Chapter 2

Epo and Non-hematopoietic Cells: What Do We Know?

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Abstract

The hematopoietic growth factor erythropoietin (Epo) circulates in plasma and controls the oxygen carrying capacity of the blood (Fisher. Exp Biol Med (Maywood) 228:1–14, 2003). Epo is produced primarily in the adult kidney and fetal liver and was originally believed to play a role restricted to stimulation of early erythroid precursor proliferation, inhibition of apoptosis, and differentiation of the erythroid lineage. Early studies showed that mice with targeted deletion of Epo or the Epo receptor (EpoR) show impaired erythropoiesis, lack mature erythrocytes, and die in utero around embryonic day 13.5 (Wu et al. Cell 83:59–67, 1995; Lin et al. Genes Dev. 10:154–164, 1996). These animals also exhibited heart defects, abnormal vascular development as well as increased apoptosis in the brain suggesting additional functions for Epo signaling in normal development of the central nervous system and heart. Now, in addition to its well-known role in erythropoiesis, a diverse array of cells have been identified that produce Epo and/or express the Epo-R including endothelial cells, smooth muscle cells, and cells of the central nervous system (Masuda et al. J Biol Chem. 269:19488–19493, 1994; Marti et al. Eur J Neurosci. 8:666–676, 1996; Bernaudin et al. J Cereb Blood Flow Metab. 19:643–651, 1999; Li et al. Neurochem Res. 32:2132–2141, 2007). Endogenously produced Epo and/or expression of the EpoR gives rise to autocrine and paracrine signaling in different organs particularly during hypoxia, toxicity, and injury conditions. Epo has been shown to regulate a variety of cell functions such as calcium transport (Korbel et al. J Comp Physiol B. 174:121–128, 2004) neurotransmitter synthesis and cell survival (Velly et al. Pharmacol Ther. 128:445–459, 2010; Vogel et al. Blood. 102:2278–2284, 2003). Furthermore Epo has neurotrophic effects (Grimm et al. Nat Med. 8:718–724, 2002; Junk et al. Proc Natl Acad Sci U S A. 99:10659–10664, 2002), can induce an angiogenic phenotype in cultured endothelial cells, is a potent angiogenic factor in vivo (Ribatti et al. Eur J Clin Invest. 33:891–896, 2003) and might enhance ventilation in hypoxic conditions (Soliz et al. J Physiol. 568:559–571, 2005; Soliz et al. J Physiol. 583, 329–336, 2007). Thus multiple functions have been identified breathing new life and exciting possibilities into what is really an old growth factor.

This review will address the function of Epo in non-hematopoietic tissues with significant emphasis on the brain and heart.

Key words Non-hematopoietic cells, Adult kidney, Fetal liver, HIF

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1 Epo Expression Is Regulated by Hypoxia-Inducible Factors

Epo expression is hypoxia inducible and regulation occurs via the hypoxia responsive element (HRE) present in the 3′ region of the gene which is bound by heterodimeric transcription factors namely hypoxia-inducible factors (HIFs). Three members of the HIF transcription factor family HIF-1, -2, and -3 have now been identified. HIF-1 was discovered in 1991 by its ability to bind and stimulate transcription of the Epo gene during hypoxia (16, 17) and for several years, was assumed to be the primary stimulus for Epo production in response to acute hypoxia. Later a second hypoxia-inducible transcription factor termed HIF-2 was discovered (18–20). Subsequent data from in vivo (21) and in vitro (22) experiments suggested that despite the fact that HIF-1 clearly binds the HRE of the Epo gene in response to hypoxia and both have the potential to bind many of the same genes, in vivo HIF-2 is the primary mediator of Epo expression in kidneys in response to hypoxia. In agreement downregulation of HIF-2 in the brain, but not HIF-1, drastically reduced hypoxia-induced Epo expression (23) and more recently Haase and colleagues (24) clearly demonstrated the primary role of HIF-2 in promoting the hypoxic renal Epo response.

The HIFs are heterodimers composed of a constitutively expressed β subunit (also known as aryl hydrocarbon receptor nuclear translocation, ARNT) and an oxygen-regulated α subunit (reviewed by ref. 25–27). Regulation of HIF activity occurs at different levels including protein stability, phosphorylation, nuclear translocation, and activity, all being influenced by alterations in oxygen levels. Under normoxic conditions the α subunit is degraded. In contrast, under hypoxic conditions the α subunit is stabilized and translocated to the nucleus where it dimerizes with ARNT and subsequently binds to hypoxic binding sites (HBS) of target genes. The HBS is a conserved consensus sequence (A/G) CGTG within the HRE present in oxygen-regulated target genes involved in cell survival, glycolysis, angiogenesis, erythropoiesis, and iron metabolism (25). Degradation of HIF-α is triggered by oxygen-dependent hydroxylation of prolyl residues located in the oxygen-dependent degradation domain by a family of prolyl hydroxylases, namely PHD1, PHD2, and PHD3. These enzymes are specific HIF prolyl hydroxylases that require Fe(II) as a cofactor as well as oxygen and 2-oxoglutarate as co-substrates (28, 29). Prolyl hydroxylation promotes the recruitment of the tumor suppressor protein von Hippel Lindau, which is part of the E3 ligase ubiquitination complex, priming HIFs for degradation in the proteosomes (reviewed by ref. 30, 31).

Other regulatory elements in the 5′ promoter of the Epo gene include a highly conserved GATA sequence as well as NFκB binding motifs (32, 33). Both these sites seem to have inhibitory

effects on Epo expression. The GATA site preferentially binds the transcription factor GATA-2, which has been reported to inhibit Epo gene expression (34, 35). NFκB binding to a site adjacent to the minimal HRE of the Epo promoter also inhibits Epo expression. Although activities of GATA-2 and NFκB in HepG2 cells decrease in hypoxia compared to normoxia conditions both transcription factors were shown to be involved in the suppression of Epo gene expression by IL-1β and TNFα (35). Thus these pathways may be responsible for impaired Epo synthesis in a variety of inflammatory diseases and cancers.

2 EpoR Is Expressed in Multiple Tissues

Hypoxia and anemia are major events known to induce Epo gene expression, however it should be noted that many different injuries induce Epo expression (36, 37). Once the signals are transduced erythropoietin is released into the circulating blood flow and finally binds cells expressing the Epo receptor (EpoR).

