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Erythropoietin: Emerging role of erythropoietin in neonatal neuroprotection

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Abstract

BACKGROUND—In the last two decades, there has been considerable evolution in the understanding role of erythropoietin (Epo) in neuroprotection. Epo has both paracrine and autocrine functions in the brain. Epo binding results in neurogenesis, oligodendrogenesis, and angiogenesis. Epo and its receptor are upregulated by exposure to hypoxia and proinflammatory cytokines following brain injury. While Epo aids in recovery of locally injured neuronal cells, it provides negative feedback to glial cells in the penumbra, thereby limiting extension of injury. This forms the rationale for use of recombinant Epo and Epo mimetics in neonatal and adult injury models of stroke, traumatic brain injury, spinal cord injury, intracerebral hemorrhage and neonatal hypoxic-ischemia.

METHOD—Review of published literature (Pubmed, Medline and Google scholar)

RESULTS—Preclinical neuroprotective data are reviewed and the rationale for proceeding to clinical trials is discussed. Results from Phase I/II trials are presented, as are updates on ongoing and upcoming clinical trials of Epo neuroprotection in neonatal populations.

CONCLUSIONS—The scientific rationale and preclinical data for Epo neuroprotection are promising. Phase II and III clinical trials are currently in process to determine the safety and efficacy of neuroprotective dosing of Epo for extreme prematurity and hypoxic ischemic encephalopathy in neonates.

Keywords

Erythropoietin; cytokine; premature; hypoxic ischemic encephalopathy; brain

Introduction

Erythropoietin (Epo) is a cytokine with an emerging role in neuroprotection. Since the existence of a primary hematopoietic growth factor was proposed by Carnot in 1906 (1),

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there has been a considerable evolution in the understanding of the multiple roles of Epo. In the last two decades, the non-hematopoietic functions of Epo and its receptor (EpoR) in the central nervous system have been elucidated. Epo and its receptor are not only expressed in the brain (hippocampus, cortex, internal capsule and midbrain (2)) in glial cells (3), neurons (4) and endothelial cells (5), they actively participate in neurogenesis and angiogenesis (6) during embryonic development and are upregulated following brain injury. These actions of Epo on nervous tissues and brain capillaries, along with its cytoprotective properties (7) are critical during neurodevelopment and recovery following brain injury. This review will describe the mechanism of action of Epo, EpoR and the therapeutic potential of Epo in brain injury.

Mechanism of Action of Epo and EpoR

Epo is a 30.4-kDa glycoprotein. The gene is located on chromosome 7 and the mature protein is 165 amino acids long after posttranslational modification (8). The Epo molecule has four glycosylation sites, and sialic acid residues that determine its biological function (9, 10). During fetal life, liver hepatocytes are the primary site of systemic Epo production. Close to full gestation, hepatocytes transition their role of Epo synthesis to peritubular fibroblasts in the kidney. Epo promotes the maturation of erythroid progenitors into red blood cells by inhibiting apoptosis and stimulating proliferation and differentiation of these cells. Subsequent studies have shown that other adult organs such as liver, spleen and brain also produce Epo (11, 12). Local Epo production is noted both during fetal development and in adult brains of rodents, nonhuman primates and humans (11-13). In the brain, astrocytes (14), and to a lesser extent neurons (15), and immature oligodendrocytes (14, 16, 17) produce Epo in a regulated manner. These cells, and others, including microglia (18) and endothelial cells (19), express EpoR to varying degrees. This supports the role of Epo as both an autocrine and paracrine hormone of the nervous system (20). Epo is not only neuroprotective *in vitro*, but in *in vivo* models of hypoxia-ischemia, it decreases neuronal and oligodendrocyte death, and promotes neurogenesis, angiogenesis and oligodendrogenesis (16, 17, 21).

