

It was initially assumed that the large, glycosylated EPO molecule could not cross the intact blood–brain barrier (BBB) [33, 46] and thus early studies of neuroprotection using recombinant human EPO (rhEPO) employed only a direct, intrathecal administration. Contrary to this assumption, we unexpectedly observed a robust effect of systemically administered rhEPO on learning in a conditioned taste aversion model following peripheral administration [47], a finding that implied that EPO penetrated the BBB. Cerebral spinal fluid (CSF) sampling after intravenous administration showed that in the rat, as much as 1% of the plasma peak of rhEPO appeared within the CSF with a delay of $\sim 1.5$ h [48], well within the neuroprotective range *in vitro*. Subsequent examination of the endothelium forming the BBB showed abundant immunoreactive EPOR protein, enriched within the luminal membrane [49], suggesting the possibility of transcytosis of an EPO–EPOR complex from within the capillary lumen into the brain parenchyma, as is known to occur for other cytokines [50]. In support of this observation, movement of rhEPO out of the circulation into the intact brain has been directly observed in human subjects using radiolabelled EPO following peripheral administration [47]. More extensive studies confirmed that systemic administration of large doses of rhEPO (500–5000 IU kg$^{-1}$ bw) in rodents, sheep and primates [51, 52] appeared within the CSF with a mean delay of about 1 h and reaching neuroprotective concentrations within 2 h. Recently, a formal pharmacokinetic study confirming the appearance of exogenous EPO in the CSF of humans has been performed, utilizing Ommaya reservoirs placed into the lateral ventricles [53]. It should also be noted that a penetration of EPO across other tight barriers has also been documented, e.g. the blood–retinal barrier [54].

Although it is clear that pharmacologically relevant amounts of EPO enter the brain, spinal cord and retina following intravenous administration, whether movement across the BBB occurs via a specific, receptor-mediated mechanism or simply by diffusion has not been resolved. In an initial study, biotin was detected within the brain parenchyma following biotin-labelled EPO administration [49]. Another study performed using an *in vitro* model of the BBB (across an endothelial cell to an astrocyte feeder layer) [55] concluded that EPO was transported in a unidirectional and saturable manner, consistent with receptor-mediated transport. However, although a subsequent *in vivo* evaluation performed in the mouse confirmed a significant permeability of rhEPO, it was equivalent to labelled albumin and not inhibited by excess unlabelled rhEPO and therefore appeared to be nonspecific [56]. In the same study, though, recombinant mouse EPO behaved differently, penetrating into the brain significantly faster and to a higher extent than albumin. Further studies are needed to directly assess the movement of EPO into the brain parenchyma, in a regional manner and correlated with the expression of EPO receptors in the endothelial cells forming the BBB in that region.

**Efficacy of peripherally administered EPO in neurological diseases**

The unexpected discovery that systemically administered rhEPO can achieve neuroprotective concentrations within the CSF opened the way for the potential use of rhEPO to treat neurological diseases, even those characterized by an intact BBB. Subsequently, our group showed that peripherally administered rhEPO is effective in a wide variety of models of brain injury, including ischaemia-reperfusion (stroke), blunt cortical trauma, neurotoxin-induced seizures and experimental autoimmune encephalitis (a model for multiple sclerosis) [49].

The pathology of the stroke model we employed follows the innate immune response programme
previously described closely. It is characterized by the formation of a large penumbral region in which massive neuronal death occurs by apoptosis. The model is established as follows: in young male rats, permanent occlusion of the middle cerebral artery and the ipsilateral internal carotid results in only a small cortical infarct due to an abundant collateral blood supply [57]. However, a subsequent reversible occlusion of the contralateral carotid [58] produces a large, ischaemic volume surrounding the infarct. After 60 min of occlusion of blood flow, neurones within this penumbra will undergo apoptosis within the following 24 h, associated with the influx of inflammatory cells (leucocytes and microglia) and production of pro-inflammatory cytokines [49, 59]. Administration of rhEPO (5000 IU kg\(^{-1}\) bw via the intraperitoneal route) immediately following restoration of the cerebral circulation dramatically reduced neuronal apoptosis within the penumbra by up to 75%. However, rhEPO did not affect the volume of the necrotic core. rhEPO also markedly reduced the influx of microglia and leucocytes and tissue levels of pro-inflammatory cytokines [59]. It is notable that a very similar tissue-protective effect can be accomplished in this model by directly neutralizing TNF-\(\alpha\) or its production by the use of an anti-TNF-\(\alpha\) antibody or an inhibitor of TNF-\(\alpha\) synthesis injected directly into the brain [60]. Thus, the sterile inflammatory response induced by ischaemia-reperfusion can be reduced by antagonizing the pro-inflammatory components, using a variety of approaches.

Exploration of the dose–response of rhEPO-mediated neuronal protection revealed that \(\sim 500\) IU kg\(^{-1}\) bw constituted a minimum effective dose. When injury volume was estimated by vital staining performed 24 h following ischaemia, EPO administered up to 6 h following reperfusion resulted in robust protection of the affected cerebral cortex [49]. Subsequent studies evaluating behavioural outcome have shown that EPO administration delayed for 24 h following reperfusion, although without effect on infarct size, still has strong beneficial, protective effects [61].

In general, the development of new treatments for stroke has been frustrating, as successful translation of positive neuroprotective effects observed in preclinical models has not been replicated in clinical trials [62]. It is encouraging, however, that Ehrenreich et al. [63] documented significant neuroprotective effects of rhEPO in a small proof-of-concept human stroke study. The study consisted of 40 patients suffering an ischaemic stroke in the territory of the middle cerebral artery with a mean time to drug administration of 5 h. Twenty-one patients were administered a high dose of rhEPO (33 000 IU i.v. over 20 min, providing an estimated peak level of \(\sim 3\) nmol L\(^{-1}\); see Fig. 2) daily for the first 3 days following stroke onset, whilst the other 19 patients received saline. End-point variables assessed included neurological outcome as determined by the US National Institutes of Health and the Scandinavian stroke scales, as well as functional outcome at 30 days as determined by the Barthel and Rankin scales. The rhEPO-treated group exhibited a significantly superior outcome in a number of these neurological assessments.