The EpoR is a member of the type 1 superfamily of single-transmembrane cytokine receptors (38, 39). Expression of the EpoR is located in progenitor cells from hematopoietic, endothelial, skeletal muscle, and neuronal compartments (40–42). EpoR is downregulated during differentiation of erythroid cells and not expressed on skeletal muscle. Interestingly, despite being significantly downregulated in developing neuronal tissues until embryonic day 17, EpoR expression persists in select vascular and neuronal compartments. EpoR has been observed in brain during development and adulthood (37, 43–46). More recent studies have demonstrated expression of EpoR on cells from a variety of tissues including heart (47), kidney (48), pancreas (49), and uterus (50).

3 Classical Erythroid EpoR Signaling

Erythropoiesis is stimulated by generating a complex network of molecular signals involved in the control of cell proliferation, differentiation, and death. EpoR homodimers are expressed on the erythroid progenitor cell surface (51) and binding of Epo to the EpoR triggers conformational changes in the receptor extracellular domain that consequently activates JAK2 by autophosphorylation (52, 53). JAK2 activation results in the phosphorylation of tyrosine residues on the cytoplasmic region of EpoR and recruits a variety of Src homology-2 (SH2) domain-containing proteins that initiate downstream cascades via different signaling pathways including signal transducer and activator of transcription (STAT), phosphatidylinositol-3 kinase (PI3K)/Akt (also known as protein kinase B) and mitogen-activated

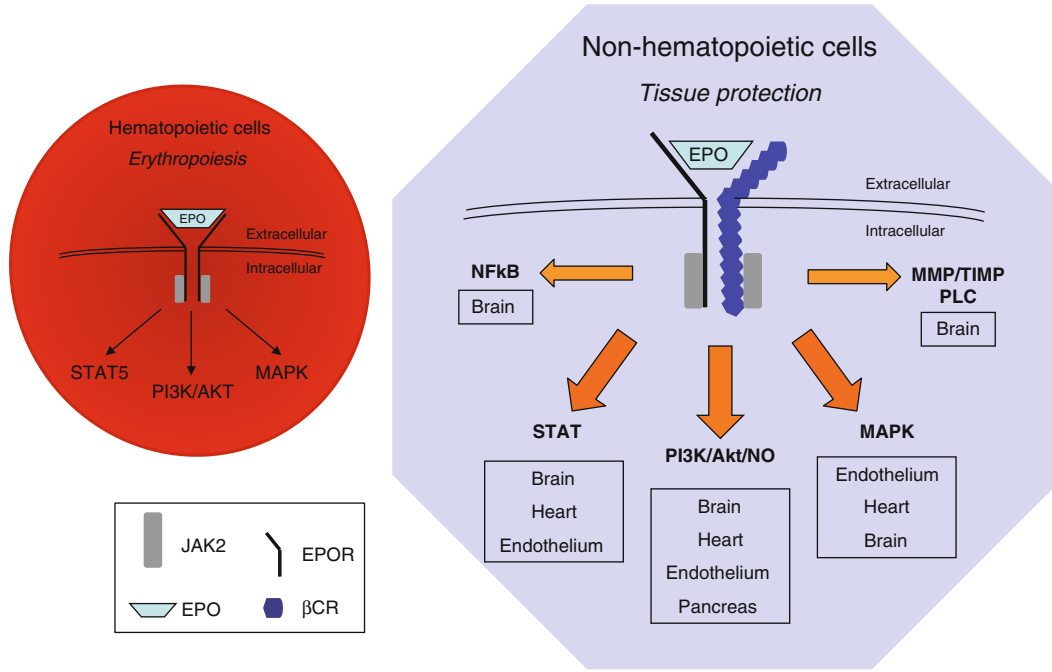

Fig. 1 Downstream pathways activated by Epo signaling in hematopoietic and non-hematopoietic cells. In non-hematopoietic cells the βCR subunit makes a functional receptor with a classic EpoR. In the absence of βCR it is postulated that the homodimer configuration will occur. Note the similarities of the downstream pathways activated by both hetero- and homodimers

protein kinase (MAPK) (54, 55). Although Epo can activate STAT1, STAT3, and STAT5a/b, JAK2/STAT5 is the classical pathway activated in erythroid cells (summarized in Fig. 1 and reviewed in ref. 56). Epo-mediated activation of this pathway leads to the upregulation of the antiapoptotic Bcl2 and Bcl-X_L gene, thereby protecting precursors from apoptosis (56, 57).

The PI3K/Akt pathway has been shown to be necessary, but not solely sufficient, for erythroid cell survival by protecting them from apoptosis (58). The PI3K/Akt cascade phosphorylates serine residue 310 of GATA-1 both in vitro and in erythroid cells thereby enhancing GATA-1 transcriptional activity (55). GATA-1 binds to a consensus GATA motif present in the *cis*-regulatory elements of most erythroid genes and is a key transcription factor for antiapoptotic Bcl-X_L and erythroid-specific gene transcription, and terminal differentiation of erythroid precursors into red blood cells (59–61). Notably PI3K can also be indirectly recruited to EpoR by other proteins such as Grb-2. PI3K-mediated Akt phosphorylation inhibits cytochrome c release from mitochondria (62) and facilitates NFκB activation by enhancing inhibitor of NF-kappaB (IκB) degradation (63). Additionally, Akt can inhibit activity of Foxo3A

thereby downregulating target proteins having antiproliferative or proapoptotic functions (64, 65).

Another important Epo-mediated signaling pathway is the MAPK pathway. MAPKs are serine/threonine kinases activated by extracellular signals of which there are at least three distinct types: the classical ERK1/ERK2 kinases, the p38MAPKs (p38), and the stress-activated protein kinase/Jun kinase (SAPK/JNK) subfamily. All play important roles in Epo-induced differentiation or apoptosis (66–70).

Soon after stimulation of the receptor by its ligand, mechanisms integral to downregulation of these signaling pathways are also activated, returning signaling proteins to their basal levels. This process is crucial to prevent hyperstimulation and, consequently, the dysregulation of cellular machinery (reviewed in ref. 63). Notably, EpoR is also synthesized in a soluble form (sEpoR) that corresponds to the extracellular domain of the complete receptor as a result of alternative splicing of EpoR mRNA (71). The sEpoR is secreted into the extracellular fluid and acts as a sink, sequestering Epo and preventing its ability to activate EpoR and downstream signaling cascades (see Fig. 2, pathway 8). The presence of sEpoR has been reported in plasma and several tissues including liver, spleen, kidney, heart, brain, and bone marrow (15, 72).

