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New Avenues of Exploration for Erythropoietin

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Abstract

Discovery that the hormone erythropoietin (EPO) and its receptor play a significant biological role in tissues outside of the hematopoietic system has fueled significant interest in EPO as a novel cytoprotective agent in both neuronal and vascular systems. Erythropoietin is now considered to have applicability in a variety of disorders that include cerebral ischemia, myocardial infarction, and chronic congestive heart failure. Erythropoietin modulates a broad array of cellular processes that include progenitor stem cell development, cellular integrity, and angiogenesis. As a result, cellular protection by EPO is robust and EPO inhibits the apoptotic mechanisms of injury, including the preservation of cellular membrane asymmetry to prevent inflammation. As the investigation into clinical applications for EPO that maximize efficacy and minimize toxicity progresses, a deeper appreciation for the novel roles that EPO plays in the brain and heart and throughout the entire body should be acquired.

PRESENTLY, THE HORMONE ERYTHropoietin (EPO) is approved by the US Food and Drug Administration for the treatment of anemia that may result from a variety of conditions, including the anemia associated with chronic renal failure, irrespective of the need for dialysis, and anemia in patients scheduled for elective noncardiovascular surgery, to lessen their requirement for allogenic blood transfusions. In addition, patients who become anemic as a result of chemotherapy administered for nonmyeloid malignancies or during zidovudine treatment of infection with human immunodeficiency virus would fall under the guidelines for the administration of EPO.

In the brief span of approximately a decade, the premise that EPO is required only for erythropoiesis has been altered by studies demonstrating the existence of EPO and its receptor in other organs and tissues outside of the liver and the kidney, such as the brain and heart. We describe herein the unique role and cellular mechanisms of EPO that shape its potential to offer novel therapy for a variety of acute and chronic disorders in both neuronal and vascular systems. To identify studies to include in this discussion, we searched the databases MEDLINE/PubMed, Current Contents/Life Sciences, Chemical Abstracts, and BIOSIS using individual entries and combinations of keywords that included the terms *anemia, angiogenesis, apoptosis, brain, cardiomyocyte, cell injury, congestive heart failure, cytokines, erythropoietin, erythropoietin receptor, heart, ischemia, infarction, inflammation, nervous system, programmed cell death, renal disease, stem cells, and vascular disease*.

Structure, Expression, and Cellular Pathways of EPO

The *EPO* gene is located on chromosome 7, exists as a single copy in a 5.4 kb region of the genomic DNA, and encodes a polypeptide chain containing 193 amino acids. The circulatory mature protein is a 30.4-kDa glycoprotein, which has a span of 165 amino acids. Erythropoietin has 4 glycosylated chains including 3 *N*-linked and 1 *O*-linked acidic oligosaccharide side chains. The glycosylated chains are necessary for the biological activity of EPO and can protect the EPO protein from damage by oxygen radicals.¹ In addition, the *N*- and *O*-linked chains are required for the production and secretion of the mature EPO. The biological activity of EPO also relies on 2 disulfide bonds formed between cysteine amino acids.²

Besides the kidney, liver, and uterus, additional organs have been found to secrete EPO, including peripheral endothelial cells, vascular smooth muscle cells, and insulin-producing cells³ (Table 1). Yet of all these newly identified sites, the presence of EPO in the nervous and vascular systems has sparked the most significant enthusiasm for further investigation. In the nervous system, the major sites of EPO production and secretion are the hippocampus, internal capsule, cortex, midbrain, cerebral endothelial cells, and astrocytes.^{4,5} Additional secretory sites for EPO in the vascular system also continue to be discovered and include peripheral endothelial cells, enterocytes, and muscle (skeletal, smooth, and cardiac).^{3,5,6} The EPO receptor (EPOR) is expressed by a variety of cells that include neurons, microglia, astrocytes, and cerebral endothelial cells and by the myelin sheaths on human peripheral nerves.^{2,4}

Although EPO is recognized as a critical modulator of erythroid production, a diminished concentration of red blood cells does not directly stimulate EPO production and secretion as would be predicted. Production and secretion of EPO and the expression of EPOR are instead regulated by the tissue oxygen supply. A deficiency in tissue oxygen results in the production of EPO and an increase in the expression of the EPOR in the kidney and liver.^{3,4} During hypoxia, EPO production also may originate in the brain, possibly crossing the blood-brain barrier to reach the systematic circulation and peripheral organs. The hypoxia-dependent gene transcription of EPO and EPOR directly results from the activation of the hypoxia-inducible factor 1 (HIF-1) pathway.⁷ Each of the HIF family members—HIF-1 α , HIF-1 β , and HIF-3 α —have a significant role in regulating the expression of EPO and the EPOR. Interestingly, a variety of stressors in addition to hypoxia may lead to activation of the HIF pathway and increased EPO expression, such as hypoglycemia, increased intracellular calcium, or the intense neuronal depolarizations generated by mitochondrial reactive oxygen species.^{2,4} Anemic stress, insulin release, and several cytokines, including insulinlike growth factor (IGF), tumor necrosis factor α (TNF- α), interleukin 1 β (IL-1 β), and interleukin 6 (IL-6), also can lead to increased expression of EPO and the EPOR.²⁻⁴