EpoR has a 66kDa protein component that belongs to the single chain cytokine type I receptor superfamily and confers Epo binding property (22). EpoR has an extracellular N-terminal domain, a single hydrophobic transmembrane segment and a cytosolic domain with no intrinsic kinase activity (22, 23). Epo binds to two adjacent EpoR on the cell surface. This leads to homodimerization and activation of receptor associated tyrosine kinase (Janus Kinase 2). The specific tyrosine sites that become phosphorylated later serve as docking sites for intracellular proteins including Signal Transducer and Activator of Transcription 5 molecule (or STAT5), which is a signal transducer and simultaneously activates several other cascades, including erythropoiesis (24). In neurons, other sites that are phosphorylated include activated phosphatidylinositol 3-kinase/akt, rat sarcoma (Ras)/extracellular signal regulated kinase (ERK1/2) and nuclear factor- kappa-B (NF-κB) (25). Nuclear factor-κB pathway plays a role in antiapoptosis in neurons and neural stem cell production (26). Epo signaling is later terminated by activation of phosphatases that dephosphorylate Janus Kinase 2 (25). Receptor density is regulated by endocytosis of the cell surface receptor followed by lysosomal degradation (27).

Brines *et al* has proposed that Epo mediates its neuroprotective effects via a heterodimeric EpoR made up of one EpoR and one common beta chain, which is similar to the signal-transducing subunit shared by granulocyte-macrophage colony stimulating factor, interleukin- 3 and interleukin-5 receptors (28). Both *in vitro* and *in vivo* experiments have demonstrated that EpoR in neural tissue has different molecular weight and lower affinity to Epo compared to the homodimeric EpoR found on erythroid progenitor cells (29). However, the role of the heterodimeric EpoR is quite controversial, as some investigators have shown antiapoptotic and neuroprotective properties of the homodimeric EpoR (30), and others have shown that expression of Epo and EpoR track together, while expression of the common beta chain is unrelated to either molecule (31).

A number of Epo variants have been developed to modify the pharmacologic properties of Epo and improve its neuroprotective function without stimulating erythropoiesis. AsialoEpo is a synthetic molecule similar to endogenous Epo made by removing the sialyted chains, and is studied for neuroprotective purposes (32, 33). Carbamylated Epo (CEPO) is another nonerythropoietic form of Epo molecule in which lysines are chemically converted to homocitrulline, which allows binding only to the EpoR heteromer in neural tissues (32, 34-36). Darbepoetin is yet another Epo variant containing additional oligosaccharide chains thereby extending the circulation duration compared to recombinant human Epo (37). Epotris is an Epo-mimetic peptide which lacks erythropoietic activity and corresponds to the C alpha-helix region (amino-acid residues 92-111) of human Epo with neuroprotective properties (38). It is of great medical interest because the small size enables it to cross the placenta or blood brain barrier efficiently compared to recombinant Epo. No studies have yet been done to assess safety or efficacy of these compounds as prenatal treatments. An endogenous neuro-active Epo molecule which is smaller than Epo produced by the kidneys due to less fully sialyted oligosaccharide chains called hypoglycosylated Epo (hypo Epo) has been recently been identified (29).

In addition to the structural variants of Epo described above, there are compounds that indirectly target EpoR by inducing Epo gene expression. Systemic Epo production is induced by transcription factors- hypoxia inducible factor (HIF) -1 and HIF-2 through tightly regulated pathways (39-42). HIF molecules are heterodimeric basic-helix-loop-helix per-aryl hydrocarbon receptor nuclear transporter-Sim (PAS) consisting of common constitutively expressed beta subunits and inducible alpha subunits (43, 44). In the central nervous system, HIF-1 α is ubiquitously expressed. These transcription factors are stimulated by hypoxia (12, 45), but other stimuli such as hypoglycemia, insulin release, reactive oxygen species and insulin-like growth factor also activate HIF (46). HIF in turn simultaneously transactivates other genes encoding glucose transporters, glycolytic enzymes, and vascular endothelial growth factor (VEGF) (47) along with Epo, the products of which either increase oxygen delivery or facilitate long term metabolic adaptation to reduced oxygen availability (43). GATA-binding transcription factor-2, directly inhibits Epo promoter during normoxia (48, 49). HIF-stabilizers and GATA binding transcription factor-2 inhibitors are being actively pursued as alternatives to recombinant Epo for stimulation of erythropoiesis, and ultimately also for neuroprotection (37, 50). The advantages and disadvantages of these agents are presently not known and further studies are warranted (51).

