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## Promises and Pitfalls in Erythropoietin-Mediated Tissue Protection: Are Nonerythropoietic Derivatives a Way Forward?

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### Abstract

The essential biological role of erythropoietin (EPO) in maintaining erythrocyte mass has been well understood for many years. Although EPO is required for the maturation of red cells, it also has strong procoagulant effects on the vascular endothelium and platelets, which limit erythrocyte losses after hemorrhage. Like other members of the type 1 cytokine superfamily, EPO has multiple biological activities. For the past 10 years, multiple investigators have shown that EPO acts as a locally produced antagonist of proinflammatory cytokines that are generated by the innate immune response in response to infection, trauma, or metabolic stress. Specifically, EPO inhibits apoptosis of cells surrounding a locus of injury, reduces the influx of inflammatory cells, and recruits tissue-specific stem cells and endothelial progenitor cells. Available evidence suggests that these multiple, nonerythropoietic effects of EPO are mediated by a tissue protective receptor (TPR) that is distinct from the homodimeric receptor responsible for erythropoiesis. Notably, activation of the TPR requires a higher concentration of EPO than is needed for maximal erythropoiesis. Unfortunately, these higher concentrations of EPO also stimulate hematopoietic and pro-coagulant pathways, which can cause adverse effects and, therefore, potentially limit the clinical use of EPO for tissue protection. To circumvent these problems, the EPO molecule has been successfully modified in a variety of ways to interact only with the TPR. Early clinical experience has shown that these compounds appear to be safe, and proof of concept trials are ready to begin.

### Keywords

inflammation; cytoprotection; apoptosis; cytokines; innate immune response

### OVERVIEW: TISSUE PROTECTIVE EFFECTS OF ERYTHROPOIETIN

Erythropoietin (EPO) was first identified as a hormone that stimulates erythropoiesis by blocking the programmed cell death of red cell precursors (reviewed by Fisher<sup>1</sup>). In its endocrine role, EPO is produced in the adult kidney in response to hypoxia and released into the circulation. After binding to the homodimeric EPO receptor (EPOR)<sub>2</sub> at picomolar concentrations, it facilitates the survival of proerythroblasts within the bone marrow (Fig. 1). Because erythrocyte precursors are continuously produced, a sustained, low concentration of EPO within the circulation is required to replace senescent red cells.

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Unexpectedly, in 1990, investigators observed that EPO also stimulated growth and migration of endothelial cells in culture.<sup>2</sup> Notably, the concentrations of EPO required for these effects were substantially higher (nanomolar) than that required for erythropoiesis. Follow-on studies showed neuronal-like PC12 cells also expressed a receptor for EPO with a lower affinity for EPO<sup>3</sup> that was biochemically distinct from the (EPOR)<sub>2</sub>. A variety of recent evidence has shown that this alternative receptor for EPO is a heteromer, composed of EPOR monomer subunits and the beta common receptor (CD131) that is also used for signaling by the type I cytokines granulocyte macrophage colony-stimulating factor, interleukin 3 (IL-3), and IL-5.<sup>4</sup> Recently, Sautina et al.<sup>5</sup> have proposed that the VEGF receptor also associates with CD131 in vascular endothelial cells.

The existence of receptor isoforms suggested that the EPO has other biological functions in addition to erythropoiesis. Indeed, for the past decade, additional work has shown that EPO is a principal component of a paracrine-autocrine system that mediates tissue protection. In this mode, EPO is locally produced in response to tissue injury but with a significant time delay. This pool of EPO subsequently signals through the heteromeric receptor (tissue protective receptor; TPR) to prevent secondary injury by suppressing proinflammatory cytokines activated by the innate immune response and blocks cellular apoptosis. In a variety of tissues,<sup>6,7</sup> EPO antagonizes both the production and the effects of proinflammatory cytokines and limits ischemia-reperfusion injury. Expression of the TPR peaks several hours after infection or injury but before EPO production. In addition, proinflammatory cytokines suppress endogenous EPO but stimulate TPR expression. It is the relative excess of the TPR with respect to EPO that provides the rationale for administration of exogenous EPO for tissue protection.

Historically, the cytoprotective effects of EPO were first demonstrated in the nervous system, which remains the most extensively studied tissue to date. Our group expanded relevance of the newly discovered neuroprotective effects of EPO into ischemic, traumatic, and inflammatory lesions in a series of publications<sup>8</sup> beginning in 2000 and as reviewed by Brines and Cerami.<sup>6</sup> Furthermore, we showed that systemically administered EPO could cross the blood-brain barrier (BBB) in therapeutically relevant concentrations and significantly reduce the volume of injury in animal models of stroke or traumatic brain injury (TBI). The discovery that EPO passes through the intact BBB and is active not only in ischemia but in a variety of injury models greatly accelerated interest in using recombinant human EPO (rhEPO) to prevent brain injury. Since then, we and many other investigators have shown that EPO possesses tissue protective effects in diverse tissues and organs, including the heart, kidney, gut, lung, and skin (reviewed by Arcasoy<sup>9</sup>). Here, we will highlight in moderate detail some of the significant findings in the nervous system as examples of EPO-mediated tissue protection.

