Erythropoietin on a Tightrope: Balancing Neuronal and Vascular Protection between Intrinsic and Extrinsic Pathways

Faqi Li^a Zhao Zhong Chong^a Kenneth Maiese^{a–d}

^aDivision of Cellular and Molecular Cerebral Ischemia, ^bDepartments of Neurology and Anatomy and Cell Biology, ^cCenter for Molecular Medicine and Genetics and ^dInstitute of Environmental Health Sciences, Wayne State University School of Medicine, Detroit, Mich., USA

Key Words

Akt · Angiogenesis · Caspases · Forkhead transcription factors · Janus kinase 2 · Microglia · Mitogen-activated protein kinases · Protein kinase C · Stem cells

Abstract

Enthusiasm for erythropoietin (EPO) as a broad cytoprotective agent continues to increase at an almost exponential rate. The premise that EPO was required only for erythropoiesis was eventually shed by recent work 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. As a result, EPO has been identified as a possible candidate in the formulation of therapeutic strategies for both cardiac and nervous system diseases. EPO has been shown to mediate an array of vital cellular functions that involve progenitor stem cell development, cellular protection, angiogenesis, DNA repair, and cellular longevity. An important requirement to achieve the goal of preventing or even reducing cellular injury by any cytoprotective agent is the ability to uncover the cellular pathways that ultimately drive a cell to its demise. We present for consideration several critical cellular pathways modulated by EPO that involve Janus kinase 2 (Jak2), the serine-threonine kinase Akt,

forkhead transcription factors, glycogen synthase kinase-3 β (GSK-3 β), cellular calcium, protein kinase C, caspases, as well as the control of inflammatory microglial activation. As we continue to gain new insight into these pathways, EPO should emerge as a critical agent for the development, maturation, and survival of cells throughout the body.

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Introduction

Erythropoietin (EPO) is initially produced in the fetal liver during development, but shortly after birth, the generation of EPO is subsequently shifted to the kidney [1]. One of the primary functions of EPO, which is fostered by the activation of the EPO receptor (EPOR) and subsequent signal transduction pathways, is to promote proliferation, differentiation, and survival of erythroid progenitors resulting in the increased production of red blood cells. Erythropoiesis was originally considered to be the only physiological action of EPO. This concept was subsequently overturned by the knowledge that EPO and the EPOR are expressed in other organs and tissues outside of the liver and the kidney, such as the brain, heart, and uterus. In addition, several cell popula-

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1424–862X/04/0136–0265\$21.00/0

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Kenneth Maiese, MD
Department of Neurology, 8C-1 UHC, Wayne State University School of Medicine
4201 St. Antoine, Detroit, MI 48201 (USA)
Tel. +1 313 966 0833, Fax +1 313 966 0486
E-Mail kmaiese@med.wayne.edu, aa2088@wayne.edu

Fig. 1. EPO acts upon a wide variety of cells that include neurons, vascular cells, and cardiac cells to prevent cellular inflammation, block apoptotic injury, and foster angiogenesis. EPO acts directly upon the survival of neurons, ECs, cardiomyocytes, vascular smooth muscle cells (VSMC), renal cells as well as promoting cell development in progenitor stem cells and the induction of angiogenesis. EPO also modulates microglial activity to block harmful cytokine release and prevent the phagocytosis of cells 'tagged' by cellular phosphatidylserine membrane exposure. GMCs = Gastric mucosal cells; PECs = prostate epithelial cells.

tions also harbor expression of both EPO and the EPOR including neuronal, cardiac, and endothelial cells (ECs) (fig. 1). As a result, the function of EPO is now known to extend broadly beyond erythropoiesis. For example, EPO may play a role in the cardiovascular system by preventing ischemic heart disease injury. The expression of EPO in the central nervous system (CNS) also may suggest a potential cytoprotective function for this protein during nervous system disorders. In this review, we will focus on the role of EPO in systems of both neuronal and cardiovascular origins as a possible protective agent that may be applicable for immediate and long-term therapeutic regimens.

The Molecular Determinants for the Biological Activity 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 [2]. During the production and secretion of EPO, a 166-amino acid peptide is initially generated following the cleavage of a 27-amino acid hydrophobic secretory leader at the amino-terminal. In addition, a carboxy-terminal arginine in position 166 is removed both in the mature

human and recombinant human EPO (rhEPO) [3]. As a result, the circulatory mature protein of EPO has a span of 165 amino acids.

EPO is a 30.4-kDa glycoprotein and the carbohydrate content contributes to approximately 40% of its molecular weight. There are four glycosylated chains including three N-linked and one O-linked acidic oligosaccharide side chains. N-linked glycosylation sites occur at the positions 24, 38 and 83 of aspartyl residues, while the O-linked glycosylation site is at Ser¹²⁶. Three N-glycan chains of human EPO consist of the tetra-antennary structure with or without N-acetylglucosamine repeating units [4]. The O-linked sugar chain is composed of Gal-GalNAc and sialic acids [5].

The glycosylated chains are required for the biological activity of EPO. Human EPO is stabilized by the carbohydrate chains [6] and the oligosaccharides in EPO may protect the EPO protein structure from oxygen radical activity [7]. The N-glycosylated chains contribute to the thermal stability of EPO [4]. In addition, the N- and O-linked chains may be necessary for the production and secretion of the mature EPO [8]. Replacement of asparagines 38 and 83 by glutamate or serine 126 by glycine can decrease the production and secretion of EPO [9]. The presence of the carbohydrates also are important in the control of the metabolism of EPO, since EPO molecules with high sialic

acid content can be easily cleared by the body through specific binding in the liver [10].

The biological activity of EPO also relies upon two disulfide bonds formed between cysteines at positions 7 and 160 and at positions 29 and 33. The requirement of these disulfide bridges has been demonstrated by the evidence that reduction of these bonds results in the loss of the biological activity of EPO. Alkylation of the sulfhydryl groups results in irreversible loss of the biological activity of EPO. Reoxidization of EPO after reduction by guanidine HCl leads to regeneration of 85% of the biological activity of EPO [11]. In addition, Cys³³ replacement with proline also reduces the biological function of EPO. These results suggest that the two disulfide bridges are critical for EPO to serve its biological function.

Organs of Production and Expression for EPO and Its Receptor

Over time, a combination of studies have shown that the main organs of EPO production and secretion are the kidney, liver, brain, and uterus (fig. 1). The primary site of EPO production and secretion is in the kidney. The peritubular interstitial cells in the kidney are responsible for production and secretion of EPO [12]. With the use of cDNA probes derived from the EPO gene, peritubular ECs, tubular epithelial cells, and nephron segments in the kidney also have been demonstrated to be vital cells for the production and secretion of EPO [13, 14].

Secondary sites of EPO production and secretion involve the liver and the uterus. Studies have indicated that hepatocytes, hepatoma cells, and Kupffer cells of the liver can produce and secrete EPO [12]. In regard to the uterine production of EPO, it is believed that the hypoproliferative neonatal anemia that invariably occurs in the early weeks after birth may partly result from the loss of EPO production and secretion by placenta [15]. As knowledge of the sites for the generation of EPO increases, other novel organs, tissues, and cells continue to surface as secretory tissues for EPO that include ECs, enterocytes, muscle (skeletal, smooth, and cardiac), and insulin-producing cells [16–19].

Yet of all the newly identified sites for the existence of EPO and its receptor, it is the production of EPO in the brain that has generated a significant level of interest and enthusiasm for further investigation. Production and secretion of EPO have been illustrated in the nervous system of rodents, primates, and humans [20–22]. In the brain, the major sites of EPO production and secretion

are in the hippocampus, internal capsule, cortex, mid-brain, cerebral ECs, and astrocytes. Interestingly, studies that involve electrical stimulation of the brain have indicated that EPO may be produced by a diverse system of secretory cells [23].

EPO must bind to a target cell surface receptor to bring into play its biological function. Once the EPO gene was cloned [2], work was initiated to identify a receptor for EPO. The EPOR was found to be expressed in both normal and transformed erythroid cells [24]. EPO regulates bone marrow erythroid cell proliferation, differentiation, and survival through its binding to an erythroid progenitor cell surface EPOR. Additional work has documented the presence of the EPOR in numerous nonerythroid blood lines including myeloid cells, lymphocytes, and megakaryocytes as well as multiple nonhematopoietic cells, such as neurons, microglia, astrocytes, vascular smooth muscle cells, cardiomyocytes, mesangial cells, prostate cells and renal cells [16, 20–22, 25] (fig. 1). Recent work has even documented the presence of the EPOR on myelin sheaths of radicular nerves in human peripheral nerves [26], suggesting both a developmental and cytoprotective role for EPO not only during CNS disorders, but also in disease entities that involve the peripheral nervous system. The EPOR also is expressed in primary cerebral ECs [20, 27]. In addition, expression of the EPOR mRNA in ECs occurs in human umbilical veins, bovine adrenal capillaries, and rat brain capillaries [16, 28]. Binding studies with radioiodinated rhEPO has revealed that approximately 27,000 EPORs with an affinity in the 10^{-9} M range are present on each EC.