In addition to the prevention and treatment of ischaemia-reperfusion damage to the nervous system, as predicted by the sterile inflammatory response to injury, rhEPO has been shown to be effective in other preclinical models of CNS injury. A noncomprehensive list includes experimental autoimmune encephalitis [64–68], subarachnoid haemorrhage [69–74], surgical [75], mechanical [76, 77] or cold-induced [78] trauma and excitotoxins [79].

As rhEPO is an approved drug that has been safely administered at haematopoietic doses to hundreds of thousands of patients, there have also been investigator-initiated trials or clinical experiments that have bypassed preclinical models and have been carried out directly in a patient population. For example, the autosomal recessive degenerative neurological disease Friedreich’s ataxia (FRDA) is characterized by spinocerebellar ataxia with variable involvement of the myocardium (hypertrophic cardiomyopathy) and diabetes mellitus. The implicated gene frataxin is a small mitochondrial protein involved with cellular iron metabolism. It has been noted that FRDA patients have reduced levels of frataxin in their fibroblasts, leucocytes as well as other cell types examined.
A conditional knock-out mouse model of frataxin reproduces the clinical syndrome confirming the role of frataxin in the aetiology of this disease. A recent study showed that the low frataxin levels in leucocytes obtained from FRDA patients increased following exposure to rhEPO in vitro. In a follow-up, small open-label clinical trial, 12 FRA patients were administered rhEPO (5000 IU subcutaneously three times weekly) for 8 weeks. Variables assessed included frataxin expression in the peripheral leucocytes, severity of neurological symptoms and markers of oxidative stress. EPO administration was associated with a significant increase in frataxin levels, correlated with an improvement in the clinical score and a reduction in markers of oxidative stress. This study has been extended to a 6-month follow-up period with sustained effects, but with increases in haemoglobin concentration severe enough to require phlebotomy in half of the patients.

Beginning with the earliest studies of EPO in the brain, it was abundantly clear that this molecule also possessed neurotrophic properties. In injured tissue, EPO enhances tissue repair by mobilizing stem cells into the region of injury and increases neurite outgrowth and synaptogenesis. Some of the neurotrophic activities appear to be secondary, arising from the action of other neurotrophic molecules that are stimulated by EPO, e.g. BDNF. A number of studies using a variety of animal models have documented the positive effects of EPO on the preservation of learning and memory following brain injury. For example, EPO (5000 IU kg$^{-1}$ bw; as well as the nonerythropoietic derivative CEPO to be discussed extensively below) administered intraperitoneally 6 or 24 h following controlled cortical contusion in rats increased brain parenchymal BDNF levels and neurogenesis within the hippocampus, resulting in enhanced spatial learning in a Morris water maze by day 32 following injury.

In addition to the beneficial effects of EPO upon the healing phase of CNS injury, EPO’s neurotrophic properties also affects the neuronal substrate of learning in normal animals. For example, rhEPO administered to normal mice as 1 week of daily intraperitoneal injections (5000 IU kg$^{-1}$ bw) produced a 30% increase in neuronal precursors and radial glia within the subgranular zone of the hippocampus. However, these increases were not maintained without continued rhEPO administration. Recently, Adamcio et al. showed that administration of EPO for 3 weeks (5000 IU kg$^{-1}$ bw intraperitoneally every other day) enhanced long-term potentiation in the hippocampus of young mice. These effects persisted for 3 weeks (but were lost by 4 weeks) following the termination of EPO treatment and were independent of changes in the haematocrit.

Of note, recent clinical experiments assessing the effects of rhEPO on cognition have been carried out in normal human volunteers by Miskowiak et al. The subjects were administered 40 000 IU of EPO intravenously and evaluated using functional magnetic resonance to assess recall memory. The peak plasma concentration of this dose is estimated to be well within the tissue-protective range. One week later, individuals who had received EPO exhibited an enhanced hippocampal response, consistent with an increase in BDNF and neurotrophic activity observed in the animal models. In follow-up experiments examining the effects of rhEPO on fear processing, rhEPO administration was associated with brain responses to emotional information very similar to those produced by conventional antidepressants. The results of these studies strongly suggest that rhEPO may be a useful treatment for some psychiatric diseases.

The retina also expresses EPO and EPOR. Within the eye, ischaemia-reperfusion injury, such as that caused by an increased intraocular pressure (acute glaucoma), triggers apoptosis of retinal ganglion cells. Systemic administration of rhEPO immediately following 45 min of ocular ischaemia is highly effective at preventing ganglion cell loss, similar to the results observed in experimental stroke. Ganglion cell loss incurred by sustained ischaemia without reperfusion induced by laser coagulation of the retinal venous system was also prevented by the administration of rhEPO. Notably, a role for endogenous EPO (primarily a product of the Müller cell, a specialized glial
One important group of ophthalmic diseases are those in which the retina is subjected to prolonged hypoxia, as for example, in the retinopathy of prematurity or diabetes mellitus. In the case of retinopathy of prematurity (ROP), exposure to hyperoxia (such as associated with ventilator support of premature infants) suppresses endogenous retinal EPO production, leading to dropout of retinal vessels and neuronal losses. When the retina is subsequently exposed to normoxic conditions, the retina becomes ischaemic, inducing HIF stabilization with the resultant activation of hypoxia-sensitive genes, including vascular endothelial growth factor (VEGF) and EPO [91]. Under these conditions, the proteins drive abnormal angiogenesis. Critically, in the rodent model of ROP, timing of the administration of exogenous EPO is critical for the development of pathology [91]. If EPO is administered during the hyperoxic phase, dropout of vessels is prevented and subsequently, the abnormal angiogenesis in normoxia prevented. Additionally, EPO prevents retinal neuronal (ganglion cell) loss, similar to its effect in ischaemic brain. In contrast, if EPO is administered in the normoxic period when endogenous EPO is elevated, neoangiogenesis is amplified. Thus, timing of administration with respect to the pathophysiology is of the utmost importance in some disease states [92].