## **ERYTHROPOIETIN IN CENTRAL AND PERIPHERAL NERVOUS SYSTEM INJURIES**

### **Stroke**

Many laboratories have assessed the effects of systemically administered rhEPO in animal models of nonhemorrhagic ischemic stroke. Recently, 2 meta-analyses of these animal studies were published. Minnerup et al.<sup>10</sup> analyzed 16 preclinical studies and concluded that when rhEPOs (or its analogues, as discussed later) were administered after the onset of ischemia, infarct size was reduced by about one-third and was associated with improved neurobehavioral outcomes. A second meta-analysis<sup>11</sup> examined 19 studies involving 346 animals for infarct size and 425 animals for neurobehavioral outcome and found that rhEPO improved infarct size by 30.0% (95% confidence interval [CI], 21.3–38.8) and

neurobehavioral outcome by 39.8% (33.7–45.9). These authors also note that studies that randomized the animals to treatment group or that blinded assessment of outcome showed lower efficacy.<sup>11</sup>

Results of the first attempt to translate these preclinical findings into a patient population suffering from nonhemorrhagic stroke in the territory of the middle cerebral artery suggested that rhEPO might be beneficial for the treatment of patients.<sup>12</sup> However, a larger double-blind, placebo-controlled, randomized multicenter EPO Stroke Trial (NCT00604630), designed to evaluate efficacy and safety of rhEPO in stroke, did not demonstrate a therapeutic effect. In fact, the overall death rate was increased in the rhEPO-treated group: 16.4% (n = 42 of 256) versus 9.0% (n = 24 of 266) in the placebo group (odds ratio, 1.98; 95% CI, 1.16–3.38; *P* = 0.01).<sup>13</sup> The major difference between these 2 studies was that the later one included a significant number of patients that also received thrombolytic treatment. Results of a recent preclinical rodent stroke model provide a plausible explanation for the different outcome of these trials: Zechariah et al.<sup>14</sup> have shown that tissue plasminogen activator amplifies EPO-mediated metalloproteinase production, leading to a breakdown of the BBB and increased tissue damage.

### Traumatic Brain and Spinal Cord Injury

In experimental models, rhEPO improves outcome after a TBI.<sup>8,15,16</sup> At high doses, rhEPO has been shown to have neuroprotective effects when given early after injury and to have effects that enhance neurological recovery even when given with a significant temporal delay.<sup>17–23</sup> The time window for EPO-induced neuroprotection has not been well defined, but EPO given as long as 24 hours post-injury has been reported to have significantly enhanced neurological recovery in TBI models. A clinical trial evaluating the effects of rhEPO in patients with TBI (NCT00313716) is ongoing at the Baylor College of Medicine and the University of Texas Health Science Center at Houston/Memorial Hermann Hospital. An additional multicenter trial is planned, but not yet initiated, by the Australian and New Zealand Intensive Care Research Centre (NCT00987454). Multiple laboratories have also demonstrated that rhEPO is neuroprotective after spinal cord injury in animal models.<sup>18–23</sup> There is one active trial (NCT00561067) evaluating the efficacy of rhEPO in patients with traumatic spinal cord injury.

### Cerebral Malaria

Cerebral malaria (CM) is a devastating neurological syndrome arising from an extreme production of proinflammatory cytokines in response to parasite-infected erythrocytes within the brain vasculature. Two laboratories have demonstrated the protective effect of rhEPO in murine models of CM.<sup>24,25</sup> The importance of endogenous EPO in human CM is underscored by a recent clinical study of 124 Kenyan children with CM, in which high endogenous plasma levels of EPO correlated with an 80% reduction in risk of developing neurological sequelae.<sup>26</sup> A clinical trial evaluating the use of rhEPO as an adjunct treatment for children with CM is currently active. This study (NCT00697164) aims to enroll 200 children of 6 months to 14 years of age admitted to the hospital with CM but without severe anemia or pulmonary involvement. The children will be treated with standard antimalarial treatment (quinine) and a 3-day course of high-dose rhEPO. The main endpoint of this trial is survival at 5 days after admission, and the study is powered to detect a 25% reduction of mortality.

### Hypoxic-Ischemic Encephalopathy

Recombinant human EPO confers neuronal protection when administered systemically to animals subjected to global brain ischemia. A trial of rhEPO treatment for 153 term infants with moderate hypoxic-ischemic encephalopathy observed a reduced disability.<sup>27</sup> Death or

moderate/severe disability occurred in 35 (43.8%) of 80 infants in the control group and 18 (24.6%) of 73 infants in the EPO group ( $P = 0.017$ ) at 18 months. In addition, a retrospective study of 82 preterm infants given 6 weeks of rhEPO therapy to prevent anemia identified an association between rhEPO doses and higher scores on the Bayley Scale of Infant Development, Psychomotor Developmental, and Mental Developmental Index at 12 months of age.<sup>28</sup>

Many additional in vivo and in vitro models of nervous system injury have been evaluated including diabetic or toxic neuropathies, experimental autoimmune encephalitis,<sup>29–31</sup> and subarachnoid hemorrhage<sup>32,33</sup> as recently reviewed by Brines and Cerami.<sup>6</sup> A small number of pilot clinical studies have also been performed in normal volunteers and in patients with depression that show effects of EPO on cognition and mood<sup>34,35</sup> or in patients with schizophrenia,<sup>36</sup> amyotrophic lateral sclerosis,<sup>37</sup> or multiple sclerosis.<sup>38</sup>

## MANY TISSUES EXPRESS EPO IN RESPONSE TO INJURY

The role of EPO as a modulator of the innate immune response is not limited to the nervous system. Virtually, all tissues examined to date respond to metabolic stress or injury by activating a local EPO response.