4 Epo and EpoR Signaling in Non-hematopoietic Tissues

Production of Epo and expression of the EpoR has been detected in non-hematopoietic tissues and emerging evidence suggests that Epo exerts cytoprotective effects on non-erythroid cells. Notably, a tissue-specific degree of Epo regulation has been reported. Depending on the severity of hypoxia, Epo mRNA levels can increase up to 20-fold in the brain in contrast to 200-fold in the kidney (5) and remain high much longer (73). Also brain Epo, purified from primary neuronal cell cultures, was shown to have lower molecular weight and be more active than recombinant Epo and serum Epo at low concentrations (74). Importantly, tissue protection in vivo and in vitro appears to require nanomolar concentrations of Epo that are not normally reached in the circulation, in contrast to low picomolar concentrations required for erythropoiesis (75) underlining the fact that paracrine/autocrine signaling likely results in high local concentrations of Epo. The EpoR expressed by PC12 cells also had lower affinity than EpoR on erythroid cells and required different accessory proteins compared to erythrocyte precursors (4). Lower binding affinities of EpoR expressed by non-erythroid cells was also reported in vivo (76). Thus, differential activity and affinity allows specific activation of erythroid and non-hematopoietic receptors thus preventing cross-talk between the endocrine and paracrine systems of Epo.

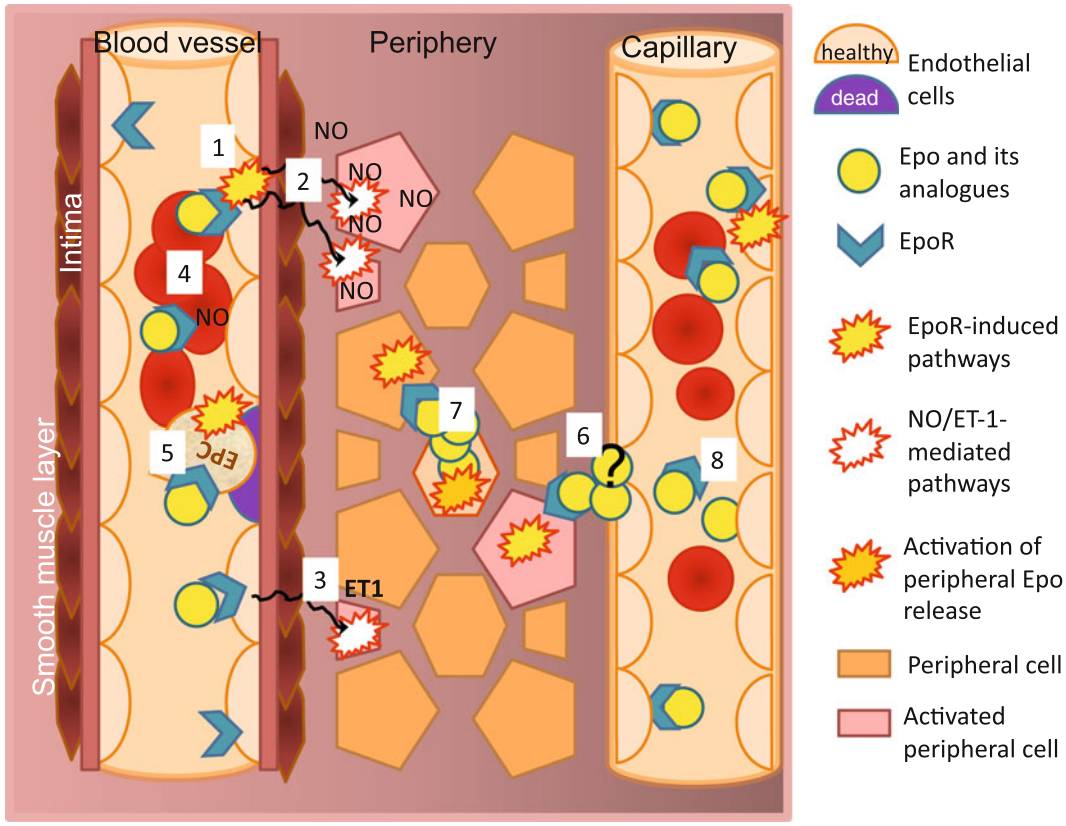

Fig. 2 Schematic representation of the multiple putative cytoprotective effects of Epo in non-erythropoietic tissues. Interaction of blood-borne Epo with heterodimeric Epo receptors on endothelial cells activates the PI3K-Akt pathway (*1*) leading to NO production by eNOS and its translocation to the periphery where it induces cytoprotective effects (*2*). Another second messenger known to be released by endothelial cells upon their stimulation with Epo is endothelin 1 (ET-1) which also elicits protective effects in peripheral cells (*3*). Further targets of circulating Epo are blood cells, including red blood cells and macrophages. Similar to endothelial cells, Epo binding to red blood cells triggers production of NO by eNOS (*4*). Endothelial precursor cells (EPCs) are very sensitive to Epo. Epo controls their number, recruitment to the site of injury, homing, and the quality of resulting mature endothelial cells (*5*). Peripheral cells were shown to respond to Epo stimulation directly. Blood vessels are largely impermeable for Epo when undamaged. However the blood-tissue barrier is less tight in capillaries and although leakage of Epo from the capillary system into the peripheral tissue has never been demonstrated convincingly, it cannot be excluded (*6*). Alternatively, peripheral cells may produce their own Epo. Indeed induction of Epo expression has been demonstrated in hypoxic brain and heart. Thus once produced the cytokine is released causing autocrine and paracrine effects (*7*). Action of Epo is transient and the cytokine is internalized and degraded upon binding to the receptor. Free Epo pools in the plasma may also be regulated by sequestration of the circulating soluble Epo receptor (*8*). For more details of these mechanisms please see main text

The functional EpoR that attenuates tissue damage is not normally, or only weakly, expressed in most tissues and is strongly induced following injury (36, 37). EpoR expression level and the number of receptors per cell is significantly lower than observed in erythropoetic precursor cells and for that reason was reported as

"undetectable" in one publication (77) - an opinion not shared by the majority of researchers working in the field (78). Recent data advocates that the tissue protective non-hematopoietic receptor is distinct from the hematopoietic receptor responsible for erythropoiesis being a heterodimer consisting of the beta common receptor subunit (βCR also known as CD131) in combination with the EpoR subunit (see Fig. 1 and reviewed by ref. 75). A variety of tissues have been found to express βCR and EpoR including the central and peripheral nervous system, retina, heart, kidney, muscle, and endothelium. Notably, the important role of the βCR in Epo-mediated protection has been demonstrated in brain injury models using βCR knockout mice (79, 80) as well as in endothelium using siRNA technology (81). However the downstream signaling mechanisms activated by βCR are still to be elucidated. When EpoR is not colocalized with βCR it presumably self-associates forming the classical EpoR homodimer that also supports signaling (reviewed by ref. 75).