Several recent studies have outlined the mechanisms by which EPO may prevent cell injury (Figure). Protection by EPO in a number of cellular systems is robust and EPO prevents apoptosis from a number of sources, including reduced or absent oxygen tension, excitotoxicity, and free radical exposure. Apoptosis involves genomic DNA destruction and the loss of cell membrane asymmetry through membrane phosphatidylserine (PS) externalization and is believed to contribute significantly to a variety of diseases, such as ischemic stroke, dementia, Alzheimer disease, spinal cord injury, and myocardial infarction.^{8,9} Erythropoietin is rare in its ability to prevent both the exposure of membrane PS residues and the committed stages of genomic DNA destruction. In several experimental models, EPO has been shown to protect potentially against microglial phagocytosis and thrombotic injury.¹⁰⁻¹⁵

Erythropoietin can prevent apoptotic injury through the Janus-tyrosine kinase 2 (Jak2) signaling pathway.¹⁶ Jak2 is activated by EPO and subsequently activates PI3-K by

phosphorylation, and PI3-K then activates protein Kinase B (AKT), also by phosphorylation.¹⁷ Erythropoietin also prevents apoptosis through the PI3-K/Akt pathway, which maintains the mitochondrial membrane potential ($\Delta\Omega_m$), prevents the cellular release of cytochrome C, and modulates caspase activity.^{12,14,18} Once activated, Akt can confer protection against genomic DNA degradation and membrane PS exposure.^{14,19,20} Up-regulation of Akt activity during injury paradigms, such as N-methyl-D-aspartate toxicity,²¹ cardiomyocyte ischemia,¹⁵ hypoxia,¹⁴ and oxidative stress,^{12,13,18} is vital for EPO's protective properties since prevention of Akt phosphorylation blocks EPO cellular protection and anti-inflammatory pathways.^{12,13,18} EPO also blocks the destruction of genomic DNA in cells^{14,18} either through the prevention of caspase 9-like activity or through the subsequent inhibition of caspase 1-like and caspase 3-like activities.

Several additional downstream pathways of Akt also have been shown to be linked intimately to the protective mechanisms of EPO.²² Protein kinase B regulates the activity of the proapoptotic fork head transcription factor (FOXO3a)^{23,24} through inhibitory phosphorylation, rendering FOXO3a ineffective to activate the transcription of nuclear genes that lead to apoptosis.²⁵ In addition, EPO suppresses glycogen synthase kinase-3 β , a serine/threonine kinase that plays a significant role in the induction of apoptosis in several cell types, including neurons, vascular smooth muscle cells, and cardiomyocytes.²⁶ Up-regulation of the antiapoptotic Bcl-2 family member Bcl-x_L in combination with apoptotic protease activating factor 1 (Apaf-1) expression also can be vital for EPO mediated cytoprotection, for without EPO, Bcl-x_L is not expressed and apoptotic cell death ensues.¹⁸

In addition to preventing apoptotic injury, EPO also has been found to play a role in neuronal progenitor cell development and through activation of nuclear factor- κ B, which fosters the production of neural stem cells.²⁷