### Myocardial Injury

The heart was the first tissue outside of the brain for which cytoprotective effects of EPO were confirmed.<sup>39</sup> Subsequent work performed by numerous investigators showed that similar to the effects within the nervous system, rhEPO protects the myocardium from ischemic, infectious, traumatic, or toxic injuries.<sup>40–45</sup> Endogenous EPO is produced locally by myocardial tissue, and as expected of a paracrine/autocrine system, a receptor for EPO is expressed by neonatal and adult rat cardiomyocytes,<sup>46,47</sup> ventricular myocytes, and endothelial cells.<sup>48</sup> Preclinical studies have shown that administration of rhEPO after ischemia/reperfusion decreases infarct size in part by inhibiting cardiomyocyte apoptosis. Recombinant human EPO and endogenous EPO may also reduce subacute ischemia by inducing neovascularization, either by stimulating endothelial cells in situ or by mobilizing endothelial progenitor cells from the bone marrow.

Clinical studies have demonstrated a beneficial effect of rhEPO administration in patients with chronic heart failure. Unlike patient populations with chronic kidney disease and cancer-related anemia (discussed later), treatment with erythropoiesis-stimulating agents (ESAs) has not been associated with higher mortality rates in patients with chronic heart failure. In fact, a recent meta-analysis of 7 randomized controlled clinical trials with 650 patients (363 and 287 patients treated with ESAs and placebo, respectively) showed a decreased risk of hospitalization for heart failure in the ESA-treated group and no difference in mortality between the 2 groups.<sup>49</sup> Although one clinical study has demonstrated that rhEPO improved cardiac function without increasing hematocrit,<sup>50</sup> in most, it has been impossible to determine if these beneficial effects on the human cardiovascular system occur independently of increases in hemoglobin levels.

In addition, in contrast to trials in which ESAs were administered chronically, acute dosing has been evaluated in the setting of revascularization procedures after myocardial infarction. Several small studies have been published that present opposite results. Binbrek et al.<sup>51</sup> observed no protective effect of EPO when used in the setting of thrombolysis. In contrast, when administered after stent placement, EPO was observed to confer benefit by reducing infarct size.<sup>52</sup>

It is important to appreciate that a number of observations derived from animal studies suggest that EPO's cardioprotective properties are independent of its erythropoietic activities. First, a single dose of rhEPO that does not increase the hematocrit is protective in rodent<sup>39</sup> and rabbit<sup>53</sup> models of cardiac infarction. Second, isolated cardiomyocytes and isolated perfused hearts are more tolerant to hypoxia when treated with rhEPO.<sup>39</sup> Third, EPO modified to possess no erythropoietic activity (discussed later) retains its cardioprotective properties in permanent ischemia and ischemia/reperfusion.<sup>54</sup> Fourth, EPO is not protective for cardiomyocytes subjected to metabolic stress that have been obtained from  $\beta$ CR (CD131) knockout mice that possess a fully intact (EPOR)<sub>2</sub>.<sup>4</sup>

## HOW DOES EPO PROTECT TISSUES?

Erythropoietin's mechanisms of protective action have been most extensively evaluated in nervous tissue for which rhEPO has been shown to possess neurotrophic,<sup>55</sup> antiapoptotic, and angiogenic properties<sup>56</sup> and to promote neural regeneration.<sup>57</sup> These effects are dose dependent<sup>58</sup> and, in some cases, depend in part on other known molecules, for example, brain-derived nerve growth factor.<sup>59</sup> In animal models, the expression of EPO and EPOR in the brain after cerebral ischemia/infarction occurs with a strict temporal sequence. Histopathological changes identify a penumbra around the lesion, that is, tissue at risk for further injury. Within the penumbra, endothelial cells, intravascular inflammatory cells, and neurons all express EPO, whereas EPOR is expressed by neurons, astrocytes, and endothelial cells.<sup>57,60,61</sup> Because the expression of EPOR precedes the up-regulation of EPO synthesis,<sup>60</sup> tissue sensitivity to EPO increases as a function of time.<sup>62</sup> Also, EPO can increase cerebral blood flow by its effect on endothelial nitric oxide production<sup>63</sup> and by preventing endothelial cell apoptosis and, in these ways, maintain the microcirculation within the penumbra. In the subacute phase, EPO can also improve tissue oxygenation by driving neoangiogenesis. These all tend to reduce infarct volume significantly.<sup>57,60,61</sup> The persistent up-regulation of EPO production by astrocytes after hypoxia also provides rapidly available sources of EPO to enable neurons to tolerate otherwise damaging ischemia.<sup>61</sup> Erythropoietin also strongly prevents the development of tissue edema by modulating the water transporter aquaporin in astrocytes.<sup>64</sup>

### Receptor Signaling Cascade

Erythropoiesis occurs when EPO binds to the preformed (EPOR)<sub>2</sub> and causes a conformational change that subsequently triggers Janus kinase/signal transducers and activators of transcription (STAT) activation. The resulting molecular cascade results in the up-regulation of antiapoptotic proteins (eg, Bcl-2) that inhibit apoptosis of erythrocyte precursors.<sup>65</sup> In contrast, EPO-mediated tissue protection uses multiple signaling pathways activated in parallel and that vary by cell and tissue type. Cell signaling pathways that are known to be used by EPO and mediate tissue protection include (1) phosphorylation and activation of Janus kinase1/2, STAT3, or STAT5A (but not of STAT1alpha and STAT5B); (2) phosphorylation and activation of phosphoinositol 3-kinase and its downstream kinase Akt; (3) activation of protein kinase C, which results in a rise in free intra-cellular calcium; (4) RAS/mitogen-activated protein kinase (Raf, MEK1/2, p42/44 MAPK, and p38 MAPK); and (5) up-regulation of Sonic hedgehog and its downstream target mammalian achaetes/scute homolog 1 (Mash1).<sup>66</sup>