Temporal and Environmental Modulation of EPO

The production of EPO and the expression of EPOR can vary significantly during the development of an organism. Elevated expression of the EPOR occurs in early embryonic murine neuronal tissues at levels similar to that observed in the adult spleen and bone marrow [29]. The level of endogenous EPOR expression is significantly reduced following the maturation of the brain [30]. In the human nervous system, the level of EPO production is also known to change with development from increased production during gestation to decreased production after birth.

Following development, the production and secretion of EPO and the expression of EPOR are regulated by the tissue oxygen supply. Plasma levels of EPO are increased

up to 1,000-fold above normal levels in response to hypoxia [1]. A deficiency in tissue oxygen results in the production of EPO and an increase in the expression of the EPOR not only in the kidney and liver [22], but also in the brain [20, 22]. During hypoxia, EPO production may originate in the brain, possibly crossing the blood-brain barrier to reach the blood and peripheral organs [31]. The hypoxia-dependent production and secretion of EPO in the brain appear to be more sustained than in peripheral organs such as the kidney [32]. Additional studies in the brains of rodents and primates subjected to systemic hypoxia also demonstrate an increase in expression of EPO and EPOR mRNA following reduced oxygenation [33]. Furthermore, neuronal cell lines have been found to retain the capacity to express the EPO gene in an oxygen-dependent manner [34]. Cerebral ischemia that leads to a deficiency of brain oxygen can result in a significant increase in the expression of EPO and the EPOR in neurons, astrocytes, and cerebral microvascular ECs in mice [35].

As expected, EPO is recognized as a critical modulator of erythroid production known as erythropoiesis. However, a diminished concentration of red blood cells does not directly stimulate EPO production and secretion as one would predict. Production and secretion of EPO for the stimulation of erythropoiesis are also oxygen dependent. Once a hypoxic stimulus is received, EPO is subsequently released into the peripheral blood circulation and upon arrival in the bone marrow, EPO binds to its receptor expressed on the surface of erythroid progenitor cells and leads to erythropoiesis [36]. This results in an elevation in the number of mature erythrocytes and the improvement of oxygen supply [37]. Interestingly, EPO also acts on the later stages of erythroid progenitor cells. EPO functions to stimulate colony-forming erythroid cells to induce these cells to proliferate and mature into erythrocytes [12].

In most tissues including the brain, hypoxia-dependent expression of EPO and EPOR are primarily regulated by hypoxia-inducible factor 1 (HIF-1), an alpha/beta heterodimeric protein that is activated by a variety of stressors, such as hypoxia. HIF-1 is essential for the production and secretion of EPO in response to hypoxia. At the transcriptional level, the hypoxia-dependent gene transcription of EPO and EPOR directly results from the activation of the HIF-1 pathway under hypoxic conditions [38]. Gene transcription of EPO is mediated by the transcription enhancer located in the 3'-flanking region of the EPO gene that specifically binds to HIF-1 [39].

HIF-1 is a basic helix-loop-helix heterodimeric transcription factor containing two subunits, HIF-1 α and HIF-1 β [39]. HIF-1 β is a constitutively expressed 91- to 94-kDa subunit that was characterized previously as aryl hydrocarbon receptor nuclear translocator (ARNT) [40]. HIF-1 α is a 120-kDa oxygen-labile subunit that undergoes rapid degradation via the ubiquitin-proteasome pathway under normoxic conditions [41]. Upon hypoxic exposure, degradation of HIF-1 α is impaired by blocking its association with von Hippel-Lindau protein that targets HIF-1 α for proteasome destruction [42]. HIF-1 α translocates to the nucleus and heterodimerizes with HIF-1 β to form a stable HIF-1 complex. The HIF complex binds to the conserved sequence (5'-RCGTG-3') near the 5' end of the hypoxia-responsive enhancer of the EPO gene to upregulate EPO gene transcription [43]. Increased DNA binding activity of HIF-1 has been observed in rat cortical neurons during oxidative stress [44, 45]. These results suggest that HIF-1 may function as oxygen sensor regulating adaptive gene transcription and resulting in the production and secretion of the EPO protein during hypoxia in the CNS. Recent studies have shown that each of the family members of HIF that include HIF-1 α , HIF-1 β , and HIF-3 α appear to play an important role in regulating the expression of EPO and the EPOR to foster protection against hypoxic cell injury [46].

Hypoxia is not the only factor involved in the expression of EPO and the EPOR. For example, the production and secretion of EPO in female reproductive organs is estrogen dependent. Administration of 17 β -estradiol (E₂), which controls the cyclic development of the uterine endometrium, can lead to a rapid and transient increase in EPO mRNA in the uterus [47]. Hypoxic-induced EPO mRNA expression in uterine tissue occurs only in the presence of E₂. This induction of EPO mRNA expression by hypoxia in the uterus is less pronounced than the EPO expression that occurs in the kidney and the brain [32]. Oviduct and ovary production of EPO is also E₂ dependent [48]. Other metabolic disturbances, such as hypoglycemia, raised intracellular calcium, or intense neuronal depolarizations generated by mitochondrial reactive oxygen species, may increase cerebral EPO expression through activation of HIF [20, 21, 49]. Anemic stress and insulin release also can lead to increased expression of EPO and the EPOR in both neuronal and nonneuronal cell populations. Finally, a variety of cytokines, including insulin-like growth factor, tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6), can regulate the production and secretion of EPO [50].

EPO Leads to Progenitor Stem Cell Development in Nonerythroid Cells

EPO can assist with the proliferation and development of progenitor stem cells (fig. 1). EPO can increase the viability of embryonic cortical neurons, promote cell survival, and upregulate the proliferative response of neuronal progenitor cells [51]. At times, EPO will selectively promote the production of neuronal progenitors at the expense of other multipotent progenitors [52]. Similar to other hematopoietic factors, EPO also can function as a neurotrophic factor for differentiated cells. For example, EPO can influence the regeneration, differentiation, and survival of central cholinergic neurons as well as mediate the differentiation of mesencephalic precursors into dopaminergic neurons [53, 54].

EPO Possesses a Robust Potential for Cytoprotection

EPO in the CNS

The adaptive response to oxygen deficiency that results in increasing production and secretion of EPO as well as the enhanced expression of the EPOR in the CNS suggests a strong potential role for EPO to prevent brain injury. Efforts to illustrate neuronal and vascular protection in the CNS by EPO have been performed using multiple in vivo and in vitro experimental models (table 1). Initial animal studies focused on the ability of EPO to reduce cerebral injury following an ischemic insult and to maintain cognitive function. One of the first experimental studies performed infused EPO into the lateral ventricles of gerbils subjected to occlusion of the common carotid artery and demonstrated that cerebroventricular administration of EPO in a concentration range of 2.5–24 units/day for 7 days prevented ischemic-induced learning disability, reduced hippocampal neuronal injury, and ameliorated neuron survival in the gerbil [55]. Infusion of EPO into the lateral ventricles of mice 24 h prior to the onset of a middle cerebral artery occlusion also resulted in a significant reduction in cerebral infarct volume [35]. Other work demonstrated that injection of EPO into the ventricles of stroke-prone spontaneously hypertensive rats with permanent occlusion of the left middle cerebral artery alleviated ischemia-induced navigation disability, supported neuron survival, and limited the degree of infarction [56, 57].

Over time, ventricular delivery systems have been considered impractical for clinical applications. As a result,

future investigations are now pursuing systemic administration of EPO in several injury models. Systemic application of EPO has been demonstrated to prevent delayed hippocampal neuronal injury following global cerebral ischemia in gerbils [58] and direct intravenous infusion of naked plasmid containing EPO cDNA is capable of rescuing neurons from lethal hypoxia-ischemia injury [59]. Application of systemic EPO following experimental subarachnoid hemorrhage also has been shown to effectively modulate the autoregulation of cerebral blood flow, reverse vasoconstriction of the basilar arteries, reduce ischemic neuronal damage, and enhance functional recovery [60].

Investigations examining the potential of EPO to prevent cortical injury have been extended to spinal cord models, the visual system, and the peripheral nervous system. During spinal cord ischemia as well as spinal cord blunt injury, EPO can prevent motor neuronal apoptosis and neurological disability, attenuate secondary inflammatory injury, and reduce lipid peroxidation [61]. In transient global retinal ischemia and ischemia-reperfusion injury models, systemic administration of EPO before or immediately after retinal ischemia can protect retinal ganglion cells from apoptosis and promote the recovery of retinal function [21, 62]. It is believed that EPO promotes neural outgrowth from retinal ganglion cells that possess EPORs [63]. Additional studies in the adult mouse hypoxic retina has shown that systemically applied EPO can cross the blood-retina barrier and prevent apoptosis during a light-induced insult [64]. These findings strongly suggest that application of EPO may be beneficial for the treatment of a variety of retinal diseases by blocking apoptotic degeneration of photoreceptors or retinal ganglion cells. In peripheral nerve injury models, therapy with EPO can significantly decrease dorsal root ganglion apoptosis, improve recovery rates from mechanical allodynia [65], and promote myelin repair [66], suggesting that EPO may play an important role in the treatment of neuropathic pain as well as diseases that involve degeneration of motor neurons.