Epo for neuroprotection

The neuroprotective role of Epo and EpoR has been elucidated through several *in vitro* and *in vivo* studies (52). Epo has cytoprotective effects on endothelial cells, glial cells and neurons. It is anti-apoptotic (53), anti-oxidative (54, 55) and anti-inflammatory (56) and also has angiogenic and neurogenic effects (16, 21, 57). Both hypoxia and proinflammatory cytokines can directly activate HIF to stimulate expression of Epo and EpoR (58, 59). Epo attenuates effects of inflammation by reducing reactive astrocytosis and microglia activation and decreases effects of cytokines by reducing the number of immune cells in the injured area (53, 60). Epo also reduces cytokine concentration through negative feedback loops (56, 60) and prevents the cellular response to inflammation from spiraling out of control (29). EpoR is expressed by neurons at the site of injury, prior to secretion of Epo by surrounding astrocytes in animal models (61), in humans (62) and *in vitro* (63). The temporal and spatial difference in production of Epo and EpoR not only aid in early neuronal recovery, it also limits the extent of ischemic injury. Hypoxic preconditioning occurs when Epo is expressed following transient hypoxia, causing reduction in cellular damage following the second hypoxic ischemic event (64-66). Pretreatment with recombinant Epo prior to an acute hypoxic event also results in less brain injury in rats compared to those without pretreatment (67). Figure 1 summarizes the complex effects Epo has during and after a hypoxic ischemic insult which combine to afford neuroprotection. In animal studies of ischemic stroke, overexpression of endogenous Epo is associated with reduction in postischemic infarct volume, brain swelling and functional deficits (68).

Furthermore, absence of endogenous Epo and EpoR augments ischemic damage and impairs neuronal survival (7, 69). In models of traumatic brain injury (70), Epo decreases white matter damage and decreases neuroinflammation in conditions of hypoxia (71, 72). Epo improves cognitive function and neuronal survival through changes seen in the hippocampus of traumatic brain injury rat pup models (73). Epo protects the vascular integrity of capillaries following injury through vascular endothelial growth factor (VEGF) (19, 74) and participates in preservation of the blood-brain barrier (6, 75). In (rat) postnatal brain, the ventricular-subventricular zone has been identified as a source of neuroblasts and oligodendrocyte progenitor cells that migrate to injured areas in the brain in response to insult. This spontaneous regeneration is insufficient to provide functional and structural recovery on its own. Epo not only promotes neurogenesis and oligodendrogenesis (16, 17), it also increases migration in and around the injured area through secretion of matrix metalloproteinases (76, 77).

Epo has additional neurotrophic effects and promotes differentiation of neurons from stem cells (78, 79). It stimulates axonal regrowth, neurite formation, dendritic sprouting, modulation of intracellular calcium and neurotransmitter synthesis and release. It is important during fetal growth as mouse embryos with absent Epo/EpoR have smaller brain size compare to their littermates (78). *In vitro*, Epo stimulates pluripotent progenitor cells to differentiate into neuronal progenitor cells (79) and later into neurons (80). One mechanism by which Epo works may be by inducing brain derived neurotrophic factor gene expression in hippocampal neurons (81), an important growth factor in fetal brain formation. Brain

derived neurotrophic factor also increases recognition-memory and neurogenesis postnatally (82).

These protective effects of Epo during ischemia/ reperfusion and prenatal brain development have prompted the use of recombinant Epo and Epo mimetics in other brain injury models (83, 84) along with transient stroke and traumatic brain injury animal models, such as autoimmune encephalitis (85), intracerebral hemorrhage (86), spinal cord injury (87, 88), neonatal focal stroke (89-92), neonatal hypoxia-ischemia (90, 93-95), intraventricular hemorrhage or periventricular leukomalacia (96) in preterm infants and cerebral palsy (97). These studies demonstrate that high dose systemic recombinant Epo, and Epo mimetics, are safe (98), and can penetrate the blood brain barrier with resulting reduction in histological damage and improvement in function. There has been growing interest in the last decade to apply these results to clinical settings in neonatal and adult populations.