The importance of EpoR specifically in non-hematopoietic tissues has been recently investigated using transgenic mice with EpoR expression restricted to hematopoietic tissues and the vascular endothelium. These mice survive without any gross abnormalities but become obese and insulin resistant due to loss of Epo regulation of energy homeostasis (82). It should be noted however that because endothelial cells have the same origin as hematopoietic cells these mice still express EpoR on vascular endothelium. Recent studies using these mice in heart ischemia–reperfusion injury model (83) and traumatic brain injury model (84) identify the endothelium as a major contributor to Epo-mediated protection and supporter of significant tissue recovery from injury. More experiments are now needed in various injury paradigms to better understand the contribution of the homoreceptor, heterodimer, and the endothelium per se to tissue protection during Epo treatment.

5 Brain

5.1 Endogenous Production of Epo in CNS

Epo and EpoR have been detected during early brain development in rodent models. Both are also expressed during human fetal development starting around 7 weeks and increase from 8 to 24 weeks (43). After birth Epo was detected in human cerebral spinal fluid and found to be induced by hypoxia (5). Notably, Epo and EpoR expression persist in the human brain throughout adulthood.

Mouse models showed that knockout of either gene caused embryonic death not only due to erythropoiesis failure but also as a result of compromised brain development. In these models the neurons exhibited intrinsic defects such as slowed proliferation and increased sensitivity to hypoxic stress (85). Additionally a specific deficit in post-stroke neurogenesis by the impaired migration of

NPC to the peri-infarct cortex was also observed in adult mice stroke models. Thus a clear role for coordinated Epo signaling in early brain development is evident.

5.2 Neuroprotection by Epo In Vitro

Different neural cells express Epo and the EpoR including neurons, astrocytes, and oligodendrocytes (6, 74, 86, 87). Epo appears to be mainly produced by astrocytes (4, 88), while EpoR is expressed by neurons (43). During injury however it seems all cells are capable of upregulating the Epo signaling cascade eliciting both autocrine and paracrine effects (see Fig. 2, pathway 7).

Epo was shown to protect neurons from hypoxic and toxic insults in different cell culture and ex vivo models (see Fig. 2, pathway 6). Epo supplementation counteracted hypoxia-induced cell death in cortical and hippocampal neurons (89–91) and protected PC12 cells from serum withdrawal (92). In toxicity models Epo pretreatment protected hippocampal and cortical neurons from glutamate (93) and NMDA exposure (46), ketamin cytotoxicity (94), kainate-induced excitocytotoxicity in cultured spinal neurons (95), as well as SH-SY5Y neuroblastoma cells from staurosprine-induced cell death (96) to name but a few. Supplementation of Epo also increased neuronal survival during oxygen glucose deprivation, the in vitro model for hypoxic-ischemia (88). Epo has also been suggested to contribute to myelin recovery by enhancing generation, proliferation, and differentiation of oligodendrocytes after ischemic injury (97, 98) and inflammatory injury (99).

Generally Epo protects neuronal cells by regulating the balance between proapoptotic and antiapoptotic pathways. Similar to erythroid cells, a major mechanism occurs through JAK2/STAT activation and induction of PI3K/Akt pathways that inhibit the pro-apoptotic protein Bad and prevent release of cytochrome c and caspase activation (see Fig. 1). Akt activation also inhibits glycogen synthase kinase 3 (GSK3) (94) resulting in inhibition of the mitochondrial permeability transition pore, a major determinant of cell death, through caspase activation. However inhibition of Akt only partially prevented neuroprotection suggesting the contribution of additional signaling mechanisms (89). A unique pathway for Epo-mediated neuroprotection in the brain seems to be induction of crosstalk between JAK2 and NFκB signaling cascades (see Fig. 1). EpoR mediated activation of JAK2 led to phosphorylation of IκB, subsequent nuclear translocation of NFκB, and NFκB-dependent transcription of neuroprotective genes (88, 100). Accordingly transfection of cerebrocortical neurons with a dominant interfering form of JAK2, or an IκB super-repressor, blocked Epo-mediated prevention of neuronal apoptosis. Epo can also modulate the activity of calcium channels through phospholipase C (PLC) (101), thereby reducing the release of excitatory neurotransmitters and augmenting nitric oxide production (92, 102). Very recent data suggests that Epo-mediated neuroprotection is also associated with increased

TIMP-1 activity and decreased MMP-9 activity in vivo and in vitro, and can be reversed by inhibition of JAK2 or TIMP-1 (103).

A couple of studies have recently implicated Epo to be a mediator of the protective effects of nitric oxide (NO) in neurons. Loss of EpoR coincided with programmed cell death in neurons (104). Neuronal NO was induced during hypoxia and correlated with protection in control cells but not increased in neurons that lacked the EpoR. However when treated with a neuronal nitric oxide synthase (nNOS) inhibitor the neurons lost their ability to induce EpoR expression in hypoxia and thus were not protected (104). In line with this finding another study demonstrated that nNOS knockout mice are more susceptible to peripheral neuropathy than their wild type counterparts due to the absence of NO-mediated activation of HIF-1 and subsequent downstream neuroprotection by Epo (105). Ex vivo experiments showed that protection recovered by using low doses of NOS donors was almost completely abrogated by Epo siRNA. Thus it appears the neuroprotective effect of Epo, as well as EpoR expression on neural cells, may also be regulated by NO.

Intriguingly, what determines the specific pathways activated by Epo, or the coordination of these multiple cascades, remains till now unknown.