Experimental Evidence Supports a Robust Cytoprotective Capacity for EPO

EPO Prevents Nervous System Function

Both in vivo and in vitro experimental models have demonstrated neuronal and vascular protection in the nervous system by EPO (Table 2). Animal models using cerebral ventricular application of EPO during cerebral hypoxia-ischemia illustrate a reduction in ischemia-induced learning disability, increased neuronal survival, and the development of ischemic tolerance.^{28,29} In particular, in models of both focal and global cerebral ischemia, EPO reduced cerebral infarction and was able to protect sensitive hippocampal neurons from injury.^{2,4,22} A number of additional studies have now pursued systemic administration of EPO, since ventricular delivery systems are impractical for clinical applications. For example, systemic administration of EPO before or immediately after a retinal insult can protect retinal ganglion cells from apoptosis.¹¹ Systemic application of EPO also can improve functional outcome and reduce lipid peroxidation immediately after spinal cord injury.³⁰ Application of systemic EPO following experimental subarachnoid hemorrhage restores the autoregulation of cerebral blood flow, reverses basilar artery vasoconstriction, and enhances neuronal survival and functional recovery.³¹ Furthermore, EPO can block microglial cell activation and proliferation to prevent phagocytosis of injured cells through pathways that involve cellular membrane PS exposure, Akt,³² and the regulation of caspases.^{12,13} Erythropoietin also directly prevents cellular inflammation by inhibiting several proinflammatory cytokines, such as IL-6, TNF- α , and monocyte chemoattractant protein 1.^{2,4} Interestingly, these effects of EPO can be mediated through both hormonal and paracrine modalities.^{12,13,28}

EPO Protects Vascular Integrity and Promotes Angiogenesis

Erythropoietin plays a dual role in vascular protection by preserving endothelial cell integrity^{3,14,18} and by promoting angiogenesis new capillary formation from preexisting vessels into an avascular area, a process known as angiogenesis.³ Erythropoietin also can prevent blood-brain barrier permeability during injury and maintain the establishment of cell to cell junctions.³³ Erythropoietin has both mitogenic and chemotactic effects that can lead to matrix metalloproteinase-2 production that is necessary for endothelial cell migration, cell proliferation, and vessel formation in endothelial cell lines. Erythropoietin stimulates postnatal neovascularization by increasing endothelial progenitor cell mobilization from the bone marrow.³⁴ Angiogenesis has been observed in rat aortic rings 4 days following incubation with EPO in reconstituted basement membrane matrix³⁵ and in endothelial cells derived from human adult myocardial tissue treated with EPO.³⁶ This angiogenesis generated by EPO may also provide indirect cellular protection in the nervous system. By causing the proliferation and migration of brain capillary endothelial cells after an ischemic brain insult, EPO may improve blood flow to ischemic cells starved for oxygen and nutrients, decreasing the amount of neuronal damage.²²

EPO Promotes Cardiomyocyte Protection and Function—Given the potentially protective effects of EPO on brain ischemia and vascular endothelial cell injury, several studies have evaluated the role of EPO during cardiac ischemia and reperfusion injury (Table 2). Erythropoietin administration either before or during myocardial ischemia-reperfusion can protect against myocardial cell apoptosis and decrease infarct size, resulting in enhanced cardiac function and improved left ventricular contractility.³⁷ In the isolated rat heart following ischemia-reperfusion experiments, beneficial effects of treatment with EPO have been shown to improve significantly the postischemic recovery of left ventricular pressure.³⁸

Early clinical studies of patients with anemia or receiving long-term hemodialysis have indicated that administration of EPO can increase left ventricular ejection fraction, stroke volume, and cardiac output, suggesting improved cardiac function secondary to the correction of anemia.³⁹ In addition to the correction of anemia, EPO can promote microvascular growth in the heart, suggesting that functional cardiac recovery with EPO may ensue also from the generation of new blood vessels.⁶ Other experimental studies have illustrated that EPO administration may protect myocardial cells directly, increasing cardiac cell proliferation in neonatal rats, reducing myocardiocyte apoptosis during ischemia or reperfusion injury, leading to improved left ventricular function.^{15,38}

More recent clinical randomized controlled studies in patients with mild anemia and severe or resistant congestive heart failure have demonstrated that EPO in combination with intravenous iron can lead to increased left ventricular ejection fraction and a reduction in hospitalization days by almost 80%⁴⁰ (Table 2). Additional investigations involving subcutaneous EPO in patients with diabetes and patients with severe resistant congestive heart failure have shown a decrease in breathlessness and/or fatigue, increased left ventricular ejection fraction, and a significant decrease in the number of hospitalization days.⁴¹ In patients with moderate to severe chronic heart failure, the peak oxygen consumption and exercise duration of patients are significantly increased following treatment with EPO, suggesting that EPO can enhance exercise capacity in patients with heart failure.⁴²

Currently 11 trials are registered with the National Institutes of Health and can be found on its Web site (www.clinicaltrials.gov) that will examine the ability of EPO to treat anemia and fatigue in individuals with renal failure or neoplastic disease. Other trials are now investigating EPO's role in cardiovascular disease, including the Studies of Anemia in Heart Failure (STAMINA HeFT), which will randomize patients with chronic heart failure to receive

darbepoetin or placebo and then assess their functional status assessed by exercise treadmill tests.⁴³