Individual specific tissue protective effects that are activated by EPO in particular cell types can depend upon different pathways. For example, EPO has both neurotrophic and antiapoptotic effects in neurons. For hippocampal neurons, EPO signals via both the PI3K/Akt and the STAT pathways to support neurite outgrowth, whereas the STAT pathway is not required for inhibition of apoptosis.<sup>67</sup> In contrast, in the SH-SY5Y differentiated

neuroblastoma cell line, both the Akt and STAT pathway activation is required to prevent apoptosis.<sup>68</sup>

## A CONUNDRUM: THE HIGH DOSES OF RHEPO REQUIRED FOR TISSUE PROTECTION CAN CAUSE SERIOUS ADVERSE EFFECTS

Despite the many attractive features of rhEPO-mediated tissue protection observed in preclinical models, translation of rhEPO-dependent protection into the clinical setting for use in neurological, renal, or cardiac disorders has posed a dilemma. The high doses of rhEPO required for tissue protection and enhancement of recovery are associated with very real risks of significant clinical complications. In fact, even moderate doses of rhEPO administered chronically have been associated with adverse consequences in patients. For example, the very large Trial to Reduce Cardiovascular Events with Aranesp Therapy trial<sup>69</sup> evaluated whether the use of an ESA to treat anemia in diabetic patients with renal insufficiency could reduce mortality and slow disease progression. In fact, the results showed an increased incidence of stroke within the ESA arm and no beneficial effects of rhEPO on disease progression.

### Survival

Large-scale clinical studies involving higher doses of ESAs administered as treatment of anemia or to improve quality of life have shown that as a class ESAs reduce overall survival and/or increase the risk of tumor progression or recurrence in patients with breast, non-small cell lung, head and neck, lymphoid, and cervical cancer (<http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm200297.htm>). A recent meta-analysis of randomized trials evaluating the association of recombinant ESAs with mortality in patients with cancer analyzed data from a total of 13,933 patients with cancer in 53 trials.<sup>70</sup> Erythropoiesis-stimulating agents increased mortality during the active study period (combined hazard ratio [cHR], 1.17; 95% CI, 1.06–1.30) and reduced overall survival (cHR, 1.06; 95% CI, 1.00–1.12), with little heterogeneity between trials. The cHR for mortality during the active study period was 1.10 (range, 0.98–1.24), whereas it was 1.04 (range, 0.97–1.11) for overall survival. There was little evidence for a difference between trials of patients given different anticancer treatments (*P* value for interaction = 0.42). The mechanism by which ESAs affect these outcomes is not entirely clear but appears to arise from thrombosis and tumor growth.

### Thrombosis

Multiple studies using both animal models and patients indicate that ESAs augment thrombopoiesis as well as platelet and endothelial cell activation.<sup>71–77</sup> The resulting thrombotic complications of EPO are dose related.<sup>78–80</sup> Almost all studies of ESAs administered to critically ill patients and in postoperative patients have shown an increased risk of thromboembolic events after ESA administration. For example, a randomized, double-blind, placebo-controlled, multicenter trial (N = 1460) in anemic critically ill patients demonstrated a 29-day survival benefit in the trauma subgroup receiving rhEPO (mortality, 6.7% vs 3.5%), although it did not result in fewer blood transfusions. Despite the improvement in the outcome of the rhEPO-treated patients, there was a significant increase in the incidence of thrombo-phlebitis, from 5.7% to 8.7% (*P* = 0.04).<sup>81</sup> The most serious complications associated with rhEPO occurred in a recent trial of rhEPO, given at 40,000 IU/d for 3 days after stroke, in which rhEPO-treated patients had a higher rate of intracranial hemorrhage and a higher mortality rate.<sup>13</sup> These results triggered the US Food Drug Administration to place all rhEPO neuroprotection trials in the United States on hold.

## Tumor Growth

A second possible mechanism by which exogenously administered rhEPO may worsen outcome in patients with cancer is by promoting vasculogenesis and tumor growth. Erythropoietin has been shown to promote physiological angiogenesis,<sup>82,83</sup> angiogenesis during wound healing,<sup>84</sup> and estrogen-dependant cyclical angiogenesis in the uterus.<sup>85,86</sup> However, it appears that EPO can also drive pathological neoangiogenesis. For example, 3 important studies in different mouse models of cancer have shown that EPO is an important angiogenic factor that regulates neovascularization of tumors.<sup>86–88</sup> An additional concern is that EPO may act as a growth factor for certain types of cancer cells, although it must be noted that there is a great deal of controversy concerning this possibility. On the one hand, results of a number of in vitro studies show that rhEPO can induce proliferation, increase cellular migration and invasion, as well as confer resistance to chemoradiation in some tumor cells.<sup>89–95</sup> On the other hand, studies using different tumor types have also concluded that rhEPO does not affect cellular proliferation or in vitro sensitivity to chemotherapeutic agents.<sup>95–97</sup> It is notable that many workers in the field have demonstrated the expression of EPOR on tumor cells and responses to EPO (reviewed by Arcasoy<sup>9</sup>). In rebuttal, several papers recently published by scientists employed by Amgen (the largest manufacturer of ESAs) have concluded that the reagents used by other scientists are faulty<sup>98</sup> and that tumor cells do not express a functional EPOR or respond biologically to rhEPO.<sup>99,100</sup> Further work will obviously be required to clarify this controversial area.