Epo in clinical trials

Phase I/II studies to evaluate feasibility, safety and establish appropriate dosing in human preterm (97, 99, 100) and term neonates (99, 101-103) have been done. Target populations include neonates at high risk for poor neurodevelopmental outcomes due to extreme prematurity, hypoxic ischemic encephalopathy, perinatal stroke, or complex cyanotic heart disease. Epo pharmacokinetics have been studied using doses ranging from 250 units/kg to 2500 units/kg (99, 101). These studies have demonstrated safety, and the potential for efficacy (104). Surprisingly, drug clearance was slower in term infants with hypoxic ischemic encephalopathy compared to preterm infants less than 1000 gm. The dose of 1000 units/kg has been well tolerated in all patients (99, 101), and corresponds best to pharmacokinetic measures of neuroprotective doses in neonatal rats (93, 105). This dose was also neuroprotective in conjunction with hypothermia in a recent nonhuman primate study (95). Studies of Darbepoetin have also been done for both safety and neuroprotective efficacy in preterm infants (NCT00334737) and in term infants with hypoxic-ischemic encephalopathy (NCT01471015). Initial results from preterm infants (NCT00334737) show erythropoietic benefit and no safety issues (106). Neurodevelopmental outcomes at two years of age are *in press*. Study with term infants (NCT01471015) has completed enrollment, and no safety issues were identified. See Table 1 with summary of other clinical trials evaluating neonatal neuroprotective role of Epo and its analogues in term and preterm infant populations.

If Darbepoetin is found to be neuroprotective, this would be advantageous because fewer treatments would be required than with recombinant Epo. None of the published studies have shown adverse side effects common to adult studies, including polycythemia, thrombosis, hypertension or retinopathy of prematurity. Side effects due to high red cell mass and polycythemia is less likely in critically ill neonates with high phlebotomy losses. Treatment in these neonatal populations is also of shorter duration than in adult populations with renal failure, where treatment is often for the lifetime.

Larger phase II and III studies to test safety and efficacy in neonatal populations are now underway (see Table 1). The Preterm Epo Neuroprotection trial (PENUT trial,

NCT01378273) is now enrolling, targeting preterm infants 24 to 27 completed weeks of gestation. Enrollment is now completed for the Swiss study involving premature infants less than 32 weeks of gestation (NCT00413946). A phase II trial combining Epo with hypothermia for term infants with hypoxic ischemic encephalopathy, the “Neonatal Erythropoietin and Therapeutic Hypothermia Outcomes in Newborn Brain Injury” (NEAT-O) NCT01913340 trial is ongoing, as is a French study “Efficacy of Erythropoietin to Improve Survival and Neurological Outcome in Hypoxic Ischemic Encephalopathy (Neurepo)”, NCT01732146. Keeping inclusion and exclusion criteria similar for different target population, along with long term follow-up up to two years will allow us to compare variations in the dose of Epo used (1000 to 3000 units/kg). These variations in the dose strength, number of doses used and their safety profiles, will provide important information regarding the optimal use of recombinant Epo, should it be found to be of benefit in these disparate populations. Comparable studies are being done in adult populations with diseases that bear high risk of cognitive or neurologic impairment. Recombinant Epo or Epo analogues are being evaluated as therapy for acute ischemic stroke, traumatic brain injury, subarachnoid hemorrhage, resistant depression, multiple sclerosis, and schizophrenia (107-113).

Conclusion

Epo is a pleiotropic cytokine with multiple applications, including cytoprotection, particularly of the nervous system. The role of Epo and its receptor in the brain has been widely studied in multiple preclinical models ranging from neonatal brain injury to adult injuries. Both Epo and Epo-analogs may prove useful for treating pathologies of the central nervous system. In neonates, the side effect profile of erythropoietin has been minimal. Treated infants have not shown common side effects seen in adults such as thrombosis and polycythemia. The safety of neuroprotective dosing of rEpo must be established for each treatment population. In addition, the combined safety and efficacy of neuroprotective treatments such as moderate hypothermia *plus* rEpo must be established. The goal for this decade is to safely take the application of this fascinating cytokine from the bench-to-the bedside, a process that is already underway.

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Figure 1.

Neuroprotective effects of Epo in brain following hypoxic-ischemic injury. Brain injury increases local astrogliosis and microglial cell activation. Astrocytes produce Epo, which enhances neurogenesis and oligodendrogenesis, while decreasing local inflammation. It also stimulates angiogenesis through local endothelial cells.

Table 1

Global clinical trials looking at effect of recombinant Epo and its analogues in neonatal brain diseases affecting both preterm and term infants. These trials are currently active, recruiting or recently completed. They are taken from www.clinicaltrials.gov.