EPO Protects Vascular Integrity and Promotes Angiogenesis

Since cerebral ischemia can impair the integrity of the blood-brain barrier, it is hypothesized that EPO can acquire access to cerebral tissue through regions of blood-brain barrier breakdown and may ultimately assist with the repair of the blood-brain barrier [60, 67]. Without injury to the blood-brain barrier, EPO may not gain access to the brain [68]. On closer inspection, recent work

Table 1. EPO in the nervous system

Experimental models	Role of EPO	References
<i>Animal model studies</i>		
Focal and global cerebral ischemia 5–5,000 U/kg i.p. or 10–50 U/kg (or 0.4 µg/kg) i.v. or 0.2–25 U/day i.c.v. applied either pretreatment or posttreatment with injury in rats, mice, or gerbils	Infarct volume, brain edema, and neuronal apoptosis decreased; neuronal survival and cerebral functional recovery increased	35, 55, 58
Retinal ischemia 5,000 U/kg i.p. or 2 µl intravitreal applied either pre-treatment or posttreatment with injury in mice and rats	Ganglion and photoreceptor cell apoptosis decreased	63, 164
Neonatal hypoxic-ischemic brain injury 300–5,000 U/kg i.p. applied either pretreatment or posttreatment with injury in mice and rats	Infarct volume, neuronal apoptosis, NO production, and caspase 3 activity decreased	56, 200
Spinal cord ischemic injury 100–5,000 U/kg i.p. or 10–1,000 U/kg i.v. application posttreatment with injury in rats or rabbits	Motor neuronal apoptosis, inflammation, and lipid peroxidation decreased; neuronal survival increased; functional recovery improved	
Subarachnoid hemorrhage 1,000 U/kg i.p. or 400 U/kg s.c. applied immediately or 5 min after injury in rats or rabbits	Rat mortality, neuronal death, and vasoconstriction decreased; functional recovery and blood flow auto-regulation improved	60, 67
Cerebral inflammation 500–5,000 U/kg i.p. applied immediately after injury in rats	Brain injury and inflammatory cytokine release decreased	61, 118
Peripheral nerve injury 1,000–5,000 U/kg s.c. or 1–10 U/kg i.v. applied pretreatment with injury in rats	Dorsal root ganglion and spinal neuronal apoptosis decreased; myelin repair; recovery from mechanical allodynia improved	64, 65
<i>Tissue culture studies</i>		
Anoxic injury 0.01–100 ng/ml applied either pretreatment or posttreatment with injury in ECs, hippocampal, or cortical neurons	DNA fragmentation and PS exposure decreased; cell survival increased	68, 117
NO injury 10 ng/ml, 20 U/ml, 25 U/day applied either pretreatment or posttreatment with injury in ECs, hippocampal and cortical neurons	DNA fragmentation, PS exposure and NO production decreased; cell survival increased	27, 55, 58, 105, 117, 123
Glutamate toxicity 50 ng/ml or 3–300 pmol/ml applied pretreatment with injury in hippocampal, cortical and cerebellar neurons	Glutamate release decreased; neuronal survival increased	35, 122, 149
BBB disruption 10 U/ml applied with VEGF (10 ng/ml) in monolayer of bovine brain ECs	BBB permeability decreased	
Stem cell development 0.5–15 U/ml applied immediately pretreatment to neuronal cholinergic or dopaminergic stem cells, human fetal neuronal cells and rat embryonic neuronal cells	Apoptosis decreased; neuronal progenitor cell number increased	51–54
Angiogenesis 0.5–100 U/ml applied immediately pretreatment in EC cultures	Intussusceptive microvascular growth, neovascularization, proliferation, and migration increased	17, 73, 74

BBB = Blood-brain barrier; i.c.v. = intracerebroventricular injection; i.p. = intraperitoneal injection; i.v. = intravenous injection; s.c. = subcutaneous injection; VEGF = vascular endothelial growth factor.

has demonstrated that EPO can prevent blood-brain barrier permeability during injury and maintain the establishment of cell-to-cell junctions [67]. More importantly, EPO can provide direct cerebral EC protection and protect against nuclear degeneration during oxidative stress [25, 27, 69].

Yet, the role of EPO during vascular protection extends beyond the direct preservation of EC integrity and significantly involves angiogenesis, a complex process that consists of new capillary formation from preexisting vessels into an avascular area [25]. This process involves vascular basal lamina formation, migration of ECs, and alignment of migrating cells for tubular formation. There are at least two types of angiogenesis. Sprouting angiogenesis is characterized by the proliferation and migration of ECs into vascular sites [70]. In contrast, nonsprouting angiogenesis or intussusceptive microvascular growth occurs by splitting the existing vasculature into transluminal pillars or transendothelial bridges [71]. Angiogenesis takes place in various physiological and pathophysiological conditions. It is physiologically active during embryogenesis [70]. In the adult, it occurs during more limited periods such as during menstruation and during some pathological conditions such as wound healing, chronic inflammation, and tumor growth [72, 73].

EPO appears to elicit both a mitogenic and chemotactic effect. In cultured human and bovine ECs, EPO not only stimulates proliferation, but also enhances the migration of ECs [74]. In neonatal mesenteric microvascular ECs, EPO can promote vasculogenesis [75]. Other investigations have illustrated the migration of ECs on the Matrigel surface to form branching and anastomosing tubes in response to EPO exposure. Furthermore, EPO can lead to matrix metalloproteinase-2 production, cell proliferation, and vessel formation in EC lines [17].

In clinical studies, EPO serum levels are significantly associated with the number and function of circulating endothelial progenitor cells. In addition, EPO can stimulate postnatal neovascularization by increasing endothelial progenitor cell mobilization from the bone marrow [76]. Angiogenesis also has been observed in rat aortic rings 4 days following incubation with EPO in reconstituted basement membrane matrix [77] and in the ECs derived from human adult myocardial tissue treated by rhEPO [78]. Intussusceptive microvascular growth also is fostered by EPO and can be abolished by the application of an EPO-blocking antibody [79].

Both the uterine endometrium and the ovaries are dependent upon EPO for the induction of angiogenesis. Angiogenesis in the uterine endometrium functions to

compensate for lost vessels during the estrus cycle. Injection of EPO into the uterine cavity of ovariectomized mice has been shown to be necessary to foster blood vessel formation in the endometrium [47]. EPO also regulates angiogenesis in the ovary and the subsequent formation of a capillary network for the development of follicles and the corpora lutea [47].

Angiogenesis generated in the vascular system by EPO also may provide indirect cellular protection in the CNS. The proliferation and migration of brain capillary ECs by EPO can occur during an ischemic brain, possibly offering enhanced blood flow and nutrients to ischemic cells starved for oxygen and nutrients [28, 35]. Furthermore, angiogenesis in the brain may be closely related to neuronal survival in patients with ischemic stroke [80]. A benefit of angiogenesis may result from the restoration of blood flow in the ischemic border through arteriolar growth and capillary formation during cerebral ischemia [81]. As new vessel formation occurs in the ischemic border of the brain several days following a stroke [80], the induction of angiogenesis by EPO may provide direct protection of brain neurons and ECs as well as indirectly contribute to the functional recovery of the ischemic brain through improved vascular perfusion [25, 35].

EPO and Cardiac Function

EPO may be considered to be a 'broad spectrum' cytoprotectant since in addition to promoting neuronal and vascular cell survival, protection by EPO involves several other cell populations including renal cells, pancreatic islet cells, and cardiomyocytes [19, 82]. EPO may impact upon cardiovascular diseases as a result of its regulation of smooth muscle cells and the protection conferred upon vascular ECs and cardiomyocytes. The ability of EPO to preserve vascular ECs and induce angiogenesis as well as protect cardiac cells likely contributes to the significant protective role of EPO during cardiovascular injury such as ischemia, myocardial infarction, ischemia-reperfusion injury, and heart failure.