## A SOLUTION TO THESE PROBLEMS: DESIGNER EPOS THAT ARE ONLY TISSUE PROTECTIVE?

If the erythropoietic effects of EPO could be functionally separated from its tissue protective effects, more specific ligands could potentially be developed that avoid adverse effects that depend upon the EPOR homodimer. In 2003–2004, our group demonstrated for the first time that the hematopoietic and tissue protective effects of EPO are, in fact, independent biological properties<sup>101,102</sup> (Table 1). This was accomplished using 2 approaches: (1) by capitalizing on the very different temporal characteristics of EPO signaling in erythropoiesis compared with tissue protection and (2) by preferentially interfering with the binding of EPO to the hematopoietic receptor.

As discussed earlier, effective erythropoiesis requires EPO to be constantly available. In contrast, tissue protection is characterized by the appearance of molecules that trigger long-lasting effects, acting in effect as molecular switches for long-lasting gene expression programs. Recombinant human EPO possesses a relatively long plasma half-life (~5–8 hours) because of the presence of multiple oligosaccharides terminated by sialic acid. Removal of these sialic acid residues drastically increases plasma clearance, such that asialo-EPO has a reduced plasma half-life of only ~2 minutes. Although a single injection of asialo-EPO has no effect on hematopoiesis, it is as potent as native EPO for tissue protection.<sup>101</sup>

Another approach to the development of specific tissue protective ligands involves considerations of EPO structure-activity relationships with respect to the hematopoietic receptor. The molecular interaction of EPO with the (EPOR)<sub>2</sub> complex has been extensively studied, and critical regions within EPO that interact (Fig. 2). Erythropoietin with (EPOR)<sub>2</sub> have been identified<sup>103–106</sup> binds to both subunits of the homodimer and, while in a bridging position, induces a conformational change that triggers receptor signaling. The specific regions of EPO involved are portions of helices A and C (site 2, Fig. 2) as well as helix D and the loop connecting helices A and B (site 1, Fig. 2).<sup>103–106</sup> Chemical or mutational modifications of amino acid residues within these 2 regions of the EPO molecule

prevent its binding to (EPOR)<sub>2</sub>. Remarkably, however, a number of these modified EPO molecules retain potent tissue protective properties.<sup>102</sup> Using this approach, a number of EPO derivatives can be synthesized that are cytoprotective but are devoid of hematopoietic, procoagulant, and vasoconstrictive activities. Carbamoylated EPO (CEPO) is one extensively studied derivative that is produced by chemically converting the positively charged lysines within the molecule (EPO has 8) into the neutral amino acid homocitrulline. Erythropoietin and CEPO differ markedly in a number of biological properties. For example, although both recruit endothelial progenitor cells, only EPO stimulates the mitosis of more differentiated endothelial cells.<sup>107</sup> Carbamoylated EPO has been extensively evaluated in multiple preclinical models in our laboratory<sup>54,102,107–110</sup> as well as by others<sup>66,111–115</sup> and confirmed to possess fully preserved tissue protective properties. For example, rodent cardiomyocytes undergo apoptosis when exposed to the toxin staurosporine *in vitro*. Inclusion of either CEPO or EPO in the exposure medium significantly attenuates this apoptosis (Fig. 3). Carbamoylated EPO is currently in clinical development for ischemic stroke (NCT00870844) and Friedreich's ataxia (NCT01016366).

Clearly, the observation of different biological effects of EPO and CEPO support the concept of receptor isoforms. As previously noted, the TPR is pharmacologically distinct from (EPOR)<sub>2</sub>. Specifically, it exhibits a lower affinity for EPO, forms distinct molecular species in cross-linking experiments,<sup>3</sup> and when immunoprecipitated under nonreducing conditions, the EPOR monomer is covalently bound to the βCR (CD131). Previous work has shown that CD131 form complexes with EPOR<sup>116</sup> and with the alpha receptor subunits specific for granulocyte macrophage colony-stimulating factor, IL-3, and IL-5. However, because normal hematopoiesis occurs in CD131 knockout mice, it was concluded that this association was not biologically relevant. Thus, there are 2 different EPOR complexes, one accounting for its hematopoietic and the other for its tissue-protective activities. As expected, in CD131 knockout mice, neither EPO nor CEPO is tissue protective. For example, Figure 4 shows that while wild-type mice respond well to CEPO in the setting of spinal cord injury, neither EPO nor CEPO is protective in CD131 knockout mice.

In an effort to further characterize the structure-activity relationships of EPO, our team produced a series of peptides based on the primary sequence of EPO as well as peptides designed to mimic its 3-dimensional spatial structure. The most interesting peptides are derived from residues of EPO that are not involved in binding to the classical (EPOR)<sub>2</sub> complex (ie, regions of EPO other than sites 1 and 2; Fig. 2). These peptides were then assayed for tissue/neuroprotective and hematopoietic potency. As a result of these studies, helix B was identified as a tissue protective region of EPO. Of note, helix B does not contain lysine so it is not modified by carbamoylation. It is the only helix not involved with binding to the homodimeric receptor.

One of the peptides, ARA 290, is currently in clinical development by Araim Pharmaceuticals, a spin-off of Warren Pharmaceuticals. ARA 290 is a remarkable 11 amino acid peptide composed of the spatially adjacent amino acids on the portion of helix B that faces outward into the aqueous medium (Fig. 5). ARA 290 was designed to mimic the tertiary structure of helix B<sup>117</sup> and thus acts as a ligand for the TPR. Despite the fact that ARA 290 has a short half-life (~2 minutes), it is at least equipotent on a molar basis to rhEPO, because like asialoEPO,<sup>101</sup> it has a rapid effect on gene expression and, in this way, triggers protection responses.<sup>118</sup> The tissue protective effects of ARA 290 have demonstrated using a large number of animal models, including models of sciatic nerve injury, middle cerebral artery ischemia, renal ischemia-reperfusion injury, and wound healing.<sup>117</sup> For example, in mice subjected to 30 minutes of ischemia followed by reperfusion, administration of ARA 290 after reperfusion is associated with preservation of



function (Fig. 6) and a reduction of tissue injury.<sup>117</sup> In addition, like EPO, ARA 290 also has positive effects in some normal tissues, for example, effects on cognition.