Target population	Trial	Investigator and site	Phase	Study design	NCT	Drug	Drug dosing	Primary outcome
Perinatal asphyxia	Neuroprotective role of erythropoietin in perinatal asphyxia	Dr. Mushiaq Bhat, MD, Sheri Kashmir Srinagar, Jammu & Kashmir, India	Phase II/III	Randomized placebo controlled	NCT02002039	rEpo	500u/kg i.v. every other day for 5 doses	Death or disability at 12-18 months of life
Perinatal asphyxia	Darbepoietin in newborns undergoing cooling for encephalopathy (DANCE)	Dr. Mariana Baserga MD, University of Utah, Salt Lake City, UT, USA	Phase I/II	Randomized placebo controlled	NCT01471015	Darbe	2 or 10mcg/kg dose i.v. 1st dose before 12hrs, 2nd dose at 7 days	Safety and pharmacokinetics
Perinatal asphyxia	Neonatal erythropoietin and therapeutic hypothermia outcomes in newborn brain injury (NEAT O)	Dr. Yvonne Wu MD MPH, University of San Francisco, San Francisco, CA, USA	Phase I/II	Randomized placebo controlled	NCT01913340	rEpo	1000u/kg i.v. every 48hrs for total 5 doses	Safety and efficacy with neurodevelopmental outcomes at 1 year
Perinatal asphyxia	Erythropoietin to improve survival and neurological outcome in hypoxic ischemic encephalopathy (NEUREPO)	Dr. Juliana Patkai MD PhD, Assistance Publique-HopAitaux de Paris, Paris, France	Phase III	Randomized placebo controlled trial	NCT01732146	rEpo	1000-1500u/kg i.v. 1st dose at <12hrs, 2nd and 3rd dose every 24hrs	Survival without neurologic sequelae at 24months
Preterm infants with intraventricular hemorrhage	Erythropoietin for repair of cerebral injury in very preterm infants (EpoRepair)	Dr. Sven Wellmann MD, University of Zurich, Zurich, Switzerland	Phase III	Randomized placebo controlled	NCT02076373	rEpo	2000u/kg i.v. 1st dose at 5 days, 2nd and 3rd dose every 24hrs; 4th dose at 10 days after start; 5th dose at 17 days	Neurodevelopmental outcome at 5yrs
Infants 32 weeks gestation	Erythropoietin for neuroprotection in very preterm Infants	Dr. Bai-Hong Su MD PhD, China Medical University Hospital, Taichung, Taiwan	Phase I/II	Randomized placebo controlled	NCT00910234	rEpo	100u/kg or 3000u/kg/dose i.v. 1st dose at 3-6hrs after birth, 2nd and 3rd dose every 24hrs for total 3 dose	Brain injury (intraventricular hemorrhage) and periventricular leukomalacia at 2 months
Premature infants 24-27 weeks gestation	Preterm Epo neuroprotection (PENUT)	Dr. Sandra E Juul MD PhD, University of Washington, Seattle, WA, USA	Phase III	Randomized placebo controlled double blind	NCT01378273	rEpo	1000u/kg i.v. every 48hrs for 6 doses, then 400u/kg s.c three times a week until 32 ⁶⁷ corrected age	Neurodevelopmental outcome at 24-26 months corrected age by Bayley III
Premature infants 26-32 weeks gestation	Does erythropoietin improve outcome in very preterm infants?	Prof. Hans Ulrich Bucher, MD PhD, Swiss Neonatal Network, Switzerland	Phase II	Randomized parallel assigned	NCT00413946	rEpo	3000u/kg given i.v. at 3hrs, 12-18hrs and 36-42hrs total 3 doses	Mental development index Bayley II at 24 month corrected age
Premature infants < 32wk gestation	Effect of erythropoietin on preterm brain injury (EPO)	Dr. Huiqing Sun, Ass Prof, Zhengzhou Children's Hospital, Zhengzhou, China	Phase IV	Prospective parallel assignment	NCT02036073	rEpo	500u/kg every other day for 14 days, beginning < 24 hours of age	Incidence of mental development index <70 at 18 month corrected age

i.v. = intravenous, s.c. = subcutaneous, Darbe = darbepoietin, rEpo = recombinant erythropoietin