Given the positive effects of EPO against brain ischemia and reperfusion injury, recent studies both in vitro and in vivo have been conducted to evaluate the role of EPO during cardiac ischemia and reperfusion injury (table 2). In isolated hearts subjected to ischemia-reperfusion, treatment with EPO 24 h following injury can reduce apoptosis in cardiomyocytes, limit myocardial infarct size, and promote functional recovery of the heart [83]. Parenteral administration of EPO also is sufficient to induce dramatic protection against ischemia-reperfusion injury in the heart [84]. A more recent study has dem-

Table 2. EPO in the cardiovascular system

Experimental models	Role of EPO	References
<i>Animal model studies</i>		
Myocardial ischemia-reperfusion injury 1,000 or 5,000 U/kg i.p. applied pretreatment with injury or 100 U/kg (0.16 U/ml) or 10 U/ml (14–15 ml/min, total 1,500 U) perfused into isolated heart either pretreatment or posttreatment with injury in rats, mice, or rabbits	Myocardial infarct volume, myocardial apoptosis, and caspase 3 activity decreased; survival of myocardiocytes, ATP levels, and functional recovery increased; Jak2, STAT3, STAT5, ERK and Akt activity increased	83, 85, 86, 93
Myocardial infarction 3,000–5,000 U/kg i.p. or 1,000–5,000 U/kg i.v. applied immediately or posttreatment with injury in rats or rabbits	Myocardial infarct size, cardiac cell apoptosis, and LV size decreased; cardiomyocyte survival, LV performance, ERK and Akt activity increased	88–90
<i>Tissue culture studies</i>		
Anoxic injury 100 ng/ml or 4–10 U/ml applied either pretreatment or posttreatment with injury in myocardiocytes, cardiac fibroblasts, or H9c2 myoblast cell line	Myocardial apoptosis, overall cardiac cell death, and caspase activity decreased; Jak1, Jak2, STAT3, STAT5, ERK and Akt activity increased	85, 88, 89, 93
<i>Clinical studies</i>		
Heart failure 2,000–30,000 U/week, s.c. or i.v. application in the patients with chronic heart failure	Number of hospitalization days decreased; functional recovery increased; clinical symptoms and signs improved	94, 95
Akt = Protein kinase B; ERK = extracellular signal-related kinase; i.p. = intraperitoneal injection; i.v. = intravenous injection; LV = left ventricle; s.c. = subcutaneous injection; STAT = signal transducer and activator of transcription.		

onstrated that EPO treatment either prior to or during myocardial ischemia/reperfusion can protect against myocardial cell apoptosis and decrease infarct size, resulting in enhanced cardiac function and recovery, including left ventricular contractility [85]. In the isolated rat heart following ischemia/reperfusion experiments, beneficial effects of treatment with EPO also have been shown to reduce cellular necrosis and improve postischemic recovery of left ventricular pressure significantly [86, 87]. At the onset of coronary artery occlusion, EPO administered also can significantly inhibit apoptosis in the central region of myocardial ischemia [88]. Even in acute scenarios following coronary artery ligation, EPO leads to a decrease in apoptotic cells by 50% in the myocardium and significantly improves cardiac function [89, 90]. Current work has illustrated that EPO can promote intussusceptive microvascular growth in the heart, suggesting that the protective effect of EPO on ischemic heart disease may be a partial result from an increase in myocardium blood supply as a result of the generation of new blood vessels [79].

In addition to the correction of anemia, the beneficial effects of EPO on heart failure result from a direct protection of the myocardial cells. As a result, EPO is considered to be appropriate for the treatment of patients with heart failure following anemia or anemia accompanying heart failure. Early studies have indicated that administration of EPO can lead to a decrease in left ventricular hypertrophy, inhibit left ventricular dilatation, and increase left ventricular ejection fraction, stroke volume, and cardiac output, suggesting cardiac function improvement in patients with congestive heart failure resulted from anemia correction [91, 92]. Other in vivo studies have illustrated that treatment with EPO can increase cardiac cell proliferation in neonatal rats, reduce myocardiocyte apoptosis during ischemia-reperfusion injury, and improve left ventricular function [89, 90, 93].

Recently, randomized control 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% [94] (table 2). In additional investigations involving subcutaneous EPO in diabetics and nondiabetics with severe, resistant congestive heart failure breathlessness and/or fatigue have been shown to decrease, left ventricular ejection fraction to increase, and the number of hospitalization days to significantly decrease [95]. 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. As a result, work has supported the premise that EPO can function as a novel cytoprotectant against acute or chronic ischemic heart disease by enhancing cardiac cell survival and proliferation, increasing cardiovascular blood flow, and improving heart remodeling and function.

EPO Drives Extrinsic Cellular Protection during Inflammatory Injury

EPO can offer cytoprotection that extends beyond the preservation of intrinsic cellular integrity, such as preservation of genomic DNA integrity. EPO can influence extrinsic cell homeostasis through the modulation of microglial activation and the control of cytokine release (fig. 1). Microglia are monocyte-derived immunocompetent cells that enter the CNS during embryonic development and function similar to peripheral macrophages for the phagocytic removal of apoptotic cells. There exist several potential mechanisms that may regulate the phagocytosis of cells that have entered the apoptotic pathway. Some studies identify the generation of annexin I and membrane phosphatidylserine (PS) exposure that appears to be necessary to connect an apoptotic cell with a phagocyte [96]. Secreted factors by either apoptotic or phagocytic cells, such as milk fat globule-EGF factor 8 [97], fractalkine [98], and lipid lysophosphatidylcholine [99], also have been shown to assist with the phagocytic removal of injured cells.

The translocation of membrane PS residues from the inner cellular membrane to the outer surface appears to be critical for the removal of apoptotic cells [100–102]. The phospholipids of the plasma membrane are normally in an asymmetric pattern with the outer leaflet of the plasma membrane consisting primarily of choline-containing lipids, such as phosphatidylcholine and sphingomyelin, and the inner leaflets consisting of aminophospholipids that include phosphatidylethanolamine and PS. The loss of membrane phospholipid asymmetry leads to the external-

ization of membrane PS residues and serves to identify cells for phagocytosis [102–105].

Both the induction of the phosphatidylserine receptor (PSR) on microglia and the exposure of membrane PS residues are necessary to activate microglia. Cells, such as neurons or ECs, exposed to injury can lead to the induction of both microglial activation and microglial PSR expression. Treatment with an anti-PSR neutralizing antibody in microglia prevents this microglial activation [106, 107] and application of PS directly results in microglial activation that can be blocked by a PSR-neutralizing antibody [102, 106], suggesting that both PS exposure in target cells and PSR expression in microglia are necessary for microglial recognition of apoptotic cells in the nervous system. Recognition of cellular membrane PS by the PS-specific receptors on microglia may require cofactors, such as Gas6 [108] or other agents, such as integrin and lectin [109].

Although vital for both cellular homeostasis as well as host defense mechanisms, microglia may aggravate a cellular insult. Studies with microglia stimulated by phorbol myristate acetate have demonstrated the release of superoxide radicals. Application of scavenger agents for reactive oxygen species, such as superoxide dismutase or deferoxamine mesylate, in the presence of activated microglia can prevent cellular injury. These studies suggest that oxidative stress generated by microglia can be responsible for cellular injury [110]. Activated microglia upregulate a variety of surface receptors and yield significant proinflammatory and neurotoxic factors, such as TNF- α and IL-1 β , free radicals such as nitric oxide (NO) and superoxide [111], and fatty acid metabolites such as eicosanoids that can precipitate cell death [112]. The secretion of cytokines by microglia also may represent another source of cytotoxicity for microglia. Microglia produce a variety of cytokines in response to toxic stimulation, such as interleukins and TNF. TNF- α production by microglia may be linked to neurodegeneration by increasing the sensitivity of neurons to free radical exposure [113].

Furthermore, several neurodegenerative disorders may progress during microglial activation. In Huntington's disease and amyotrophic lateral sclerosis, significant microglial activation occurs in areas of the nervous system that are specific for these disease entities [114, 115]. During cerebral ischemia, activation of microglia parallels the induction of cellular apoptosis and correlates well with the severity of the ischemic insult [116]. In patients with Alzheimer's disease, microglial cells colocalize with the perivascular deposits of β -amyloid (A β). In addition,

Fig. 2. EPO offers neuronal, vascular, and cardiac protection through a series of cellular pathways. The ability of EPO and the EPOR to enhance cell survival and block cell inflammation originates with upstream pathways that involve the Janus-tyrosine kinase 2 (Jak2) protein and protein kinase B (Akt). Downstream from the activation of Jak2 and Akt, EPO modulates STAT5, FOXO3a, GSK-3 β , Bad, Bcl-x_L, IKK, NF- κ B, Gadd45 β , and possibly MAPKs. Closely tied to the ability of EPO to maintain cellular integrity and prevent inflammatory activation that ultimately can lead to cellular apoptosis are the maintenance of $\Delta\Psi_m$, the modulation of Apaf-1, the release of cytochrome c (Cyto-c) and the activation of caspases 1, 3, 8 and 9.

microglial activation has been observed to occur in concert with the evolution of amyloid plaques [117].

Recently, EPO has been demonstrated to reduce or prevent cellular inflammation during oxidative stress [106, 118], cerebral ischemia [119], and trauma [61]. The protein Akt can modulate the spatial regulation of actin assembly, suggesting a relationship between Akt and the coordination of cytoskeletal organization [120]. In addition, Akt appears to be a necessary component for the modulation of membrane PS externalization and prevent microglial activation [102, 106, 107, 118] (fig. 2). Recent work also has shown that Akt can directly control cellular membrane asymmetry and microglial activation through the prevention of cysteine protease degradation of Bcl-x_L and the specific inhibition of caspase 1-, 3- and 9-like activities [121] (fig. 2). EPO can control microglial activation through the modulation of Akt and cellular mem-

brane PS exposure [27, 118]. As a result, the shedding of membrane PS residues that is known to occur during apoptosis may be prevented [122]. Prevention of inflammatory microglial activation by EPO also may be dependent upon caspase activation and cytokine release. EPO can directly inhibit the activities of caspase 1, 3, 8 and 9. Caspase 1 is believed to be principally responsible for the externalization of membrane PS residues in several cell populations and required for microglial activation [27, 106, 118]. EPO also may directly address cellular inflammation by blocking the release of several proinflammatory cytokines, such as IL-6, TNF- α and monocyte chemoattractant protein 1 [23].