## SAFETY OF ARA 290

Extensive preclinical safety studies have been carried out by Araim Pharmaceuticals. The results of these studies show that ARA 290 even at very high dosages has no effect on hematopoiesis or any toxic activities in rabbits, rats, or mice. Safety trials have been carried successfully through phase 1a and 1b. ARA 290 is currently poised to enter proof-of-concept studies.

## CONCLUSIONS

Erythropoietin locally produced in response to cellular stress plays many roles in the prevention, attenuation, and termination of injury. Erythropoietin also promotes healing and restoration of function in diverse tissues. However, the strong suppressive effects of proinflammatory cytokines produced by activation of the innate immune response tend to limit the beneficial effects of locally produced EPO. In contrast, the TPR is strongly up-regulated in the vicinity of injury, providing the rationale for systemic exogenous EPO administration. Tissue protection requires a high local concentration of EPO because of the relatively low affinity of the TPR. Engineered molecules that bind only to TPR avoid hematopoietic complications and appear well suited for translation of EPO-mediated tissue protection into the clinic.

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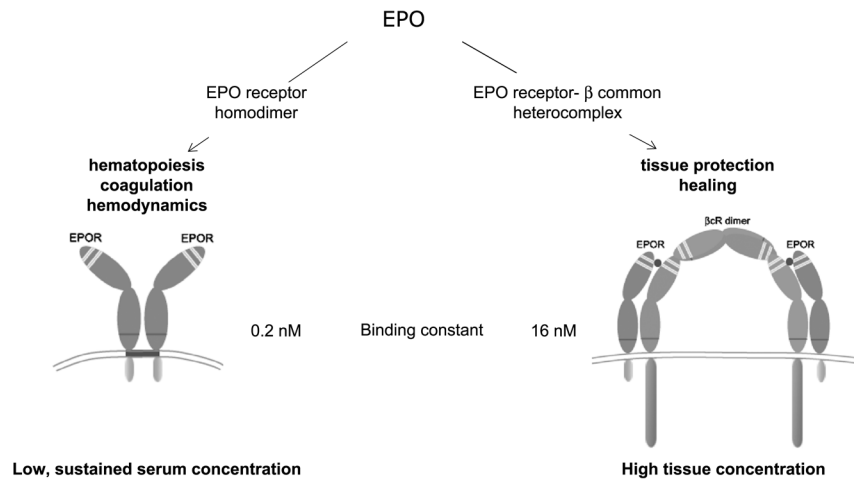
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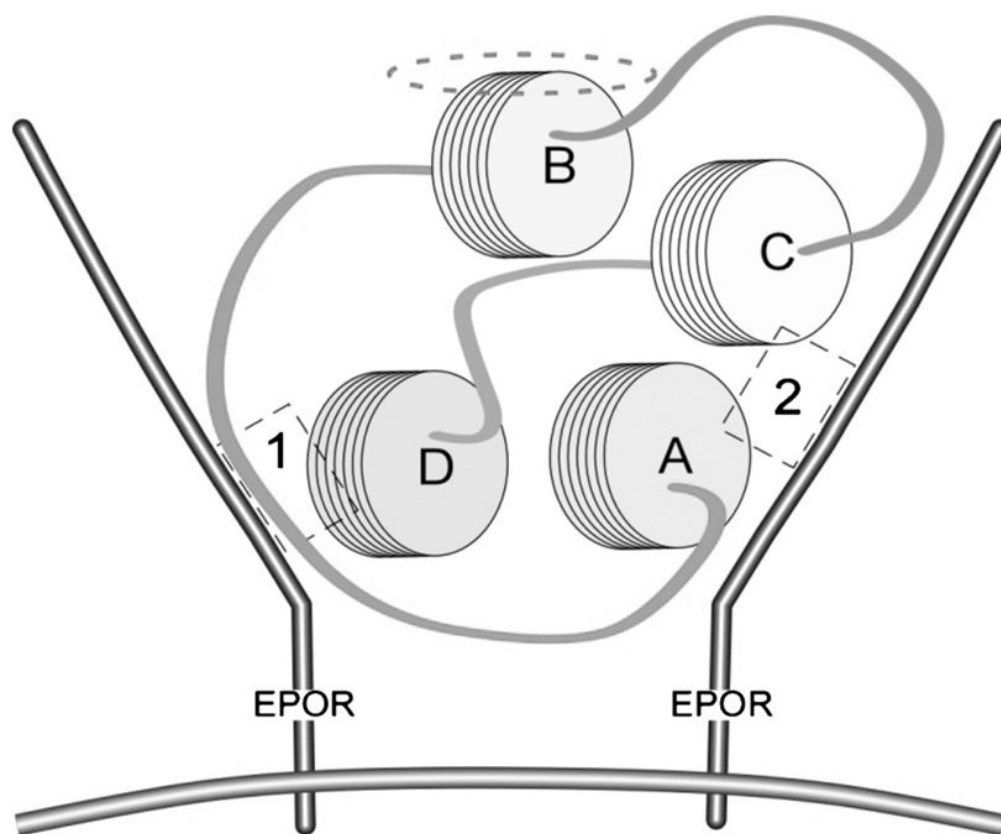
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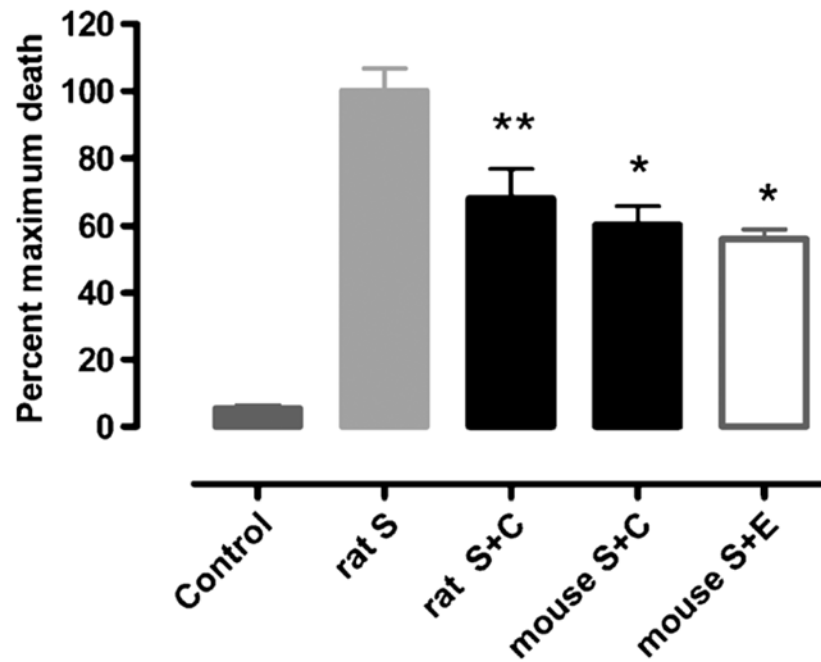