Like ROP, proliferative diabetic retinopathy (PDR) is triggered by retinal ischaemia. Vitreal sampling of diabetic patients with proliferative retinopathy has documented that VEGF and EPO levels are increased and it has been assumed that EPO is a driver of the ensuing angiogenesis [93]. Animal models have shown that administration of rhEPO at this stage worsens pathology [94]. However, early in the development of PDR when the retina is mildly ischaemic, exogenous EPO prevents aberrant angiogenesis. For example, in a rat model of early diabetes, either systemic administration [95] or a single intravitreal injection [96] of rhEPO delivered at the onset of hyperglycaemia prevented subsequent neuronal dropout, as well as retinal oedema, another hallmark of diabetic retinopathy. In sum, these results confirm that the particular stage of the retinal ischaemic disease determines whether EPO administration is beneficial or harmful. This clinical dilemma may be solved by the development of nonerythropoietic tissue-protective molecules (see below). Fortunately, the progression of retinal diseases can be carefully followed noninvasively so that therapy can be timed to correspond to the phase of the pathophysiology which responds positively to EPO.

Erythropoietin also likely plays tissue-protective roles in many other specialized neural systems. For example, complex organs such as the cochlea express EPO and EPOR [97] and rhEPO is protective of injury produced by ischaemia [98] or by the toxic antibiotic gentamycin [98].

Other tissues and organs: inducers and amplifiers of tissue injury

As discussed above, multiple processes trigger the innate injury response, activating the local expression of pro-inflammatory cytokines and triggering potential self-amplifying damage. In general, this response is not tissue dependent. In all organs examined to date, EPO is synthesized in the border zone, reducing metabolic stress and antagonizing inflammation as well as programmed cell death. Similar to its roles in the nervous system, exogenously administered EPO is effective in a wide variety of conditions and tissues. Many observations have confirmed that late administration of EPO is highly effective in many animal models of tissue injury, making rhEPO or tissue-protective mimetic therapy a realistic possibility in many disease states.

Infection/inflammation

A number of studies have demonstrated that EPO directly inhibits the production of pro-inflammatory cytokines by inflammatory cells which are the effector arm of the innate injury response. For example, Strunk et al. [99] assessed the activity of EPO on stimulated cytokine production by human leucocytes.
Using heparinized whole blood obtained from neonates or adults, these investigators observed that IL-2, -6, -8, γ-interferon (IFN-γ) and TNF-α stimulated by lipopolysaccharide (LPS) or phorbol ester could be significantly inhibited by rhEPO. These observations have been confirmed using the human monocytic/macrophage cell line U937 [100]. In these experiments, rhEPO antagonized the direct, cytotoxic effects of LPS on these cells besides reducing the secretion of TNF-α and to a lesser extent IL-6. In another recent experiment, this group has shown that rhEPO also inhibits cytokine production in vitro by monocytes exposed to Neisseria meningitidis [101]. Thus, EPO can directly affect the secretion of pro-inflammatory cytokines by leucocytes.

In vivo models of inflammation have also provided evidence for an anti-inflammatory effect of EPO, as, for example, collagen-induced arthritis [102]. In the mouse model, arthritis was induced by intradermal injection of type II collagen along with Freund’s complete adjuvant. In untreated animals, clinical rheumatic disease was evident by day 27, progressing over an ensuing 35 day period characterized by weight loss, joint articular cartilage erosion associated with leucocyte invasion and focal bone resorption. Beginning on day 25, one group received daily injections of EPO subcutaneously (1000 IU kg$^{-1}$ bw). After 10 days of therapy (day 35 from immunization), the treated group had gained twice as much weight as the control group and exhibited a less severe arthritis score. Macrophage inflammatory proteins 1 and 2, and myeloperoxidase (a measure of macrophage and neutrophil infiltration respectively) sampled from joint space were only about 50% of the values of the control group. Similarly, the histological and radiographic damage scores were markedly reduced.

Severe falciparum malaria is a very common parasitic disease with extremely high mortality in extensive regions throughout the world. A dreaded complication is cerebral malaria, a condition characterized by breakdown of the BBB, seizures, coma and high mortality, precipitated by pro-inflammatory cytokines activating cerebral vascular endothelial cells (reviewed in Gimenez et al. [103]). From a pathological perspective, there is a similarity to ischaemic stroke. Because this condition is driven by inflammatory processes, EPO would be predicted to reduce its pathology. The potential cytoprotective potential of EPO in the development of cerebral malaria has been tested in a mouse model by several groups [104, 105]. Mice were infected by an intraperitoneal injection of Plasmodium berghei-infected erythrocytes. In one experiment, all saline-treated mice died by day 11 following inoculation, whereas about half of the mice receiving rhEPO survived [105]; administration of rhEPO on days 4–7 following infection provided optimal protection. Notably, EPO administration did not affect the parasite load. Additionally, TNF-α, IL-1β and IFN-γ gene expression were all increased in the brains of animals with cerebral malaria and associated with an increase in neuronal apoptosis. Administration of rhEPO markedly reduced mRNA levels for these pro-inflammatory cytokines and reduced neuronal apoptosis within the cerebral cortex by ~75%. The duration of the experiment was sufficiently long to observe an erythropoietic effect of EPO, leading to an increase in haematocrit. However, no association was noted between the haematocrit value, parasite load and the severity of cerebral malaria. Interestingly, a recent clinical investigation has identified a relationship between high circulating EPO levels and superior prognosis of neurological sequelae in children with cerebral malaria [106]. A prospective clinical trial evaluating the treatment of cerebral malaria with rhEPO is currently in progress in Africa.