EPO and Apoptotic Oxidative Stress

Protection against oxidative stress by EPO can be quite robust and involves preventing apoptosis from a number of sources, such as reduced or absent oxygen tension, excitotoxicity, and free radical exposure. For example, during hypoxia or glutamate exposure, administration of EPO can result in a significant increase in neuronal survival in cultured hippocampal neurons [118, 123]. Several other studies have demonstrated that EPO elicits cellular protection against NO exposure and endothelial NO production to maintain blood-brain barrier integrity [58, 67, 106, 118, 124].

Apoptosis is a primary component of cellular injury in neuronal, vascular, and cardiac cell populations. Apoptotic injury is believed to contribute significantly to a variety of neurological disorders such as ischemic stroke [104, 125], dementia [126], Alzheimer's disease [127], Parkinson's disease [128], and spinal cord injury [129, 130]. Circumstances such as the lack of trophic support, exposure to neurotoxins, and the induction of oxidative stress and DNA damage can become critical for the initiation of apoptosis [131].

EPO offers cellular protection at the levels of DNA degradation and membrane PS exposure. Membrane PS exposure and DNA fragmentation are two functionally independent processes that lead to apoptotic cellular injury. The biological role of membrane PS externalization can vary in different cell populations. In many cell systems, membrane PS externalization can become a signal for the phagocytosis of cells [102, 103, 106, 132]. In the nervous system, cells expressing externalized PS may be removed by microglia. An additional role of membrane PS externalization in the vascular cell system is the activation of coagulation cascades. The externalization of membrane PS residues in ECs can promote the formation of a procoagulant surface [100, 101, 133]. In contrast to the early externalization of membrane PS residues, the cleavage of genomic DNA into fragments is a delayed event that occurs late during apoptosis [101, 134]. Application of EPO during injury can prevent the exposure of membrane PS residues and also inhibit the committed stages of genomic DNA destruction [64, 65, 69, 106, 118]. Thus, EPO offers early cellular protection by maintaining genomic stability and also provides a more long-term protection by maintaining membrane PS asymmetry to block microglial phagocytosis and prevent the formation of a procoagulant surface in ECs [102, 109] (fig. 2).

Posttreatment paradigms visualized in 'real time' with living cellular systems also have demonstrated a 'window

of opportunity' to prevent early apoptotic changes and the progression of membrane PS residue exposure once an injury has been initiated [105, 132, 135]. Several studies that have examined the efficacy of cytoprotectants have supported the premise that cellular apoptosis is reversible. For example, the application of growth factors [136], benzothiazole compounds [137–139], metabotropic glutamate receptor agonists [104, 140], and enhanced Bcl-2 expression [141] have been shown to either prevent or reverse membrane and nuclear changes associated with apoptosis. As a result, the cellular mediators that are responsible for the induction of PS exposure and DNA fragmentation may function as critical targets for cytoprotective strategies. In this regard, the protective capacity of EPO is complex in nature and appears to require a 'therapeutic window' following the onset of a toxic exposure. This confined period for protection by EPO, which can vary up to 6 h in some models, most likely coincides with the progressive induction of secondary cellular pathways such as cytochrome c release and cysteine protease induction [21, 25]. Several other factors may determine both the concentration and temporal parameters that regulate the protective ability of EPO, since chronic administration of EPO can result in the formation of anti-EPO antibodies [142] and decrease the expression of the EPOR on the cell surface [143].

EPO and the Modulation of Cellular Pathways

An important requirement to achieve the goal of preventing or even reducing cellular injury by any cytoprotective agent in either neuronal or vascular systems is the ability to uncover the cellular pathways that ultimately drive a cell to its demise. Recent work for EPO has begun to focus on mechanisms that involve a host of cellular signal transduction pathways. In the following sections, we present for consideration novel cellular pathways that originate from Janus kinase 2 (Jak2) and the serine-threonine kinase Akt and involve cellular pathways related to phagocytic microglia, FOXO3a, glycogen synthase kinase-3 β (GSK-3 β), Bad, Bcl-x_L, nuclear factor κ B (NF- κ B), mitochondrial permeability, apoptotic protease-activating factor-1 (Apaf-1), and caspases. These cellular pathways continue to shape our understanding of the significant role EPO plays that appears to be almost exclusive of its originally described function to stimulate erythropoiesis (fig. 2).

EPO Is Dependent upon Jak2 Activity and Its Substrates

Of course, cellular signal transduction initiated by EPO begins with the activation of the EPOR. The EPOR is part of the type 1 superfamily of cytokine receptors and is activated via homodimerization [144, 145]. Members of this receptor family share a common domain structure consisting of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain. The extracellular domain is necessary for the initial binding of EPO and the intracellular domain is responsible for the transduction of intracellular signaling [146]. The cytoplasmic portion of the EPOR contains a Box 1 motif that specifically binds to and activates Jak2 through phosphorylation [147].

EPO can block apoptotic injury through phosphorylation of Jak2. Jak2 is a member of a family of Janus-type protein-tyrosine kinases including Jak1, Jak2, Jak3, and Tyk2 that are characterized by a kinase domain in the carboxyl terminal region, a kinase-like domain, and a large amino-terminal domain [148]. The amino-terminal domain of Jak2 is responsible for the binding of Jak2 with the β -subunit of the EPOR at a region proximal to the membrane that contains Box 1 sequence [149]. EPO appears to prevent neuronal cell apoptotic injury through its reliance on Jak2 phosphorylation [150], since a loss of Jak2 activity abrogates the cytoprotection by EPO [151]. As a result of an increased Jak2 phosphorylation, EPO also can reduce peripheral pain sensation [65].

The signal transducer and activator of transcription (STAT) proteins are direct substrates of Janus kinases. Activation of Jak kinases results in tyrosine phosphorylation and dimerization of STATs. The active STATs then translocate to the nucleus and bind to specific DNA sequences in the promoter regions of responsive genes to lead to gene transcription. Six primary mammalian STAT genes have been identified. They consist of STAT1, 2, 3, 4, 5 and 6 and are essential for performing pleiotropic cellular functions in response to cytokines and growth factors.

Activation of STAT5 also can modulate EPO proliferation as well as protection against cellular apoptosis [152]. In erythroleukemic cell lines, EPO-dependent cell survival is accompanied by sustained STAT5 DNA-binding activity. Stable expression of the truncated STAT5a has been shown to enhance STAT5-DNA binding activity and reduce the induction of apoptosis [153]. In contrast, induction of apoptosis can be observed in cells that lack STAT5 (STAT5a^{-/-}/5b^{-/-}) function [154]. For example, STAT5a^{-/-}5b^{-/-} fetal liver erythroid progenitors show

higher levels of apoptosis and are less responsive to the presence of EPO [155].

Activation of Protein Kinase B (Akt) Promotes Cellular Survival through EPO

In addition to Jak2, EPO is dependent upon the activation of phosphoinositide 3-kinase (PI 3-K) and protein kinase B (PKB), also known as Akt [156]. Akt has been identified as a principal component in a variety of pathways to promote cell survival and block apoptotic degradation. In mammals, three family members of PKB have been identified, which are termed PKB α or Akt1, PKB β or Akt2, and PKB γ or Akt3. Akt belongs to the cAMP-dependent kinase/protein kinase G/protein kinase C (PKC) superfamily of protein kinases and consists of three functionally domains [157, 158]. The N-terminal pleckstrin homology (PH) domain provides binding sites for membrane phospholipids, which is involved in the recruitment of Akt to the plasma membrane. The catalytic domain of Akt has specificity for serine or threonine residues of several Akt substrates. The C-terminal hydrophobic motif functions to provide a docking site for the activation of kinases.

Expression for Akt1 and Akt2 is present at high levels in the brain during development, but gradually is decreased during postnatal periods [159]. Following stimulation by agents such as trophic factors or cytokines, PI 3-K is recruited to the plasma membrane, phosphorylates glycerophospholipid phosphatidylinositol 4,5-bisphosphate, and results in the production of phosphatidylinositol 3,4-bisphosphate (PIP₂) and phosphatidylinositol 3,4,5-trisphosphate (PIP₃). Akt then translocates from the cytosol to the cell membrane after its binding to PIP₂ and PIP₃ through their respective PH domains, and subsequently becomes activated through phosphorylation by phosphoinositide-dependent kinase 1 [160].