**FIGURE 1.** Erythropoietin signals via 2 distinct receptor isoforms. The hematopoietic receptor is a high affinity homodimer that mediates hematological and vascular effects. In contrast, current evidence suggests that the TPR is a lower affinity heteromer composed of EPOR and  $\beta$ CR (CD131).



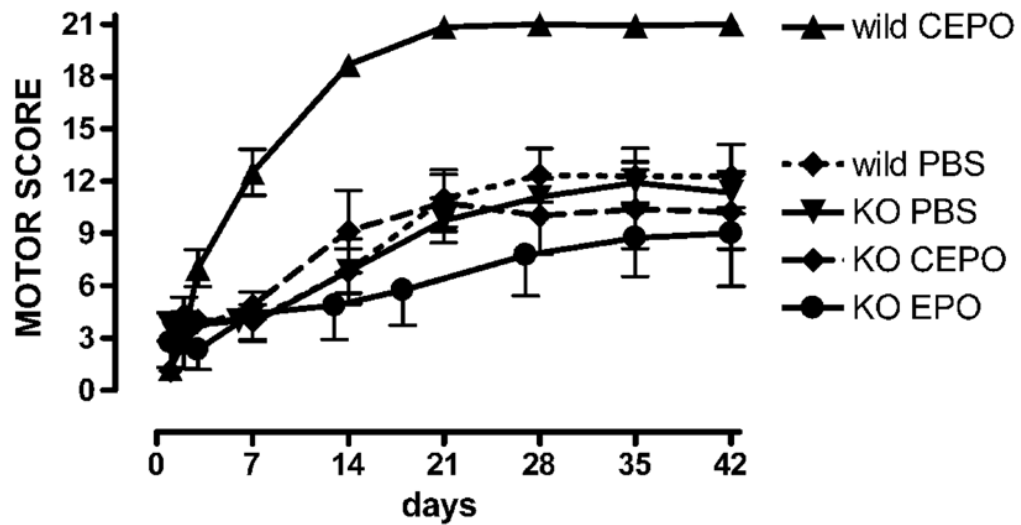


**FIGURE 2.** Erythropoietin binds to  $(EPOR)_2$  by bridging across an assembled homodimer. Helices A–D of EPO associate via hydrophobic interactions to form a compact, globular configuration. Sites 1 and 2 (indicated by dashed boxes) within the topography of the EPO molecule bind with high affinity to localized regions on each EPOR monomer. The aqueous face of the helix B is oriented away from the interior of the receptor, indicated by the dashed ellipse. Reprinted with permission from *Proc Natl Acad Sci U S A*.<sup>117</sup>



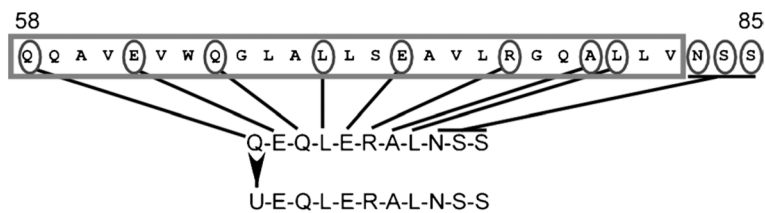
**FIGURE 3.**

The nonhematopoietic derivative carbamoylated EPO protects cardiomyocytes from staurosporine-induced apoptosis in vitro. Mean percentage of myocytes having undergone apoptosis after isolation from rat or mouse hearts. Shown are data for rat control ( $n = 9$ ), S (Staurosporine,  $n = 10$ ), rat S plus C (S plus CEPO,  $n = 10$ ), mouse S plus C ( $n = 4$ ), and mouse S plus E (S plus EPO;  $n = 4$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus staurosporine. Reprinted with permission from *Proc Natl Acad Sci U S A*.<sup>54</sup>