As a final (but not exhaustive) example of the anti-inflammatory effects of EPO, consideration of a cancer cachexia model is instructive. Lean body mass wasting associated with malignancy is one of the most problematic complications of cancer. Although no single factor has been implicated, pro-inflammatory cytokines are thought to play a prominent role. A mouse model has been developed in which colon 26 adenocarcinoma cells injected into the footpads produce a lethal cachexia, with death beginning around day 12 [107]. This model is characterized by a small tumour burden (~350 mg/mouse when the animals begin to die) yet produces an extreme cachexia driven
by pro-inflammatory cytokines. Animals receiving 5000 IU kg\(^{-1}\) bw of rhEPO intraperitoneally daily exhibited an increased survival time, as well as a significant decrease in serum IL-6 and TNF-\(\alpha\) concentrations. There was no difference in the tumour size between the treated and untreated animals at the end of treatment, although the IL-6 concentrations were depressed in the tumours of EPO-treated mice, and TNF-\(\alpha\) was undetectable.

**Myocardium**

Following the demonstration of tissue-protective effects of EPO in the nervous system, historically ischaemic myocardium was the next organ evaluated for tissue-protective effects of EPO. Like the brain, ischaemic injury in the heart produces a central necrotic area bordered by a penumbral region ‘at risk’ for further injury. Also, secondary injury caused by inflammation is a prominent component of the pathophysiology of a variety of myocardial injuries. Interestingly, it had been known for a number of years that the heart could be ‘preconditioned’ by exposure to brief periods of nondamaging hypoxia, increasing greatly myocardial resistance to subsequent exposure to ordinarily damaging hypoxia [108].

Based on the concept of the injury response, the knowledge that the heart expressed EPOR, and the possibility that preconditioning involved induction of endogenous EPO, we determined whether rhEPO would protect the myocardium in a manner similar to the role of EPO in the nervous system [109]. The first experiments were performed *in vitro* using freshly isolated cardiomyocytes obtained from the adult rat. After 28 h of hypoxia (\(~3\%\) \(O_2\)), about 50% of untreated cardiomyocytes underwent apoptosis and 10% necrosis. Addition of rhEPO (100 ng mL\(^{-1}\)) at the onset of hypoxia rescued about 50% of the apoptotic cells, but did not affect the necrotic population. In follow-up studies *in vivo*, adult rats were subjected to a 30-min nontraumatic occlusion of the left anterior descending coronary artery followed by reperfusion. One group was administered rhEPO (5000 IU kg\(^{-1}\) bw intraperitoneally each day) beginning at reperfusion and followed for 7 days. At the end of this time period, haemodynamic studies demonstrated a significant functional improvement in the rhEPO group, characterized by a smaller left ventricular end diastolic volume, i.e. closer to normal. This outcome was associated with an \(\sim 50\%\) difference in the number of surviving cardiomyocytes in the left ventricle: about a 65% loss in the saline group compared with only 35% in the rhEPO group.

Similar findings have been reported by other groups [110–113], which also extend to models of permanent ischaemia [114–116]. Evaluation of whether EPO exposure in advance of a myocardial ischaemia is protective, i.e. in a preconditioning mode, did demonstrate protection, but it was only partial compared to EPO administered immediately following reperfusion [117]. The therapeutic window for beneficial effects of EPO in experimental myocardial infarction (MI) is wide. For example, using a permanent occlusion model in the rat, Moon *et al.* [118] determined that a single 3000 IU kg\(^{-1}\) bw dose of rhEPO administered at the time of occlusion was equivalent to the same dose administered with a time delay of up to 12 h. A dose administered at 24 h, however, was not effective. A dose–response analysis showed that 150 IU kg\(^{-1}\) bw, the minimum effective dose, was as effective as 3000 IU kg\(^{-1}\) bw when given immediately following occlusion. However, the temporal window of the minimum effective dose was markedly reduced, being only 4 h, suggesting that the critical time for treatment is quite late following injury. Finally, as in many other models, a single 3000 IU kg\(^{-1}\) bw dose of EPO in this model provided the same efficacy (as determined by infarct size) as 3000 IU kg\(^{-1}\) bw administered daily for 7 days following infarction [119].

The experiments described above were all acute or of short-term (\(\leq 1\) week) duration. Experiments with longer time periods following infarction have revealed additional beneficial effects that are associated with delayed treatment with EPO or its derivatives. As a result of these experiments, it has been recognized that another prominent role for EPO in the injury response is the recruitment of EPCs from the bone marrow to travel via the circulation to regions of injury and thus...
participate in repair angiogenesis. van der Meer et al. [116] hypothesized that late treatment by EPO would be beneficial following MI independent of its effects on myocardial apoptosis in the acute setting. To test this idea, a permanent coronary occlusion was performed and high dose therapy (40 $\mu$g kg$^{-1}$ bw week$^{-1}$) with the long-acting EPO analogue darbepoietin was initiated 3 weeks following infarction. At 6-week follow-up, the late treatment group as expected exhibited no reduction in infarct size compared with the saline group. Nonetheless, these animals possessed significantly improved cardiac function. Evaluation of the myocardium showed that the treated group exhibited an improved capillary-to-cardiomyocyte ratio, consistent with neoangiogenesis. In a follow-up study [120] using transgenic EPCs containing an enzyme marker that allowed for localization of the cells within tissue, EPO administration produced an increase in the number of circulating EPCs which were incorporated into existing vascular endothelium throughout the body. However, EPO-stimulated EPCs formed new vessels only within the ischaemic border zone of a MI and significantly improved endothelial function. In this manner, EPO preferentially ‘targets’ ischaemic regions for neoangiogenesis. Similar effects presumably also underlie the positive effects of rhEPO in wound healing and tissue repair.

The first safety study in patients with an acute MI [121] was performed using 22 patients randomized into no additional treatment or a single dose of 300 $\mu$g of darbepoietin (a moderately high dose) delivered before primary coronary intervention. No adverse effects were observed following darbepoietin administration. The group receiving the EPO analogue exhibited a significant increase in circulating EPCs at 3 days following infarct, similar to observations in the animal model. Although the EPO treatment group was characterized by a longer time to treatment and more extensive baseline area at risk (as judged by ST-segment elevation), LV ejection fraction after 4 months was similar to that of the control group. Although this finding suggests a possible positive effect of EPO administration, the small size of the study precluded a firm assessment of benefit in the EPO-treated group. Based on these promising results, an open-label multi-centre trial of EPO in acute MI is currently in progress [122].