Once activated, Akt can provide protection against cellular injury. Maximal activity of Akt is achieved through phosphorylation by phosphoinositide-dependent kinase 1 at Ser⁴⁷³ to confer protection against genomic DNA degradation [69, 161, 162] and membrane PS exposure [69, 102, 106]. During a number of injury paradigms, such as toxic insults involving excitotoxicity [163], free radical exposure [106, 121, 164], neuronal axotomy [165], hypoxia [69], or trauma [166], Akt is phosphorylated leading to increased activity and protection against apoptosis induction.

EPO elicits phosphorylation of Akt and this is dependent upon the activation of PI 3-K and Jak2 [156]. The phosphorylation of two residues, Thr³⁰⁸ and Ser⁴⁷³, is con-

sidered necessary for the activation of Akt [167]. Activation of Jak2 promotes the phosphorylation of tyrosine residues in the intracellular portion of the EPOR [147]. Phosphorylation of the last tyrosine of the EPOR initiates binding of the 85-kDa regulatory subunit of PI 3-K, a heterodimer consisting of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit. As a result of the binding of the 85-kDa regulatory subunit, the 110-kDa catalytic subunit becomes active and leads to the phosphorylation of Akt [168].

Central to the ability of EPO to prevent cellular apoptosis is the activation of Akt by EPO. In primary human erythroid progenitors, apoptosis is blocked through the activation of Akt [169]. During anoxia or free radical exposure, expression of the active form of Akt (phospho-Akt) is increased [102, 107]. EPO can significantly enhance the activity of Akt during oxidative stress and prevent inflammatory activation of microglia [27, 106, 118]. This upregulation of Akt activity during injury paradigms appears to be vital for EPO protection, since prevention of Akt phosphorylation blocks cellular protection by EPO [27, 106, 118]. Through the regulation of the PI 3-K/Akt-dependent pathway, EPO can prevent cellular apoptosis following N-methyl-D-aspartate toxicity [170], neuronal axotomy [165], hypoxia [69], and oxidative stress [27, 106, 118].

The Forkhead Transcription Factor, GSK-3 β , Bad, Bcl-x_L, and NF- κ B Are Essential for EPO to Afford Cellular Protection

The forkhead transcription factor (FOXO3a, FKHL1) represents one cellular pathway that is centrally controlled by Akt. Activation of FOXO3a can result in apoptotic cellular degeneration in a transcription-dependent manner following its translocation to the nucleus [171–173]. FOXO3a activation has been demonstrated to disrupt mitochondrial membrane permeability ($\Delta\Psi_m$) and may result in cytochrome c release [174]. Akt inhibition of FOXO3a requires its phosphorylation that results in the association of FOXO3a with 14-3-3 protein and retention of FOXO3a in the cytoplasm, rendering it ineffective in regulating nuclear genes, resulting in the blockade of apoptosis. During periods of oxidative stress in the nervous system, an initial inhibitory phosphorylation of FOXO3a at the regulatory phosphorylation sites (Thr³² and Ser²⁵³) can occur [173, 175, 176]. However, loss of phosphorylated FOXO3a expression appears to subsequently result over a 12-hour period, possibly by caspase degradation, which potentially can enhance the vulnerability of cells to apoptotic injury [176]. EPO may function

to prevent apoptotic injury, possibly through an Akt-dependent mechanism, by preventing the association of FOXO3a with the transcriptional coactivator p300 that would normally lead to the induction of apoptosis [177].

Akt also regulates the activity of GSK-3 β , a serine/threonine kinase. Although phosphorylation of GSK-3 β at Ser⁹ by Akt results in its inactivation, it is important to note that phosphorylation of GSK-3 β at Thr²¹⁶ results in an enhanced activity of the enzyme, which can occur during neuronal degeneration [178]. GSK-3 β plays a significant role in the regulation of apoptosis in several cell types, such as neurons [179], vascular smooth muscle cells [180], and cardiomyocytes [181]. For example, GSK-3 β has been shown to be involved with the neurotoxicity of A β during Alzheimer's disease and its expression coincides with the development of neurofibrillary changes [182]. GSK-3 β also can regulate amyloid precursor protein (APP) processing and the phosphorylation of tau [131, 135]. GSK-3 β facilitates A β release by increasing the cellular maturation of APP [183], a process believed to occur during the early onset of Alzheimer's disease [184]. Although overexpression of GSK-3 β can trigger apoptosis possibly through the transcription factors cAMP response element binding protein, c-myc, c-jun, and β -catenin, inhibition of GSK-3 β activity by Akt can prevent cell injury. In regard to EPO, GSK-3 β activity is suppressed by EPO and may be associated with a conformational change in Bax to prevent cell death [185].

Akt can inactivate Bad, a proapoptotic Bcl-2 family member, through phosphorylation of its serine residues. Bad is a Bcl-2 homology 3 (BH3)-only subfamily member of Bcl-2 proteins that are associated with the regulation of apoptosis. Three phosphorylated serine sites have been identified on Bad, including Ser¹¹², Ser¹³⁶, and Ser¹⁵⁵. Akt preferentially phosphorylates the residue Ser¹³⁶ of Bad [186]. A fourth phosphorylation site of Bad has recently been identified at Ser¹⁷⁰ that also results in the blockade of proapoptotic activity of Bad [187]. The endogenous dephosphorylated Bad is localized in the outer mitochondrial membrane and binds to the antiapoptotic Bcl-2 family member Bcl-x_L through its BH3 domain. Subsequent phosphorylation of Bad by Akt leads to the binding of Bad with the cytosolic protein 14-3-3 to release Bcl-x_L and allow it to block apoptosis. Bcl-2 and Bcl-x_L prevent Bax translocation to the mitochondria, maintain the potential of the mitochondrial membrane, and prevent the release of cytochrome c from the mitochondria [188].

EPO is linked to Bad and the Bcl-2 family through Bcl-x_L. In erythroid cells, the Bcl-2 member Bcl-x_L has been shown to be strongly expressed and necessary for EPO to

prevent apoptosis in the later stages of erythroid progenitor cell life [189]. In addition, expression of Bcl-2 and Bcl-x_L has been demonstrated to be dependent upon EPO. For example, in a murine erythroid progenitor cell line, EPO can specifically maintain the expression of Bcl-2 and Bcl-x_L [190]. EPO also is able to maintain the expression of Bcl-2 and Bcl-x_L and alter the Bcl/Bax ratio towards a net 'antiapoptotic' effect, thereby preventing cellular injury [191]. EPO may require Bcl-x_L expression for cytoprotection, since without EPO, Bcl-x_L is not expressed and apoptotic cell death results in hematopoietic cells [190]. Similar results in neurons and ECs illustrate that upregulation of Bcl-x_L by EPO may be necessary for the prevention of apoptosis [27] in combination with the modulation of Apaf-1 expression and cytochrome c release, similar to other 'antiapoptotic' proteins, such as heat shock proteins [192–194].

Expression and cytoprotection of EPO also are dependent, in part, upon Akt and the activation of NF- κ B. The induction of several antiapoptotic genes by NF- κ B accounts for its cytoprotective action. NF- κ B has been shown to induce the expression of the inhibitors of apoptotic protein (IAPs) c-IAP1, c-IAP2, and x-chromosome-linked IAP. IAPs can specifically inhibit active forms of caspase 3, 7 and 9 [195]. Induction of IAP1 and c-IAP2 by NF- κ B also suppresses TNF- α initiated apoptosis through the inhibition of caspase 8 activation [196]. In addition, xIAP activation by NF- κ B is associated with the downregulation of JNK [197]. Growth arrest and DNA damage protein 45 (Gadd45 β) also has been identified as another downstream target of NF- κ B. Gadd45 β is a member of the Gadd45 family associated with cell cycle and DNA repair [198]. The induction of Gadd45 β protein by TNF- α is NF- κ B dependent and responsible for the downregulation of JNK activation and suppression of apoptosis [199]. NF- κ B also may prevent apoptosis through the direct activation of Bcl-x_L [200].

NF- κ B plays a key role in the induction of EPO during HIF-1 induction. Akt can significantly increase NF- κ B and HIF-1 activation resulting in the enhancement of EPO expression [201]. Through a regulatory loop, EPO also can promote I κ B kinase (IKK) activity, resulting in the degradation of I κ B and the subsequent liberation of NF- κ B. EPO may require NF- κ B activation to foster the production of neural stem cells [52] and prevent neuronal apoptosis [202].

Modulation of Mitochondrial Membrane Permeability through EPO

Cytoprotection by EPO is closely related to the maintenance of mitochondrial membrane potential ($\Delta\Psi_m$). Loss of $\Delta\Psi_m$ through the opening of the mitochondrial permeability transition pore represents a significant determinant for cell injury and the subsequent induction of apoptosis [27, 203, 204]. Studies that involve free radical injury, anoxia, or oxygen-glucose deprivation demonstrate that EPO prevents the depolarization of the mitochondrial membrane by maintaining $\Delta\Psi_m$ and preventing the release of cytochrome c [69, 102, 205]. Yet, EPO also may maintain $\Delta\Psi_m$ by activating Akt and regulating caspase-mediated pathways. EPO appears to employ Akt to prevent apoptosis at either a pre- or a postmitochondrial level. At a premitochondrial level, Akt can directly prevent the activation of caspase 9 [20, 27]. At a postmitochondrial level, Akt can inhibit the release of cytochrome c from mitochondria and block both caspase 9 and caspase 3 activity [205]. Feedback of these systems also can exist that modulates the half-life of Akt. Activity of Akt can be eliminated by caspase 3 induction, since caspase 3 has been shown to cleave Akt leading to the inhibition of Akt kinase activity [206].