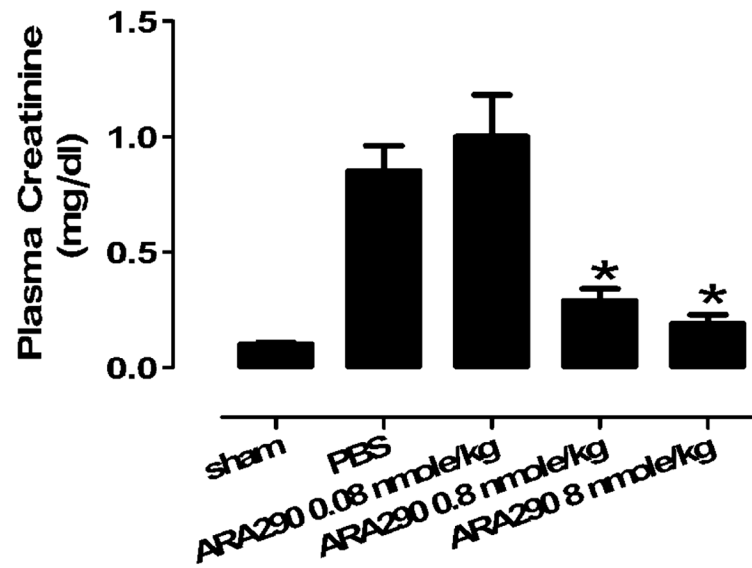


**FIGURE 4.**

$\beta$ CR knockout mice subjected to spinal cord compressive injury do not respond to either rhEPO or CEPO. Normal or CD131 knockout male mice of 8 to 16 weeks of age received a moderate compressive lesion of the spinal cord, followed immediately by a single intraperitoneal dose of rhEPO or CEPO (10  $\mu$ g/kg of body weight: the equivalent of 1000 IU of EPO) and were subsequently evaluated for motor function for 6 weeks. Mortality was similar between groups ( $\approx$ 10%–20%). Wild-type mice responded to CEPO with a complete recovery within 4 weeks. In contrast,  $\beta$ cR knockout animals exhibited little recovery in motor function among the CEPO, rhEPO, or saline groups after 6 weeks.<sup>4</sup> Reprinted with permission from *Proc Natl Acad Sci U S A*.<sup>4</sup>

**FIGURE 5.**

ARA 290 was synthesized based on the linear sequence of helix B indicated by the dashed ellipse in Figure 2 (boxed region; single letter amino acid code; U: pyroglutamate). Circled residues show those amino acid residues on the aqueous face of helix B and a linear peptide comprising only these residues was manufactured. Reprinted with permission from *Proc Natl Acad Sci U S A*.<sup>117</sup>



**FIGURE 6.**

ARA 290 improves renal ischemia-reperfusion injury. Renal function was assessed by measuring plasma creatinine in mice ( $n = 12$  each group) 24 hours after being subjected to sham operation or renal ischemia–reperfusion injury (bilateral renal pedicle occlusion for 30 min). Phosphate-buffered saline (PBS) or ARA 290 (8.0 nmol/kg of body weight) was administered intraperitoneally 1 minute, 6 hours, and 12 hours into reperfusion. Data represent mean and SEM; \*\*\*,  $P < 0.001$  versus PBS. Reprinted with permission from *Proc Natl Acad Sci U S A*.<sup>117</sup>

TABLE 1

## Hematopoietic and Neuroprotective Activities of rhEPO and Its Derivatives

rhEPO Derivative	Hematopoietic Activities (pM)		Neuroprotective Activities ( $\pm$ SD for Compounds at 300 pM)	
	Erythropoietin Receptor Binding* (IC <sub>50</sub> )	UT7 Proliferation <sup>†</sup> (EC <sub>50</sub> )	% Neurons Protected <sup>‡</sup>	% P19 Cells Protected <sup>§</sup>
Wild-type rhEPO	10	10–30	78 $\pm$ 13	49 $\pm$ 12
Asialo-rhEPO <sup>¶</sup>	14	10–30	71 $\pm$ 15	n.d.
Carbamoylated rhEPO (CEPO) <sup>//</sup>	> 10,000	> 10,000	70 $\pm$ 9	49 $\pm$ 10
Asialo-CEPO	> 10,000	> 10,000	69 $\pm$ 16	not done
S100E-rhEPO <sup>**</sup>	100	> 10,000	66 $\pm$ 9	55 $\pm$ 15
R103E-rhEPO <sup>††</sup>	> 10,000	> 10,000	55 $\pm$ 13	not done

\* Inhibition of rhEPO binding to (EPOR)<sub>2</sub>.

<sup>†</sup> UT7 is an EPO-sensitive leukemic cell line.

<sup>‡</sup> Percent rat hippocampal neurons protected after induction of apoptosis with the neurotoxin *N*-methyl-D-aspartate.

<sup>§</sup> Percent P19 (neuronal precursor cells derived from murine teratocarcinoma cells) protected after induction of apoptosis by serum withdrawal.

<sup>¶</sup> Asialo-rhEPO was generated by total enzymatic desialylation of rhEPO, possesses a very short plasma half-life and is fully neuroprotective in vivo.

<sup>//</sup> Carbamoylated EPO is a chemically modified rhEPO in which all of the lysines were transformed to homocitrulline by carbamoylation.

<sup>\*\*</sup> S100E-rhEPO is a mutein of EPO in which the S<sup>100</sup> is changed to E<sup>100</sup>. Amino acid 100 is within site 2 (Fig. 2).

<sup>††</sup> R103E-rhEPO is a mutein of rhEPO in which the R<sup>103</sup> is changed to E<sup>103</sup>.