5.3 Neuroprotection by Epo In Vivo

Different animal models have suggested potential clinical uses of Epo to combat ischemia or trauma. Cerebroventricular infusion of Epo was shown to reduce ischemia-induced learning disabilities and rescue hippocampal CA1 neurons from lethal ischemic damage in gerbils whereas infusion of EpoR abolished neuroprotection. In various mouse and rat models of ischemia, intracerebral injection of Epo also attenuated brain damage by reducing infarct volume by up to 50% (6, 106, 107) and improved cognitive function (108–110). This was further underlined by the fact that cerebral administration of soluble EpoR reduced the protective effect of hypoxia preconditioning by up to 80% in other models (111, 112). Overall exogenous Epo administration (see Fig. 2, pathway 6) has been shown to be protective in multiple cerebral tissue injuries including neonatal ((113) and reviewed by ref. 114, 115) or adult rodent focal brain ischemia, brain trauma (116), animal models of multiple sclerosis (117, 118) as well as spinal chord injury (119, 120). Increased oligodendrogenesis and attenuated proinflammatory cell infiltration was also observed in mouse models of EAE suggesting Epo positively stimulates oligodendrogenesis and reduces the autoimmune response (117, 118). In the neonatal brain, Epo significantly reduced white matter damage during hypoxia/ischemia and increased oligodendrogenesis and maturation of oligodendrocytes despite being applied in a delayed manner (113). Notably, in models of prolonged hypoxia, Epo secretion from astrocytes was shown to play an important role in

neuronal survival (4, 5) highlighting the paracrine functions of Epo (see Fig. 2, pathway 7).

Mechanistically Epo reduced infarct volume via JAK2, ERK, and PI3K/Akt pathways by elevating Bcl-xL and lowered both neuronal and inducible NOS levels in neurons (121). Upregulation of anti-apoptotic pathways was also observed in neonatal rodents submitted to focal cerebral ischemia (122). Epo-induced VEGF and BDNF have also been suggested to have an important role in angiogenesis- and neurogenesis-associated brain repair in rats treated with Epo after embolic stroke (110) similar to observations from in vitro studies (123). Epo was also shown to inhibit iNOS expression preventing the formation of excess NO and protecting facial motor neurons from death (97).

As in other neural cells Epo protects retina against cell death during injury but in contrast to other CNS regions where basal Epo is located mainly to astrocytes (4, 86), retinal neurons may express both Epo and EpoR (12). Epo prevented death of neurotrophic factor-deprived rat retinal ganglion cells (RGCs) in vitro, rescued axotomized RGCs in vivo, and prevented caspase-3 activation (124). Recently it was demonstrated that exogenous Epo significantly attenuates retinal neuronal cell death induced by glyoxal advanced glycosylation end products (AGEs) by promoting antiapoptotic and suppressing apoptotic proteins (125). Systemic administration of Epo before or immediately after retinal ischemia reduced histopathological damage and promoted functional recovery (12). When given therapeutically after light insult, Epo also mimicked the effect of hypoxic preconditioning by crossing the blood-retina barrier and preventing light-induced apoptosis via caspase-1 activation interference (11). Although transgenic overexpression of Epo with constitutively high levels of Epo in the retina protected photoreceptors against light-induced degeneration, the course or extent of retinal degeneration in genetic models was unaltered suggesting different apoptotic mechanisms exist (126).

Overall current evidence suggests that similar to erythroid cells, and as indicated by in vitro studies, phosphorylation of JAK2 is the initial step in Epo-mediated protection in the injured brain (9). Subsequently, downstream signaling modulates the transcription and activity of proteins involved in cell survival.

5.4 Neurotrophic Effects of Epo

In contrast to its neuroprotective properties, putative regeneration-enhancing effects of Epo have been less well studied. Epo was first shown to augment the activity of choline acetyltransferase in central cholinergic neurons in vitro and in vivo (127) and to enhance dopamine generation and differentiation of neuronal precursors in hypoxia. In agreement Epo was demonstrated to act directly on neural stem cells and promote the production of neuronal progenitors in forebrain (42) thus suggesting a direct contribution to neurogenesis after hypoxia. Epo-related functional recovery after spinal cord injury has also been described (119) and

correlated with behavioral improvements following Epo treatment (120). During stroke models Epo also significantly improved neurogenesis and functional recovery by increasing cerebral BDNF levels (110). Epo also enhanced oligodendrogenesis (117) and recovery of neurological function after neonatal hypoxic/ischemic brain (113). In the retina, Epo promoted neurite extension from postnatal retinal ganglion cells in vitro (128), induced JAK2/STAT3 phosphorylation and activated PI3K/Akt (see Fig. 1). Inhibition of JAK2/STAT3 abolished Epo-induced growth verifying the pathway is involved in conferring regeneration-enhancing Epo functions in the retina (129).

Thus the positive effects of Epo are not limited to neuroprotection but extend to neurogenesis and differentiation. Indeed more research needs to be performed in this area.

5.5 Epo in Treatment of Brain Diseases

Studies using Epo to combat brain disease progression have been largely encouraging. In 2002 the Göttingen Epo stroke pilot study demonstrated the neuroprotective effectiveness of Epo in human stroke patients (130). Epo-treated patients showed significantly better recovery than the control group regarding the clinical outcome parameters, the evolution of infarct size, and the profile of circulating damage markers. Disappointingly, the recent German multicenter Epo Stroke Trial revealed an increased risk of serious complications such as death, intracerebral hemorrhage, brain edema, and thromboembolic events (131). This study emphasized the point that when used in combination with other drugs (in this case recombinant tissue plasminogen activator used for hemodialysis) Epo may even be detrimental for patient outcome.

Epo therapy was effective in reducing progressive atrophy and loss of gray matter in patients diagnosed with schizophrenia (132). Also in healthy volunteers Epo improved cognitive and neural processing of emotional information showing similar effects to those of serotonergic and noradrenergic antidepressant drugs (133). Together these trials suggest future clinical applications for Epo in the treatment of psychiatric disorders characterized by cognitive dysfunction. During the first phase I/IIa study of high dose Epo treatment in patients with chronic progressive multiple sclerosis significant improvement in clinical and electrophysiological motor function as well as cognitive performance was achieved (134). Epo treatment also somewhat improved outcome for patients after subarachnoid hemorrhage (135). However, in contrast, the first randomized trial of Epo in moderate traumatic brain injury patients during the resuscitative phase showed Epo did not reduce neuronal cell death compared to placebo and disappointingly injury severity was worse in the Epo group (136).