EPO Can Oversee the Execution of Cells through Modulation of Caspase Activity

Intimately associated with the disruption in $\Delta\Psi_m$ during neuronal and vascular injury is the induction of caspase activity. Caspases are a family of cysteine proteases that cleave their substrates after aspartic residues. They are usually synthesized as inactive zymogens that are proteolytically cleaved into subunits at the onset of apoptosis and function as active caspases after reconstitution to molecular heterodimers. Caspases are composed of three domains including an N-terminal prodomain, a large subunit, and a small subunit [207]. As a result of their activation sequence, caspases are classified as either initiator caspases (also known as apical caspases) or effector caspases [208]. An initiator caspase cleaves and subsequently activates an effector caspase. The apoptotic-associated caspases include initiator caspases, such as caspase 2, 8, 9 and 10, that activate downstream effector caspases, resulting in an amplification of cascade activity. The initiator caspases consist of long N-terminal prodomains that contain caspase recruitment domains (CARDs) in caspase 2 and caspase 9, or death effector domains (DEDs) in caspase 8 and caspase 10 [209]. The effector caspases consist of caspase 3, 6 and 7 that function to directly cleave crucial cellular protein substrates that result in cell destruc-

tion. The effector caspases contain short prodomains or have no prodomains.

Activation of caspases proceeds through extrinsic and intrinsic pathways. The extrinsic pathway is initiated by death receptor activation at the cell surface, resulting in the recruitment and activation of the initiator caspase 8 upon apoptotic stimuli [210]. The intracellular death domain of death receptors, such as the TNF superfamily, CD95/Fas/Apo-1, and the death receptor 3, undergoes conformational change upon binding to extracellular ligands and forms an intracellular death-inducing signaling complex following recruitment of adaptor molecules, such as the Fas-associated death domain (FADD). FADD recruits caspase 8 through its DED domain and this leads to caspase 8 activation [211, 212]. Caspase 8 can subsequently activate caspase 3. In addition, caspase 8 activation also may result in the cleavage of Bid, a proapoptotic member of Bcl-2 family, allowing the truncated Bid (tBid) to translocate to the mitochondria [213]. This leads to cytochrome c release through Bax resulting in the subsequent activation of executioner caspases [214].

The intrinsic caspase pathway involves mitochondrial dysfunction. The mitochondrial pathway is associated with the release of cytochrome c and subsequent activation of caspase 9 followed by activation of caspase 3 [215]. The process is regulated by the Bcl-2 subfamily BH3-only proteins, which are normally located in cellular compartments other than mitochondria, but translocate to the mitochondria in response to apoptotic stimuli [216]. The translocation of these proteins delivers an apoptotic signal to mitochondria through the interaction with Bax to induce the release of cytochrome c that then binds to Apaf-1. Apaf-1 consists of three different domains that include CARDs, repeats of tryptophan and aspartate residues (WD-40 repeats), and a nucleotide-binding domain CED-4. Binding of cytochrome c to Apaf-1 results in the removal of the WD-40 domain, masking the CED-4 and CARDs, and leads to the oligomerization of Apaf-1 with the requirement of dATP/ATP [217]. The oligomerization of Apaf-1 promotes the allosteric activation of caspase 9 by forming the Apaf-1 apoptosome [218]. Caspase 9 can subsequently activate caspase 3 [218] as well as caspase 1 through the intermediary caspase 8 [219]. Together, caspase 1 and caspase 3 lead to both DNA fragmentation and membrane PS exposure [69, 101, 218].

Modulation of caspase activation by EPO may offer several avenues for protection against cell injury. The ability of EPO to prevent specific caspase 1- and caspase 3-like activities appears to play a significant role in its cellular protection [27, 69, 88]. The caspases 1 and 3 have

each been linked to the independent apoptotic pathways of genomic DNA cleavage and cellular membrane PS exposure [27, 118, 219]. With respect to caspase 1, EPO prevents PS externalization primarily through the inhibition of caspase 1-like activity and, to a lesser degree, through other caspases such as 3, 8 and 9 [27, 69, 106, 118]. These caspases are also tied to the direct activation and proliferation of microglia [102, 106, 107]. Caspase 1 is believed to be principally responsible for the externalization of membrane PS residues in several cell systems that can subsequently activate microglial phagocytosis [101, 220].

EPO may prevent genomic DNA degradation through the inhibition of cytochrome c and the subsequent blockade of caspase 3-like activity [106]. Modulation of caspase 3-like activity by EPO also may be linked to a unique regulatory mechanism that blocks the proteolytic degradation of phosphorylated FOXO3a by caspase 3. Given that FOXO3a has been shown to be a substrate for caspase 3-like proteases at the consensus sequence DELD³⁰⁴A [221], current work demonstrates that blockade of caspase 3-like activity prevents the destruction of phosphorylated FOXO3a during oxidative stress [176]. In light of the ability of EPO to directly inhibit caspase 3-like activity [20, 69, 118] and maintain inhibitory phosphorylation of FOXO3a [177], EPO may control FOXO3a through caspase 3.

In addition to preventing the activation of caspase 1 and caspase 3, EPO also can prevent caspase 8 and caspase 9-like activities [27, 118]. Caspase 9 is activated through a process that involves the cytochrome c-Apaf-1 complex [62, 218]. Interestingly, EPO can prevent cell injury by inhibiting caspase 9-like activity either through Apaf-1 or directly [27]. In addition, caspase 8 serves as an upstream initiator of executioner caspases, such as caspase 3, and also leads to the mitochondrial release of cytochrome c [222, 223]. Following caspase 8 and caspase 9 activation, caspase 3 directly leads to genomic DNA degradation. EPO prevents cellular apoptosis through parallel pathways that prevent the induction of Apaf-1 and preserve mitochondrial membrane potential in conjunction with enhanced Bcl-x_L expression [27]. Consistent with the modulation of Apaf-1 and the release of cytochrome c, EPO inhibits the activation of caspase 9 and caspase 3-like activities [27]. Therefore, EPO functions at both intrinsic and extrinsic pathways to prevent caspase activation and promote cellular integrity with maintenance of membrane PS asymmetry.

Phosphorylation of the Mitogen-Activated Protein Kinases by EPO

The mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that include the extracellular signal-related kinases (ERKs), the c-Jun amino-terminal kinases (JNKs), and p38 MAP kinase (p38). These kinases usually represent the terminal stages of signaling cascades that are activated by survival factors or death promoting receptors. Following activation, the MAPKs translocate to the cell nucleus to activate transcription activity and modulate cell differentiation, growth, or death. The ERK pathway is primarily associated with the regulation of apoptosis and can be cytoprotective, especially through activity of the ERK1/2 pathway [224, 225]. JNK and p38 activation has been associated with the induction of apoptosis during growth factor deprivation [226] and inflammatory cytokine application [227]. In neuronal and EC cultures subjected to oxidative stress, phosphorylation of p38 and JNK has been linked to apoptotic injury [20, 228, 229].

The phosphorylation of MAPKs may contribute to the cytoprotection offered by EPO. EPO can phosphorylate ERKs [230] and increase the activity of JNK and p38 [231]. This enhancement of JNK and p38 activity is believed to mediate both erythroid proliferation and differentiation [232]. Yet, modulation of JNK and p38 activity by EPO may be cell specific. In several cell systems during toxic insults, protection by EPO does not appear to require the phosphorylation of p38 or JNK, suggesting that cellular protection by EPO against apoptosis may be independent of the modulation of p38 and JNK activity [204, 231, 233].

EPO Is Closely Tied to PKC and Cellular Calcium Homeostasis

The PKC family contains eleven PKC isoenzymes that consist of three groups that can modulate apoptosis. The classical (PKC- α , β _I, β _{II}, and γ) and atypical (PKC- ζ , μ , ι/λ) groups are associated with the inhibition of apoptosis and cell survival. In contrast, the novel PKC isoenzymes (δ , η , ϵ and θ) are proapoptotic in function [234, 235]. The classical or 'calcium-dependent' isoenzymes require calcium for activation, while the novel and atypical types are calcium independent. PKC- α is the best-characterized antiapoptotic isoenzyme and has been shown to exert its apoptosis-inhibiting effects through the phosphorylation of Bcl-2 [236].

PKC is involved in the signal transduction pathways of EPO. PKC regulates erythroid proliferation and differentiation that is initiated by EPO. PKC- α has been identified as the only calcium-dependent PKC subtype ex-

pressed in erythroid progenitors. The PKC- α isoform also functions in mediating EPO-induced erythroid differentiation of CD34(+) progenitor cells [237]. Furthermore, inhibition of PKC activity also interferes with the phosphorylation of the EPOR, suggesting that PKC may function as an upstream modulator of the EPOR [238]. Investigations in myocardial ischemia further suggest that PKC is a necessary component for protection in the cardiovascular system since recovery of left and right ventricular developed pressure during EPO administration is abrogated by inhibition of PKC activity [239].