Future of EPO in Clinical Medicine

Cytoprotective agents that are effective, tightly targeted to desired disease entities, and safe are extremely attractive potential therapies, especially in the neuroscience and cardiovascular arenas. Years of clinical use in patients with anemia and chronic kidney diseases as well as new clinical work involving acute ischemic stroke and cardiac disorders have shown EPO to be safe and well tolerated,^{4,22,44} suggesting that EPO can fulfill the role as an ideal cytoprotective agent. Yet the clinical application of many cytoprotective agents has been hampered by evidence of subsequent clinical toxicity and unfortunately EPO is not exempt from this issue. Some studies suggest that elevated plasma levels of EPO independent of hemoglobin concentration can be associated with increased severity of disease and a poor prognostic value in individuals with congestive heart failure.⁴⁵ The use of EPO in patients with uncontrolled hypertension is contraindicated because both acute and long-term administration of EPO can precipitate hypertensive emergencies. Maintenance treatment with EPO has been associated with nonfatal myocardial infarction, vascular thrombosis, pyrexia, vomiting, shortness of breath, paresthesias, and upper respiratory tract infection.⁴⁶

The development of toxic effects during EPO therapy, such as increased blood viscosity during the treatment of cerebral ischemia,⁴⁷ could severely limit or halt the use of EPO for diseases of the nervous system. Furthermore, despite its cytoprotective attributes, EPO therapy has been correlated with the alteration of red blood cell membrane properties leading to a cognitive decrement in rodent animal models.⁴⁸ Therefore, alternate strategies have been suggested to develop derivations of EPO to reduce erythropoietic activity and potential toxicity; however, sometimes these preparations yield no efficacy against specific disorders^{4,22} and can lead to the loss of desirable effects of EPO, including its ability to promote angiogenesis, which is critical for cytoprotection.^{3,6,14}

In addition to the attempts to reduce potential toxicity during EPO administration, future strategies also must seek to optimize the timing of EPO administration. Experimental in vitro and in vivo studies indicate that the greatest cell survival is achieved with administration closest to the period of an acute toxic insult, suggesting that protection by EPO most likely coincides with the inhibition of early apoptotic cellular pathways that can involve mitochondrial dysfunction and caspase activation.^{10-15,29} Yet prolonged administration of EPO, such as for congestive heart failure, may not offer an additional degree of efficacy since this can lead to the formation of anti-EPO antibodies, occasional red blood cell aplasia,⁴⁹ and the decreased expression of the EPOR on the cell surface,⁵⁰ resulting in the loss of biological function of EPO at any concentration level. Such considerations become significant for any agent that may alter cellular physiology during different administration paradigms.

Enthusiasm coupled with rigorous investigation have led to the discoveries that EPO can prevent the loss of neurons, endothelial cells, and cardiomyocytes. It is now clear that EPO modulates an array of vital cellular functions that involve progenitor stem cell development, cellular protection, angiogenesis, DNA repair, and the promotion of cellular longevity. However, the ambitious prospects for the clinical applications for EPO that extend beyond the treatment of anemia and related disorders must be tempered with the knowledge that development of such therapeutic strategies presently remain in their infancy. As we continue to acquire further insight into the cellular pathways modulated by EPO, EPO and its derivatives should take center stage as potential agents for promoting and protecting cellular development, maturation, and survival during both acute and chronic diseases.

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Figure. Potential Mechanism of Erythropoietin Cytoprotection

Erythropoietin (EPO) and the EPO receptor (EPOR) prevent apoptosis and cellular inflammation through a series of pathways that originate with the binding of EPO to the EPOR to activate Janus-tyrosine kinase 2 (Jak2), phosphoinositide 3 kinase (PI 3-K), and protein kinase B (Akt). The signal transducer and activator of transcription (STAT) proteins are direct substrates of Jak2. Activation of the specific gene product STAT5 can regulate EPO mediated cell proliferation and protect against apoptosis. Downstream from activation of Jak2 and Akt, EPO modulates phosphorylation of the fork head family member FOXO3a, glycogen synthase kinase-3 β (GSK-3 β), and nuclear factor- κ B (NF- κ B). Hypoxia increases the expression of hypoxia-inducible factor-1 (HIF-1) resulting in the direct enhancement of EPO expression and a hypoxic-driven increase in EPOR expression. Erythropoietin maintains cellular integrity and prevents apoptosis through a number of pathways, such as those involving Bcl-x_L (for the maintenance of mitochondrial membrane potential), the modulation of apoptosis protease activating factor (Apaf)-1, the release of cytochrome C, and the activation of caspases 1, 3,

and 9. Erythropoietin also modulates cellular inflammation by inhibiting cellular phosphatidylserine membrane exposure and the subsequent targeting of cells for phagocytosis.