Many of the clinical studies performed show promise, however they also have a number of limitations. For example frequently the patient numbers have been small and some of the studies not blind.

Also the doses used in the different injury paradigms as well as the routes of administration vary considerably. The mechanisms that improve function, enhance regeneration and/or slow deterioration remain undetermined and similarly the reasons why some studies have been less successful or even failed is also unclear. Indeed many questions remain open and the jury is out as to whether Epo will fulfill its putative potential - based on animal studies - to be a "universal" therapy for brain diseases.

6 Heart

6.1 Endogenous Epo Acts on the Heart

Epo is important during myocardial development and knockdown of Epo or EpoR in mice results in reduction in the number of cardiomyocytes (hypoplasia) and enhanced susceptibility to left ventricular dilatation and cardiac death (137, 138). However this phenotype may be largely rescued by restoration of EpoR production in hematopoietic tissue (139). Attempts to localize the EpoR within the heart have been made by dissecting the chick embryonic heart into epicardium, myocardium, and endocardium (140). These experiments revealed that endogenous Epo is most likely produced by the epicardium whereas EpoR is present in embryonic myocardium. However, positive inotropic and lusitropic effects of Epo have been later recorded in isolated human epicardial stripes indicating that adult human and mouse epicardium responds to Epo (141). Changes in contractile force, but not in contractile rate, were reported for isolated denervated rat heart perfused with Krebs-Henseleits saline (142).

6.2 EpoR in the Heart

Epo receptors and functional responses to Epo were shown in isolated cardiomyocytes (141, 143–146) coronary endothelial cells (83, 147) and fibroblasts (148). The cardiac EpoR was shown to respond equally efficiently to Epo, carbomylated Epo (CEPO), and ARA-290 (141, 149, 150), a synthetic Epo mimetic comprised only of helix B part of the cytokine. This synthetic non-erythropoietic peptide was shown to activate the heteroreceptor, composed of an EpoR subunit and βCR, but not the classical EpoR homodimer (79). These findings suggest that the effects of Epo in the heart are most likely mediated by such a heteroreceptor. Indeed expression of βCR in the heart and the lack of Epo effect in βCR knockout myocardium was shown (79).

Whereas in hematopoietic lineage EpoR expression is induced by GATA-1, Sp1, and Wt1 transcription factors (151, 152), expression of the common EpoR subunits in the heart is under control of GATA-4 and Sp1 transcription factors (145). The role of Wt1 expressed only in epicardium in regulation of EpoR expression remains to be clarified (153). Induction of EpoR expression has been observed in the failing ischemic heart and is most likely linked to the stabilization of HIF that is downregulated in aging tissues.

In agreement heat-induced stabilization of HIF1α in the heart is also associated with an increase of EpoR in the heart (151). Thus down-regulation of various transcription factors may reduce the efficiency of myocardial Epo treatment. Changes in EpoR expression during myocardial development and as a function of age remain to be investigated. Regulators of expression of βCR in the heart have also not been studied.

6.3 Where Does Epo Act and What Are Its Targets?

The source of Epo for receptor activation in the myocardium remains unknown. Plasma-borne Epo most likely does not reach cardiomyocytes (147). Thus, the cytokine should be generated by one or more cell types within the myocardium and then be released for autocrine/paracrine receptor activation similar to that in the brain (Fig. 2, pathway 7). In zebrafish, heart and liver were shown to be the major Epo-producing organs (154). Although myocardial Epo expression may be induced by hypoxic exposure (155) the origin of endogenous Epo secreting cells in the mammalian heart is unknown.

Localization of Epo action depends on the route of its administration/secretion. When applied intravenously Epo interacts primarily with EpoR of endothelial cells of coronary vessels (Fig. 2, pathway 1) (83, 147). Thereby, cardioprotection of the plasma-borne Epo is mediated by factors secreted from the endothelium upon activation of endothelial EpoR (Fig. 2, pathways 2 and 3). Amongst these factors are endothelin-1 and NO (156). When applied directly to isolated cardiomyocytes, Epo was shown to promote mitogenesis of neonatal cardiomyocytes, affect Ca^{2+} handling in isolated cells causing an increase in the amplitude and reduction in duration of calcium transients, and protecting them from oxidative stress and doxorubicin-induced apoptosis (Fig. 2, pathways 6 and 7) (141, 157–159).

An exhaustive overview of the molecular mechanisms of cardioprotective effects of erythropoietin can be found in recent reviews (160–162). As mentioned above, the cardiac-specific receptor is most likely a heterodimer. The downstream elements of signaling cascades induced by activation of such a heteroreceptor remain largely unknown. Also current data on the molecular mechanisms of the cardioprotective action of Epo comes from observations of the downstream effects of Epo in the heart. This is characteristic of most of the studies performed to date in which observations fit into the pre-existing model of homodimer function in erythroid precursor cells (see Fig. 1). To what extent activation pathways for the homo- and heterodimer are similar remains unknown.

6.3.1 Acute Responses: PI3-Akt-eNOS Signaling

Several studies indicated that the action of Epo in the heart is associated with activation of PI3K-Akt pathway with subsequent up-regulation of NO production (83, 160, 163, 164). Endothelial NO synthase (eNOS) is localized in the caviolae of cardiomyocytes