Renal injury

The kidney is another organ with a high sensitivity to hypoxia, trauma, toxins and other injuries. Westenfelder et al. [123] were the first to demonstrate that the kidney expresses functional receptors for EPO by finding EPOR transcripts in all regions of the kidney examined and observing specific binding of radiolabelled EPO to mesangial, proximal tubular and collecting duct cells. Further, these investigators found that EPO stimulated mitosis of collecting duct cells in vitro.

A tissue-protective role for EPO in renal injury was directly demonstrated using a rat model of 45 min of bilateral ischaemia-reperfusion [124]. Animals that received rhEPO (300 IU kg$^{-1}$ bw) as an intravenous bolus 30 min before ischaemia or just before onset of reperfusion showed about a 40% reduction in the injury-related serum creatinine increase, maintained a normal urine flow and a creatinine clearance about six-fold higher than saline-treated controls. Additionally, rhEPO administration reduced tubular cell apoptosis and was associated with less degeneration of tubular architecture and reduced infiltration of neutrophils.

These results have been confirmed by a number of workers in a variety of species, including the pig [125]. A minimum effective dose has been estimated using an in vitro model of ischaemia-reperfusion injury [126]. In these experiments, proximal tubular cells were subjected to 24 h of 1% O$_2$, which caused an increase rate of apoptosis that a high-dose rhEPO ($\geq$200 IU mL$^{-1}$) was able to antagonize completely. The observation that the high but not low dose was effective is consistent with an interaction with the lower affinity tissue-protective receptor.

Other organs and tissues

A large number of studies, including clinical trials, have been performed to date that have documented significant, positive tissue-protective effects of rhEPO.

**Signalling pathways involved in tissue protection**

Receptor-initiated cell signalling by EPO occurs via multiple molecular cascades (Fig. 4) which differ in importance depending on the specific tissue or cell type, as well as the type of injury. The molecular mechanisms of EPO’s tissue protective and restorative activities have been most extensively studied in the nervous system and the heart, and to a more limited extent, the kidney. Similar to the trigger in erythropoiesis, most tissue-protective responses are initiated by the phosphorylation of JAK-2 [124, 142, 143], although JAK-1 has been implicated in the heart [144]. STAT protein (Signal Transducers and Activator of Transcription; type 5 in the nervous system [145, 146] or type 3 in the heart [113, 147]) is subsequently phosphorylated. STAT then dimerizes and translocates into the nucleus, inducing the synthesis of the protein Bcl-xL which inhibits apoptosis [148]. Additionally, JAK-2 phosphorylates phosphatidylinositol 3 (PI3) kinase which then activates Akt (Protein Kinase B) [113, 124, 146, 149], a central player in cell survival. Akt subsequently stimulates a variety of tissue-specific pathways. For example, in the endothelial cell, Akt activates endothelial nitric oxide synthase (eNOS) whilst in neurones, BCL2-associated agonist of cell death (BAD), glycogen synthase kinase 3β and nuclear factor κB (NFκB) are modulated, leading to a reduction in apoptosis via effects on mitochondrial energetics and gene activation. In contrast, in some systems, the PI3K/Akt

![Fig. 4](https://example.com/fig4.png)

**Fig. 4** Multiple signalling pathways mediating tissue protection are activated in parallel by EPO or tissue-protective compounds in a tissue-specific manner. The bulk of signalling for tissue protection is initiated through the phosphorylation of JAK-2. Major downstream mediators include the STAT-Bcl-XL, PI3K-Akt and the MAPK pathways. Different protective effects of EPO, e.g. anti-apoptosis, anti-inflammation or a decreased threshold of excitability, have been assigned to specific pathways. In a number of systems, however, parallel pathways are required for a full tissue-protective effect. In excitable tissue, a JAK-2 pathway also has been reported to modulate voltage-dependent Ca++ channels to modulate nitric oxide production and neurotransmitter release. See text for additional information.
pathway does not appear to be essential to EPO’s protective functions in the heart, with the mitogen-activated protein kinase pathway involved instead [117], ultimately inhibiting caspase activation and decreasing apoptosis.

Additionally, EPO-dependent functions that are characteristic of different cell types are activated by specific pathways. For example, in the nervous system, EPO has strong neurotrophic and antiapoptotic effects. In hippocampal neurons, both the PI3K/Akt and STAT pathways are required for its neurotrophic actions [150], whereas the STAT pathway is irrelevant for antiapoptosis. In contrast, the differentiated neuroblastoma SH-SY5Y cell line requires both the Akt and STAT pathways to prevent apoptosis [146]. How specific pathways can be selectively activated is currently not known. For the homodimeric receptor (EPOR)₂, a selective activation of individual molecular pathways has been observed to depend upon the specific membrane conformation of its subunit proteins [151]. It is likely that a similar regulation can also be exerted by the tissue-protective receptor.

In the protection of neurones from excitotoxic or nitric oxide-induced apoptosis, EPO activates NFκB in a JAK-2-dependent manner [152]. Further, voltage-dependent Ca²⁺ channels in neurones are critical for neurotransmitter release and nitric oxide production. EPO via JAK-2 inhibits neurotransmitter release [143] and augments nitric oxide production [13, 153]. In contrast, in the kidney, EPO signalling modulates voltage-insensitive Ca²⁺ channels via phospholipase C [154]. As a final (but not exhaustive) example of tissue-specific effects, cardioprotection depends critically on the activity of ATP-sensitive K⁺ channels [155]. EPO activates these channels by at least two pathways: protein kinase C (PKC) and NFκB. Further adding to the complexity of EPO’s signalling pathways, the mechanisms of tissue protection differ according to when EPO is administered with respect to the injury. In the heart, the cardioprotective effect of EPO in preconditioning depends upon the PI3K and PKC pathways, whereas when EPO is administered following injury, only the PI3K pathway is involved [156]. In summary, multiple molecular pathways are involved in EPO-mediated tissue protection that varies by cell type. Further study is needed to understand more fully the interactions of these signalling pathways that ultimately transduce tissue protection in vivo.