Given that the classical PKC isoenzymes are calcium dependent for activity, it is reasonable to assume that EPO may be involved in the modulation of cellular calcium homeostasis. Early work has demonstrated that EPO can elicit a rapid increase in intracellular free calcium in human bone marrow mononuclear cells [240]. Recent patch-clamp studies have shown that EPO can stimulate the activity of T-type voltage-dependent calcium channels [241]. EPO also can directly enhance intracellular calcium and monoamine concentrations in pheochromocytoma neuronal cell lines [242]. This work suggests that EPO can influence calcium homeostasis through either an increase in calcium influx via plasma membrane T-type voltage-dependent calcium channels or through extracellular sources [123, 243]. A rapid transient increase in $[Ca^{2+}]_i$ concentration by EPO may be necessary for cytoprotection during excitotoxicity, since a decrease in intracellular calcium can negate any protection by EPO during glutamate administration [123]. EPO may promote neuronal viability by enhancing the cytosolic concentration of free calcium and inducing cell membrane depolarization as a result of the activation of calcium channels [242, 244]. This increased function and viability of neurons through a potential calcium mechanism by EPO has been suggested to be linked to enhanced NO generation [244]. Such observations concerning a potential synergistic function for cellular protection that require $[Ca^{2+}]_i$ are not unique and have been reported with other cellular receptor systems, such as those that involve G protein-related pathways [140, 245].

EPO and Its Future for Clinical Medicine

Considerations of Clinical Efficacy, Safety, and Toxicity for EPO

Given its ability to foster cell survival in multiple biological systems, EPO should be considered as a novel clinical agent ripe for the development as robust and effica-

cious therapy for an array of disorders. Cytoprotective agents that can provide both safe and efficacious treatment would be considered extremely attractive, especially in the neuroscience and cardiovascular arenas. In this light, EPO appears to fill such a role as a nontoxic cytoprotective agent. Years of clinical administration in the patients with anemia and chronic kidney diseases have shown EPO to be well tolerated and safe [1]. More recently, one clinical trial has demonstrated safety for EPO in a limited number of patients with acute ischemic stroke and additional trials will seek to examine the role of EPO during neonatal ischemia and psychiatric disorders [20, 21].

Yet, the clinical application of many cytoprotective agents has been hampered by subsequent evidence of clinical toxicity and unfortunately EPO is not exempt from this issue. Adverse effects in patients receiving EPO for approved indications have been reported. Usually, toxic effects occurring after EPO administration are related to associated disease states, such as chronic renal failure, cancer, human immunodeficiency viral infection, or sequela following surgery [246]. Yet, both acute and long-term administration of EPO can precipitate hypertensive emergencies. As a result, the use of EPO in patients with uncontrolled hypertension is contraindicated [247].

Several mechanisms have been proposed to account for the elevation in vascular resistance and the subsequent development of high blood pressure during EPO administration. Early studies recognized that increased blood viscosity as a result of rising hematocrit values contributed to high blood pressure during chronic treatment with EPO [248]. The correction of anemia by EPO resulted in an increase in erythrocyte mass and blood viscosity [249] and the reversal of hypoxic vasodilation in uremic anemia [250]. Further studies demonstrated that constant dosage and chronic administration of EPO in iron-deficient renal anemic patients did not increase blood pressure despite a dramatic increase in hematocrit by iron repletion [251]. Thus, EPO can lead to hypertension during chronic treatment that appears to be independent of hematocrit levels.

Changes in vasoactive substances also may contribute to the hypertensive effect of EPO. Treatment with EPO enhances vascular responsiveness to norepinephrine in renal failure [252] without an alteration of plasma catecholamine levels [253]. Further experiments that demonstrate an increase in $[Ca^{2+}]_i$ in vascular smooth muscle during EPO administration suggest that calcium mobilization also may contribute to the hypertension associated with rhEPO treatment [254]. In addition, EPO may impair the balance between vasodilatory prostaglandin

and vasoconstrictive components by reducing prostacyclin production and increasing the formation of prostaglandin $F_{2\alpha}$ and thromboxane B_2 [255].

Enhanced NO production may serve to counteract the hypertension associated with EPO administration. An increase in NO production together with hypertension has been observed during EPO treatment in rats, suggesting that impairment of endogenous NO activity is not responsible for EPO-associated hypertension. More likely, endogenous NO activity may function as a mechanism that limits the hypertensive effect of EPO [256]. This hypothesis is supported by the observation that an NO synthase inhibitor can abolish renal vasodilation and result in hypertension during treatment with EPO in rats [257]. Other reports illustrate that EPO therapy can impair the vasodilatory response to NO donor sodium nitroprusside and S-nitroso-N-acetyl-D,L-penicillamine in chronic renal failure in rats [258]. Investigations into the mechanisms underlying vasodilatory resistance to NO found that treatment with EPO raised resting $[Ca^{2+}]_i$ in rats with chronic renal failure [259]. The concurrent administration of the calcium channel blocker felodipine normalized resting $[Ca^{2+}]_i$, increased NO production, and abolished hypertension induced by EPO. These results suggest that an elevation of $[Ca^{2+}]_i$ by EPO may explain an impaired vasodilatory response during the presence of NO.

Depending on the specific disease entity, administration of EPO can lead to other disabilities. EPO can result in the formation of anti-EPO antibodies, red cell aplasia [142], and decrease the expression of the EPOR on the cell surface [143], setting the stage for a vicious cycle of increased administration of EPO with the subsequent occurrence of increased toxic side effects. Maintenance treatment with EPO also has been associated with nonfatal myocardial infarction, vascular thrombosis, pyrexia, vomiting, shortness of breath, paresthesias, and upper respiratory tract infection [246].

In regard to nervous system and cardiovascular applications, development of toxic side effects during EPO therapy, such as for cerebral ischemia [260], could severely limit or halt the use of EPO for diseases of the nervous system. Therefore, strategies have been suggested to develop derivations of EPO, such as asialo-EPO and neurotrophic sequences of EPO [230], to remove erythropoietic activity and potential toxicity. Yet, present work on such approaches suggests that derivatives of EPO may possess only limited utility [261] and lack the ability to promote angiogenesis which could be critical for cytoprotection, prompting the need for further investigations [25, 35, 69].

EPO: Clinical Development on a Tightrope

Current investigations have suggested a new therapeutic potential for EPO as a cellular protectant and an anti-inflammatory mediator, rather than as an agent necessary only for the induction of erythropoiesis. EPO can prevent the loss of neurons, ECs, and cardiomyocytes and promote their functional recovery in acute and chronic diseases. As a cytoprotective agent, EPO may have applicability in a host of disorders that range from cerebral ischemia, Alzheimer's disease, Parkinson's disease, psychiatric disorders, and diabetic neuropathies [20, 21, 262] to disorders that impact upon cardiac function such as acute myocardial ischemia and chronic congestive heart failure [87].

Initially described as hematopoietic growth factor, but now considered to interface with a variety of biological cell functions, EPO modulates an array of vital cellular functions that involve progenitor stem cell development, cellular protection, angiogenesis, DNA repair, and cellular longevity. Cellular protection offered by EPO through the preservation of membrane PS asymmetry alters acute cellular injury as well as secondary thrombosis, clot formation, and inflammation.

EPO fosters cellular function and survival through a series of distinct pathways that involve Jak2, STATs, and the serine-threonine kinase Akt with its downstream substrates of FOXO3a, GSK-3 β , Bad, Bcl-x_L, and NF- κ B. Particularly attractive is the ability of EPO to use the Akt pathway for protection of cells from inflammatory injury

through the direct modulation of cellular membrane PS externalization. Intimately associated with the protective ability of EPO is the maintenance of $\Delta\Psi_m$, the central modulation of Bax, and cytochrome c release. Ultimately, EPO is able to govern Apaf-1 as well as extrinsic and intrinsic caspase pathways to preserve genomic integrity and prevent early apoptotic loss of membrane asymmetry that can lead to microglial disposal.

New investigations must uncover the cellular mechanisms that determine whether EPO can fulfill its potential as a broad and safe cytoprotectant for multiple cell systems or ultimately leads to disability, such as with hypertensive complications or autoimmune toxicity. It is clear that the development of EPO for clinical applications rests on 'tightrope' that must be carefully negotiated with a fine balance that maximizes treatment efficacy and minimizes toxic outcomes. As our knowledge of EPO becomes more refined, we should be able to appreciate in greater depth the role that EPO plays during the development, maturation, and survival of cells not only in the nervous system, but throughout the entire body.

Acknowledgments

This research was supported by the following grants (KM): American Heart Association (National), Janssen Neuroscience Award, Johnson and Johnson Focused Investigator Award, LEARN Foundation Award, MI Life Sciences Challenge Award, and NIH NIEHS (P30 ES06639).

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