and is known to regulate the activity of L-type calcium channels by phosphorylation and S-nitrosylation. Upon eNOS activation and NO binding to soluble guanylyl cyclase, PKG-induced phosphorylation of contractile protein machinery is induced (165). These effects of Epo were confirmed for isolated cells as well as in vivo in hearts after intravenous Epo administration. In the latter case Akt and eNOS phosphorylation is restricted to the endothelial cells of coronary vessels (147). In cardiomyocytes the direct cytoprotective effect of Epo is mediated by its regulatory action on calcium handling and stabilization of the mitochondria. Epo induces activation of eNOS in cavioli by its phosphorylation at Ser 1177 by Akt. The generated NO then modulates activity of L-type Ca^{2+} channels via cGMP-sensitive phosphorylation and S-nitrosylation. Along with the Ca^{2+} release from the sarcoplasmic reticulum and SERCA2A the calcium pump is activated in response to stimulation of iNOS by Epo (166, 167). The exact molecular mechanisms of the action of Epo on calcium dynamics in the heart tissue are still unknown, however in myocardial stripes and in isolated cells (not on the vessels) they were tracked down to the PI3K-sensitive activation of PKCε (141). Stabilization of mitochondrial function in ischemic/injured myocardium by Epo is mediated by the activation of the mitochondrial KATP channels by Epo (166, 167). Furthermore, uncoupling of the mitochondrial electron transduction chain is reduced due to the interaction of iNOS-derived NO with the mitochondrial cytochromes. Mitochondrial biogenesis in cardiomyocytes is promoted by Epo which in turn induces enhancement of nuclear respiratory factor-1, PGC-1α (peroxisome proliferator-activated receptor ϒ coactivator 1α), and mitochondrial transcription factor-A gene expression in wild-type but not in $eNOS^{-/-}$ or $Akt1^{-/-}$ mice (168). Thus till now, most of the cardioprotective effects of Epo interaction with its receptor in cardiomyocytes seem to be mediated via PI3K-Akt-eNOS pathway (see Fig. 1).

Systemic induction of endogenous Epo production and release is known to occur in response to hypoxic stimulation. All the above mentioned responses of heart to Epo increase the survival probability during injury.

6.3.2 Chronic Responses: Changes in Gene Expression

Long-term activation of PI3K/Akt pathways in the heart induces activation of insulin-like growth factor binding protein-5 and downregulates peroxisome proliferator activated receptor-ϒ (PPAR-ϒ) coactivator-1 shifting metabolism from oxidative to aerobic glycolytic during long-term ischemia (169). Similar reprogramming of metabolism was observed in hypoxic heart and during pathological hypertrophic remodeling (170). Glucose delivery in cardiac myocytes is up-regulated accordingly as expression of Glut4 glucose transporter is induced along with metabolic reprogramming (171). Whether long term Epo treatment causes similar effects remains unclear. Epo binding to its receptors induces

phosphorylation of Akt and eNOS - its effects are seen within 5 min (147) and can be observed for the first hour and thereafter the Epo-EpoR complex is internalized and degraded (Mihov, Tavakoli, Bogdanova unpublished observations). The internalization rate constant for Epo-EpoR complex in UT-7/Epo cells is 0.06 min^{-1} (172). Upon internalization 60% of Epo gets dissociated from the classical EpoR homodimer and recycled, whereas 40% undergoes degradation (172). This observation suggests the effect of Epo is transient with the amount of surface-based receptors decreasing upon interaction with the cytokine.

6.4 Epo in Treatment of Cardiovascular Diseases

Recent trials were performed in which very high doses of Epo were administered percutaneously in patients after they were diagnosed for myocardial infarction. The expected cardioprotective effects included pro-angiogenic, anti-inflammatory, anti-apoptotic, and anti-oxidative action of Epo which have been reported in animal models of myocardial infarction (173–175). However these trials showed no beneficial effects of Epo, and in several cases an increase in mortality and morbidity was observed due to an increased risk of thrombosis (176–179).

Possible reasons for the lack of Epo effect include the inadequate route of the cytokine administration (intravenous vs. intramyocardial vs. intraperitoneal vs. subcutaneous); lack of cofactors and ligands of NO synthases (L-arginine, tetrabiopterin, oxygen) (180–182) and a limited "window of cardioprotective effect," which was claimed to be wide, but has never been properly determined in the heart. Epo-induced activation of NOSes in their uncoupled mode, due to the shortage of substrates and cofactors, turns these enzymes from cardioprotective anti-oxidative ones to cardiotoxic and pro-oxidative (181, 183, 184). Ischemia-reperfusion of coronary vessels is associated with activation of arginase-1 in the endothelium and local reduction in arginine availability (185). Oxygen deprivation inhibits eNOS and nNOS since their affinity to this substrate is rather low (186).

As the outcome of the first Epo trials appeared to be so discouraging an alternative approach has been suggested to increase the cytokine efficacy. Cardioplegic solutions widely used in cardiac surgery to cause heart arrest are now designed to induce activation of endogenous Epo production in the arrested organ (187).

7 Pancreas

Epo deficiency and higher incidence of anemia in individuals with diabetes gave the first inkling of potential beneficial effects and therapeutic applications of Epo use in the diabetes setting. Several clinical studies reported a beneficial effect of recombinant Epo on glucose metabolism in patients undergoing hemodialysis. Epo treatment of patients with end-stage renal disease corrected lipid

abnormalities and increased insulin sensitivity, with the duration of the treatment positively correlating with insulin sensitivity in these patients (188–190).

To date Epo expression by pancreatic cells has not been observed. However EpoR was expressed on islets of both human and non-human primates following Epo supplementation, or after transduction with an Adenoviral vector expressing high levels of Epo, affording protection of the islets from cytokine-induced destruction (49, 191). In addition, performance assessment of transduced islets transplanted into diabetic immunodeficient mice showed that overexpression of Epo conferred a functional advantage (191) and is also associated with a decrease in body weight (192). A number of in vitro and in vivo papers have now provided evidence that Epo is beneficial for β cell survival. In NIT-1 pancreatic cells, the PI3K inhibitor LY294002 abrogated the anti-apoptotic activity of Epo, indicating that activation of Akt was required for Epo-induced inhibition of cytokine-induced apoptosis (see Fig. 1) (193). In another study upregulation of Bcl-2, and concomitant downregulation of Bax and caspase 3, has also been suggested as a mechanism through which Epo can protect neonatal islet cells. In vivo diabetic rodent models also advocate direct effects of Epo on pancreatic β cells (see Fig. 2, pathway 6) promoting anti-apoptosis, proliferation, and angiogenesis signaling through its cognate receptor and downstream effector, JAK2, thus increasing β-cell mass (194). A very recent study administering a single dose of the novel Epo receptor agonist CNTO 530 to diet-induced obese mice resulted in improved glucose tolerance and insulin sensitivity at least in part from increased uptake of glucose by skeletal and cardiac muscle (195). The molecular mechanism(s) responsible for translating Epo receptor signaling into improved glucose tolerance are yet to be revealed and much more data is required to better understand its beneficial mechanism of action in general. However it is clear that Epo-induced pathways involving JAK2, Akt phosphorylation, and altered expression of several downstream apoptosis-related proteins, such as Bcl-2 and Bax as seen in other tissues, are likely to be a recurrent theme.