Complications of using EPO for tissue protection

Although EPO exhibits strong tissue protective and restorative activities in a wide range of in vitro and in vivo preclinical models, concerns of potential adverse effects arise when contemplating its use in specific clinical scenarios. Because tissue protection requires higher concentrations of EPO than does erythropoiesis (Fig. 2), systemic administration of EPO will invariably fully activate its haematopoietic and vascular activities, including strong pro-coagulant and haemodynamic effects. These adverse effects are likely more relevant in particular patient groups. For example, patients characterized by the excessive production of pro-inflammatory cytokines, e.g. those with cancer, infection, or trauma, appear especially likely to encounter thrombotic complications that are frequently life threatening. A strong association between administration of rhEPO, especially at higher doses, to cancer patients and thrombotic complications and increased mortality has been observed in a number of clinical trials [157]. Further, a recent clinical trial evaluating the potential for rhEPO to eliminate transfusion requirements in intensive care unit patients found that although rhEPO administration did not affect transfusion need, rhEPO administration did significantly decrease death by 50% in the subgroup of patients with trauma [158, 159]. However, this survival benefit was accomplished at the cost of an increase in clinically relevant thromboses.

Another major adverse effect of rhEPO administration is the potential to support tumour growth and/or to confer resistance to anti-tumour therapy [160]. Results of a number of studies have documented EPOR expression by tumours, although what proportion of these are actually functional is presently unclear (reviewed in Jelkmann et al. [161]). It is important to note, however, that recent work has shown that EPO’s tumour-promoting effects do not necessarily arise.
from its action on the tumour itself, but can also occur via the stimulation of angiogenesis in the surrounding, normal tissue [162, 163].

A related area concerning EPO’s angiogenic potency is the current debate concerning a potential role for exogenous EPO in exacerbating the abnormal retinal angiogenesis of the retinopathy of prematurity [164] and diabetic retinopathy [165]. As a result of the above-noted concerns, the U.S. Food and Drug Administration has recently required a black box warning label that advises that rhEPO and darbepoetin use is associated with ‘Increased mortality, serious cardiovascular and thromboembolic events, and tumour progression’.

Development of nonerythropoietic, tissue-protective molecules

To eliminate the unavoidable cross-talk between the haematopoietic and tissue-protective systems when using systemically administered rhEPO, we have sought ways to modify the EPO molecule such that the haematopoietic activity via (EPOR)₂ is selectively eliminated whilst preserving its tissue-protective functions. Our initial approach was to drastically reduce the serum half-life of rhEPO by removing the sialic acid moieties (a maximum of 14) capping the four multi-antennary oligosaccharide chains. Desialylation of EPO providing asialoEPO was previously known to reduce plasma half life from ~6 h to only a few minutes, and this modified EPO was not erythropoietic in vivo [166]. However, in vitro asialoEPO was more potent than EPO at promoting erythropoiesis due to an increased affinity for (EPOR)₂ resulting from a loss of the negatively charged sialic acids [167]. We reasoned that a derivative of EPO that was present in the circulation for only a few minutes could trigger tissue-protective responses, but would not provide the requisite sustained levels to promote erythropoiesis, or other (EPOR)₂-dependent effects, such as enhanced platelet production and thrombosis. AsialoEPO was subsequently shown to be fully tissue protective in models of stroke, spinal cord compression and sciatic nerve crush injury with a potency comparable to EPO [168]. Moreover, this molecule was not haematopoietic when administered parenterally to mice, even up to the equivalent of 50 000 IU kg⁻¹ bw twice a week. The positive tissue-protective effects have been confirmed and extended to other organs. For example, asialoEPO (80 µg kg⁻¹ bw) administered intraperitoneally to neonatal mice with unilateral carotid ligation and subjected to 50 min of hypoxia (7.7% O₂) leads to improved infarct volumes, and neuropathological and behavioural scores identical to that observed with rhEPO [169]. Further, in the kidney asialoEPO was as potent as rhEPO (at 500 IU kg⁻¹ bw i.p.) in preventing injury in mice subjected to 60 min of a bilateral ischaemia, when administered 30 min before the restoration of renal blood flow [170]. In this study, however, only the asialoEPO group demonstrated an increase in survival, an outcome potentially due to the adverse effects of rhEPO.

The success of a single dose of asialoEPO in a wide variety of in vivo preclinical models underscores the mechanism of action of EPO in tissue protection is one in which EPO triggers, but is not needed for a sustained biological response. Prior in vitro work employing neurones treated with excitotoxins demonstrated that only a 5 min exposure to rhEPO was required for the full neuroprotection afforded by continuous exposure to rhEPO [171]. The triggering of neuroprotection by EPO occurred via gene expression, as it depended upon both RNA and protein synthesis. Because of asialoEPO’s very short plasma half-life, this agent has proven useful as a reagent for assessing the precise window during which the tissue protection system is activated, as for example, in a rat model of spinal cord compression [172]. It is important to note that the spinal cord, unlike many other tissues, normally expresses a high density of EPO receptors and is, therefore, a candidate for protection conferred in a ‘preconditioning’ mode before injury. In this study, rats were subjected to 60 s of spinal cord compression and followed neurologically for 6 weeks. AsialoEPO (1000 IU kg⁻¹ bw) was administered intravenously 24 h before or immediately following the compressive injury and compared with a saline group as well as an rhEPO group treated immediately following compression. Plasma obtained just before injury confirmed
that no asialoEPO remained within the circulation. The groups that received asialoEPO or rhEPO exhibited superior neurological scores at the first time-point assessed following injury, which was maintained for the 6-week follow-up period. Although the group pretreated with asialoEPO exhibited less neurological injury than untreated control animals, the pretreatment group was inferior to the group receiving asialoEPO immediately following injury. Additional groups received repeated doses of either asialoEPO or rhEPO following injury, consisting of three daily doses followed by doses twice weekly. The neurological outcome of these groups was identical to those that had received only a single dose immediately following injury. The neurological scoring was confirmed by histological analysis. Therefore, in this injury model although treatment in a preconditioning mode was moderately effective, posttreatment produced a superior outcome. Similar to many other injury models, early single-dose treatment produced the maximum benefit. Additional experiments have demonstrated that in this model the therapeutic window extends for days, closing by day 5 following injury (M. Brines, S. Erbayraktar, Z. Erbayraktar, G. Grasso, A. Sfacteria and A. Cerami, unpublished observations).