Table 1

Sites of Expression of Erythropoietin and Its Receptor

Cellular Expression					
Tissue	Erythropoietin		Erythropoietin Receptor		Source*
	Cell Type	Animal Model	Cell Type	Animal Model	
Brain	Neurons	Rodent, primate, human	Neurons	Rodent, primate, human	2, 4, 22, 46
	Astrocytes	Rodent, primate, human	Astrocytes	Rodent, primate, human	2, 4, 22, 46
	Microglia	Mouse, rat, human	Microglia	Mouse, rat, human	2, 4, 22, 46
			Myelin sheaths	Rat	2-4, 22
Peripheral nerve			Endothelial cells	Mouse, human	2, 4, 5, 21
			Vascular smooth muscle cells	Rat	2, 3, 22, 46
Heart	Cardiomyocytes	Mouse, rat, human	Cardiomyocytes	Mouse, rat, human	2, 46
	Kupfer cells	Mouse, primate, pig, human			2, 3
Liver					
	Hepatocytes	Mouse, primate, pig, human			2, 3
Renal	Peritubular cells	Rat, sheep, human	Peritubular cells	Rat, sheep, human	2-4
	Mesangial cells	Mouse, primate, human	Mesangial cells	Mouse, primate, human	2-4
Bone marrow	Erythroid progenitors	Human	Erythroid progenitors	Human	2, 22, 46
			Endothelial progenitor cells	Human	2, 3, 22
			Myeloid cells	Mouse, rat, human	2, 3, 22, 46
			Megakaryocytes	Mouse, rat, human	2, 3, 22, 46
Gastro-intestinal			Gastric mucosal cell	Rat	3, 46
			Insulin producing cells	Rat, primate, human	2, 3, 22
Pancreatic islets					
Reproductive organ	Uterine cells	Mouse			2, 3
	Oviductal cells	Mouse			2, 3
Placenta	Placental cells	Sheep	Placental cells	Sheep	2, 3
	Sertoli cells	Rat, pig	Leydig cells	Rat, pig	2, 3
Testis	Peritubular myoid cell	Rat, pig			2, 3

Cellular Expression					
Tissue	Erythropoietin		Erythropoietin Receptor		Source *
	Cell Type	Animal Model	Cell Type	Animal Model	
Prostate			Prostate epithelial cells	Human	2, 3

* The source number represents references in this article.

Table 2
Biological Functions of Erythropoietin in the Nervous and Cardiovascular Systems

Experimental Models With EPO			
Clinical Condition	Animal Model	Function	Source *
Neurons			
Cerebral ischemia	Rat, mouse, gerbil	Neuronal survival increased; learning ability improved; ischemic tolerance developed	2, 4, 22, 28, 29
Retinal ischemia	Rat, mouse	Photoreceptor and retinal ganglion cell apoptosis decreased	11, 22
Spinal cord injury	Rat	Motor neuronal apoptosis and inflammation decreased; neuronal function improved	30
Subarachnoid hemorrhage	Rabbit	Neuronal function and blood flow improved	22, 31
Peripheral nerve injury	Rat	Spinal neuronal apoptosis decreased; neuronal myelin repair fostered	2, 3, 22
Oxidative stress injury	Rat	DNA fragmentation, PS exposure, free radical production, and caspase activity decreased	12, 13, 28, 29
Glutamate toxicity	Rat	Glutamate release decreased; neuronal survival increased	16
Development and maturation	Mouse	Apoptosis decreased; neuronal progenitor stem cell number increased	27
Microglia			
Cerebral inflammation	Rat	Cellular inflammation decreased; cytokine release diminished	2, 4, 22
Vascular cells			
Oxidative stress injury	Rat	DNA fragmentation, PS exposure, free radical production, and caspase activity decreased; EC survival increased	14, 32
BBB disruption and angiogenesis	Calf/murine, rat, human	BBB permeability decreased; new capillary formation fostered	33–36
Cardiomyocytes			
Myocardial ischemia-reperfusion injury	Rat, rabbit	Myocardial necrosis, apoptosis, and caspase activity decreased; myocardial energy preservation and function improved	10, 37, 38
Myocardial infarction	Rat, rabbit	Myocardial necrosis and apoptosis decreased; left ventricular function improved	15, 22, 38
Heart failure	Human	Myocardial function and ejection fraction improved	39–42

Abbreviations: BBB, blood brain barrier; EC, endothelial cell; EPO, erythropoietin; PS, phosphatidylserine.

* Source represents references in this article.