8 The Endothelium

Epo was shown to act on endothelial cells in vivo and in vitro having growth and chemotactic effects (40). In fact it has been suggested that many of the observed non-erythroid cytoprotective effects of Epo are mediated by second messengers released from endothelial cells (see Fig. 2) (196). The observation that development of the conditional non-hematopoietic EpoR knockout mouse is normal further supports this view. Equally important, Epo has been shown to facilitate vascular repair and thereby

to improve blood supply to injured organs by acting on endothelial progenitor cells (EPCs; Fig. 2, pathway 5) (196). CD34+/Flk-1 (also known KDR or VEGFR2) positive cells are hematopoietic progenitor cells that may differentiate into endothelial cells and contribute to neovascularization and vascular repair (197, 198). Epo promotes proliferation (40, 196), inhibits apoptosis (199), and facilitates differentiation of EPCs (200–203). Furthermore, Epo induces mobilization of EPCs into the circulation (204, 205), and their homing (155, 206, 207). Increased eNOS expression and BH4 biosynthesis has been shown in Epo-treated EPCs and vascular cells (Fig. 2; pathway 4) (205, 208). Interestingly, recent studies on hypoxic endothelial cells have shown that VEGFR2 can also become an additional component for the EpoR/βCR complex that is essential for NO production (reviewed by ref. 75). Similar to other non-hematopoietic cells PI3K/Akt signaling cascades, induction of mitogen-activated protein kinase (MEK)/extracellular signal regulated kinase (ERK) signaling pathways (83, 147) and NO production are known to mediate Epo effects in endothelial cells in animal models and humans patients (see Figs. 1 and 2, pathway 1).

Thus indeed augmented endothelial function may play a major role in Epo-mediated protection in non-hematopoietic cells and underlie a significant amount of tissue recovery from injury. Certainly more research needs to be carried out regarding this possibility and the consequences for the future use of Epo as a treatment strategy.

9 Risks Associated with Epo Therapy

Although Epo is considered a clinically safe-to-use drug (due to its long term use by anemic patients), a number of worrying risks have been associated with its more general use as a therapeutic. The frequent use of Epo mimetics in patients with chronic kidney disease (CKD) has recently declined as randomized trials demonstrated increased incidence of cardiovascular complications and mortality without a marked benefit in quality of life (reviewed by ref. 209). Safety concerns were raised during treatment of anemia in diabetic patients with CKD when they showed a twofold higher risk of stroke, an increased risk of venous thromboembolism and cancer-related deaths (210). Several studies have suggested that exposure to high doses of Epo mimetics, when needed to achieve higher hemoglobin levels, is harmful and explains this phenomenon (211, 212). Very high doses of Epo, in conjunction with hypoxia, have also been associated with a paradoxical neurotoxic effect suggesting dose–response conditions need to be optimized. In the clinics there are also considerable concerns about potential thrombotic complications. Recent trials in which very high doses

of Epo were administered to patients diagnosed with myocardial function showed an increased risk of thrombosis (176–179). Thrombotic events were also increased in critically ill patients although Epo therapy significantly reduced mortality particularly in trauma patients (213), and increased risk of venous thromboembolism was also noted in cancer patients (214). Another trial provided evidence of a possible negative interaction between short-term administration of Epo and aspirin due to its ability to modulate endothelial activation and platelet reactivity, von Willebrand factor antigen levels and factor VIII activity (215, 216). Although largely shown to improve neurodevelopmental outcome for preterm infants, Epo has been associated with a significant increase in the rate of retinopathy and may increase hypertension, coagulation, and even interfere with neuronal development in neonates (reviewed by ref. 84). Finally the therapeutic use of Epo in cancer patients remains highly controversial. A number of trials have shown that Epo treatment increases the risk for progressive disease and death although this may be dependent on the type and stage of the cancer (reviewed by ref. 217, 218). Potentially Epo could have a direct growth-promoting effect on cancer cells as they have been shown to express EpoR.

Thus it is apparent that our knowledge of the Epo signaling cascade needs to be significantly improved to be able to harness the benefits of using Epo and its mimetics as treatment for injury and disease. To a great extent its beneficial effects seem to be related to timing (the so-called "therapeutic window of opportunity"), dose and type of injury. A better understanding of these parameters would bring us significantly forward in our quest.

10 Conclusions and Outlook: What Don't We Know?

A wealth of preclinical data shows that the Epo signaling cascade is an important mediator of protection and cell survival in many different non-hematopoietic tissues as part of an innate response to injury. Many similarities exist between the mechanisms underlying its hematopoietic and non-hematopoietic functions but there are also some key differences that functionally lead to distinct outcomes. Not unexpectedly it was thought that Epo, a drug considered clinically safe, would be a trump card in most injury paradigms, however to date results from patient trials have been varied and more recently tip the balance to being negative. However, the pleiotropic and potentially beneficial biological effects of Epo signaling in non-hematopoietic tissues warrants in depth investigations of new therapeutic protocols. Clearly the generation of Epo mimetics such as asialo-Epo, CEPO, and others that are non-erythropoietic derivatives (75, 79, 149) will be instrumental in providing new options for treatment.

There are perhaps many things we do not yet know that need to be considered before being able to reliably use Epo and/or its derivatives as therapeutic drugs in different disease paradigms. For example what are the relative contributions of endogenous derived Epo and EpoR compared to exogenous recombinant Epo that is administered therapeutically? Do multiple tissue-specific Epo or EpoR isoforms exist? Is the endogenous balance between pro- and anti-apoptotic elements differentially altered by exogenous derivatives and how? What are the side effects of using low or high doses of Epo in terms of signaling pathways and negative outcomes? Can the Epo/EpoR axis be targeted clinically for therapeutic intervention in a cell or tissue-specific manner? What is the therapeutic window for treatment considering the receptor may not always be active? Is the route of administration critical to outcome? Can we prime the tissue before treatment or stimulate endogenous Epo production? And so on. The list is very long because we do not yet know enough about the non-hematopoietic mechanisms of Epo/EpoR in different tissues, or the short- and/or long-term effects of modulating the system

As more research is performed and new therapeutic applications for Epo are explored, careful consideration of potential adverse effects will need to be factored into the design of prospective clinical studies. Clearly to effectively harness the promise of Epo-an old but now pleiotropic growth factor-questions such as these need to be addressed now.

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