AsialoEPO has also been shown to be tissue protective in a number of other models, including ischaemia-reperfusion injury of the kidney [170] or intestine [131], and in contrast-induced nephropathy [173]. Additionally, asialoEPO recruits EPCs with an increased potency compared with EPO and is highly active in promoting angiogenesis in the hypoxic muscle in the mouse [174].

Although asialoEPO is highly potent as a tissue-protective agent, it is currently unclear how many clinical situations might require an agent with a sustained plasma half-life. For example, a pathological process characterized by ongoing or repetitive injury presumably will likely be suboptimally treated with a compound possessing a half-life of only a few minutes. As one example, asialoEPO has been reported to be ineffective in a chronic, neurodegenerative mouse model of Huntington’s disease [175]. To address this issue, we reasoned that changing the EPO molecule in a way that reduced its affinity for the homodimer receptor, whilst preserving the oligosaccharide chains to prolong plasma half-life, might not necessarily abolish binding to the tissue-protective receptor. The interaction of EPO with (EPOR)₂ has been studied extensively and it is known which regions of the EPO molecule interact with the homodimeric receptor. Two distinct regions, termed site 1 and site 2, bind sequentially to each of the homodimers [176–179]. Further, specific chemical or mutational modifications to key amino acids within the EPO molecule that interact with the receptor were known to eliminate binding to the homodimer and therefore produce nonerythropoietic molecules [178]. The key question was whether these molecules might retain tissue-protective qualities.

One attractive approach was suggested by prior work which showed that changing the charge configuration within EPO sites 1 or 2 drastically reduced the affinity of EPO for (EPOR)₂. The positively charged amino acid lysine (of which EPO has eight) is relatively concentrated around binding sites 1 and 2. We noted that in vivo, lysine reacts with cyanate in equilibrium with urea to form the neutral amino acid homocitrulline, resulting in carbamylated proteins. Additionally, published data [180] showed that converting lysines within the binding sites abolished binding to the homodimer. A particularly attractive feature was that the carbamylation reaction occurred endogenously, a fact that could reduce the likelihood of provoking an immune response against the modified EPO molecule. To test this idea, we completely carbamylated EPO, forming CEPO. Formal binding studies performed using CEPO showed that this compound possessed no affinity for the (EPOR)₂ in vitro using UT-7 or TF-1 erythroleukaemic cells, BaF/3 cells transfected with EPOR, or dimerized Fc-EPOR fusion proteins [181]. However, nonhaematopoietic cells, e.g. astrocytes and neurones, bound CEPO with an affinity equal to that of EPO (~1 nmol L⁻¹). To further confirm the hypothesis that the haematopoietic binding sites are not involved in tissue protection, charge-altering mutational derivatives, for example, a change in amino acid 100 (serine, a critical residue within site 2, to glutamate), have been produced and
these have been confirmed to be tissue-protective, nonerythropoietic molecules [181].

A comprehensive comparison of EPO and CEPO was subsequently carried out utilizing in vitro and in vivo systems which revealed marked biological differences in these two proteins in haematopoietic [182] and other activities [183]. As expected, CEPO exhibited no haematopoietic activity in human bone marrow stem cells either alone or with thrombopoietin, in contrast to EPO with which it was synergistic. Further, EPO stimulated platelet production in the rat, whereas CEPO did not. An important difference was noted for the vascular endothelium: although in vivo both compounds released EPCs into the circulation, only EPO induced human umbilical vein endothelial cells to undergo mitosis to an extent equivalent to VEGF. EPO also elicited pro-coagulant behaviour in a rat model of brain death, whereas CEPO did not [183].

Markedly divergent haemodynamic responses were also observed in the rat. Both chronic and acute administration of EPO (4000–5000 IU kg⁻¹ bw intravenously) elevated the systemic blood pressure. In contrast, chronic administration of equimolar amounts of CEPO did not alter systemic blood pressure. Within the circulation of the kidney, acute infusion of 50 μg kg⁻¹ bw EPO (~5000 IU kg⁻¹ bw) produced an immediate and progressive decline in renal blood flow that was maintained throughout a 1-h observation period, whereas CEPO produced a sustained increase in renal blood flow. In aggregate, CEPO lacked the pro-coagulant, pro-angiogenic, and vasoactive activities of EPO that could be problematic if used clinically and, in particular, chronically for tissue protection [183].

In an extensive experimental evaluation, CEPO was found to be fully tissue protective in a wide variety of preclinical models including a three-vessel middle cerebral artery stroke, sciatic nerve injury, spinal cord compression, experimental autoimmune encephalitis and diabetic neuropathy [181]. The results for stroke were confirmed and extended [184] using the same three-vessel model in the rat in which intravenous doses of CEPO (50 μg kg⁻¹ bw) administered 24 and 48 h after reperfusion provided a significantly enhanced behavioural recovery and markedly reduced inflammation within the penumbra. These beneficial effects were maintained during a subsequent 60-day observational period. It is important to note that the full tissue-protective effect occurred even with a delay in administration of CEPO for 24 h following infarction. Additional confirmation of CEPO’s tissue-protective properties in the setting of delayed administration following cerebral ischaemia has been reported for models of embolic stroke in the rat [185] and rabbit [186]. CEPO is currently in clinical development by H. Lundbeck A/S (Copenhagen) in the field of ischaemic stroke [187].

A variety of other animal models utilized by a number of research groups have been evaluated using CEPO, all showing significant tissue-protective effects. These include radiation-induced brain injury [188], brain trauma [85, 189], spinal cord hemisection [190], excitotoxicity [191], amyotrophic lateral sclerosis [192], experimental autoimmune encephalitis [67], MI [193, 194], survival of stem cell-derived myocardial grafts [195], renal ischaemia-reperfusion [196, 197], ureteral obstructive kidney injury [198], cisplatin-induced neuropathy [199] and diabetic autonomic neuropathy [200]. Additionally, CEPO has been found to activate the proliferation of adult neural progenitor cells, presumably a major component in its reparative properties [201]. Finally, CEPO has been shown to reduce metabolic stress within the brain in mice subjected to chronic hypoxia [193].

What part of the EPO molecule is tissue protective?

Fully carbamylated EPO has all eight lysines (positions 20, 45, 52, 97, 116, 140, 152 and 154) converted to homocitrulline, as well as its amino terminus. These locations occur in every major secondary structure except for helix B (amino acids 58–82), which in the EPO–(EPOR)₂ complex faces out into the aqueous environment, i.e. away from the cell membrane. The other three helices contain critical sites required for the binding of EPO to (EPOR)₂. The observation that alteration in charge could be made throughout the molecule, yet preserving tissue-
protective activity, suggested that a smaller fragment or portion of EPO could constitute a cognate ligand for the tissue-protective receptor. Synthesis of a helix B 25-mer peptide (HBP) resulted in a tissue-protective molecule, which, as expected, was not erythropoietic [202]. For example, HBP promoted the survival of rat primary motor neurones in vitro and at a concentration of 1.8 nmol L$^{-1}$ and completely prevented a kainic acid-induced 40% apoptotic neuronal loss. HBP was also active in an in vivo stroke model in the rat (1.5 nmol kg$^{-1}$ bw), reducing infarct volume by 30% in a three-vessel middle cerebral artery occlusion model and preventing the development of retinal oedema in a diabetes model [202].

Of note, a 17-mer peptide has been previously described that consists of a portion of EPO’s AB loop, beginning within the small cystine loop present in human (but not rodent) protein and extending to include part of binding site 1 [203]. This peptide is not haematopoietic in vitro or in vivo and has been shown to have neurotrophic and neuroprotective properties. These are blocked in vitro by the addition of an antibody against the extracellular portion of EPOR. Whether this peptide acts via a similar mechanism as the tissue-protective peptides described here remains to be determined.

Although the 25mer of peptide helix B is a potent tissue-protective molecule, a further consideration of the compact, globular structure of EPO (a result of the hydrophobic interaction of its helices) provided the realization that the aqueous surface of helix B consisted of spatially adjacent amino acids that might act as a tissue-protective molecule if synthesized as a linear peptide. Accordingly, helix B surface peptide (HBSP; QEQLERALNSS) was synthesized to mimic the aqueous face of helix B. HBSP proved as potent a tissue-protective molecule as HBP. However, as a significant proportion of the N-terminal glutamine spontaneously formed pyroglutamate by cyclization, pyroglutamate HBSP (pHBSP) was synthesized. Testing confirmed it possessed the same spectrum of tissue-protective activities and potency as HBSP. For example, in a three-vessel occlusion rat stroke model, pHBSP reduced infarct size and improved behavioural recovery. In contrast, a ‘sequence-scrambled’ peptide that no longer recapitulated the aqueous face of helix B was not tissue protective.

Additional studies showed that HBSP was stable in rat, rabbit and human plasma. However, like other small, unmodified peptides, the plasma half-life of HBSP was only several minutes, similar to asialoEPO. Nonetheless, HBSP has proved remarkably effective in a number of model systems [202]. For example, in mice subjected to 30 min of bilateral renal ischaemia followed by reperfusion, intraperitoneal administration of pHBSP at 1 min, 6 h and 12 h produced a dose-dependent protection of renal function and injury, with the highest dose (8 nmol kg$^{-1}$ bw) exhibiting nearly normal serum creatinine and aspartate aminotransferase levels 24 h later.

Pyroglutamate HBSP also proved efficacious in subacute-chronic injury, as, for example, wound healing [202]. In this experiment, full thickness punch biopsy wounds placed on the dorsum of rats healed faster if administered pHBSP daily (24 nmol kg$^{-1}$ bw subcutaneously). Finally, in a test of cognitive function, this peptide was as active in mice as the positive control galantamine in a novel object recognition paradigm. This test is based on the greater spontaneous exploration of a novel object, compared with a familiar object [204]. In summary, short peptides derived from the structure of helix B of the EPO molecule possessed tissue-protective activities comparable to EPO, but totally lacked potentially adverse activities mediated by (EPOR)$_2$.

**Future prospects**

The discovery of tissue-protective, nonhaematopoietic peptides that are derived from the three-dimensional structure of EPO opens the door to a potentially exciting new therapeutic arena. As small molecules rather than biologics, peptides offer many advantages over EPO. Chief amongst these are ease in manufacturing, stability, potential antigenicity and cost. The experimental validation that the tissue-protective activities of helix B-derived peptides appear to be as broad and as potent (on a molar basis) as EPO, but
without (EPOR)\textsubscript{2} activity, encourages testing in the clinic. It is remarkable that over only a few short years an understanding of the biology of EPO has matured such that EPO, or more likely its derivatives, will very likely constitute a first-line therapy for a wide variety of tissue injuries.

Conflict of interest

Michael Brines and Anthony Cerami are employees of Warren Pharmaceuticals which is developing non-erythropoietic tissue protective compounds for clinical use.

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Work within the field of EPO-mediated tissue protection has exploded exponentially over the last few years. We apologize in advance to our colleagues whose work we could not discuss due to space constraints. We especially thank Dr Michael Yamin for his critical reading of this manuscript and his thoughtful suggestions.

Note

Ortho Biotech, L.P. announced 17 September 2008 that the preliminary results of a 512 patient multicenter trial evaluating the neuroprotective potential of rhEPO in ischemic stroke show an increased mortality in the rhEPO treatment arm (http://www.orthobiotech.com/orthobiotech/press_releases/Preliminary_Data_from_Experimental_Study_Demonstrate_Increased_Mortality_in_Stroke_Patients_Treated). Further information about this study is not yet available.

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