Epo, what is it good for?
Absolutely everything!!!

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I. Epo background

In response to environmental stresses, organisms respond with induction of cellular, biochemical, and molecular processes so as to alleviate the adverse effects of the inciting stressor. One environmental stress encountered during normal physiological states, such as development, and in pathophysiological states, such as stroke or myocardial infarction, is hypoxia. When exposed to systemic hypoxia or in clinical states associated with tissue hypoxia, including anemia or congenital heart disease with right-to-left shunting, mammals and other vertebrates exhibit physiological alterations in a variety of organ systems. One such response is an increase in red blood cell mass.

Physiologists postulated a century ago that a humoral substance regulates erythrocyte production in response to hypoxia[1]. Investigators devised innovative and creative experiments in an attempt to discern the mechanistic basis for this physiological response. They learned that the circulating substance that regulates red blood cell production in the adult is made in the kidney and, in cases of severe anemia, in secondary sites such as the liver and spleen. The subsequent identification of a systemic factor, erythropoietin (epo), which possesses an ability to increase the number of circulating erythrocytes was a biochemical tour-de-force as it required the purification of epo from hundreds of liters of urine[2].

Circulating or endocrine epo is made in the kidney and exerts its hematopoietic effect on hematopoietic stem and progenitor cells in the bone marrow. The cellular basis for the pro-erythrogenic effect of epo involves reduced apoptosis of erythroid progenitors. However, investigators later recognized that epo is synthesized in other organs besides the kidney and liver. In addition, it was noted that the receptor for epo was more ubiquitously expressed and not restricted to organs that produce local or paracrine epo. After the peptide sequence of epo was deduced, the cloning of the cDNA encoding epo was one of the first successes of the biotechnology industry and made Amgen a medical household word. The initial use of epo has been in the treatment of anemia.

Since epo is a cytokine with cell protective effects, investigators postulated that epo might have more widespread cytoprotective effects. Translational investigations using animal models have provided useful information regarding potential use of epo in various human disease states. Derivatives of epo that are long-lasting may provide increased efficacy for single-dose treatment strategies whereas newer derivatives that lack the erythrogenic effect, but retain the cytoprotective effect of native epo have also been developed[3,4]. A region of epo that resides in a short peptide, interacts with the epo receptor, and confers neurotrophic activity has also been identified[5]. Clinical trials employing several of these forms of epo are underway and may provide novel treatment strategies with a safe and time-proven agent.

II. Epo and its role in cytoprotection

Neuroprotection

The cytoprotective effects of epo may have widespread applications, particularly in diseases affected by ischemia or infarction. Cerebral and myocardial infarction are leading causes of morbidity and mortality in the US and western world. For example, in the US, ~750,000 patients are affected by stroke annually. Of these, ~200,000 patients die from complications of stroke. Currently, there are ~5,000,000 stroke survivors in the US. The treatment and care of stroke patients is currently estimated at $50,000,000,000 per year. Current stroke treatment modalities are limited. Improving the outcome and functional status of stroke patients would have enormous humanitarian and economic benefits.

Identifying more effective treatment of stroke and other ischemic diseases may lead to improved prevention or treatment of stroke. Damage from stroke comes not only acutely, but also from delayed effects of prolonged hypoxia in the brain[6] that vary according to insult type as well as location of infarct[7,8]. However, hypoxia exposure does not always result in cell death. Exposure of experimental subjects to sub-lethal doses of hypoxia or ischemia induces an ensuing protective response in cells, organs, and organisms. Preconditioning results in remarkable improvement in functional outcome as well as neuronal survival in experimental animal models.

There are two phases of ischemic preconditioning: an immediate or early phase that is initiated within minutes to hours after exposure to the preconditioning ischemic event, and a delayed or late phase that is initiated within hours to days after the priming event. The early preconditioning phase of preconditioning involves existing molecular triggers whereas the late phase requires *de novo* synthesis of molecular mediators that confer various aspects of preconditioning. The adaptive aspects of late preconditioning are also induced by systemic hypoxia, an environmental stress typically encountered during tissue ischemia.

The induction of gene products during hypoxic or ischemic preconditioning includes glycolytic enzymes to provide energy during states of oxygen deprivation, proangiogenic factors to stimulate blood vessel formation and thereby increase hence oxygen delivery, and the erythrogenic factor epo as well as the epo receptor. Although one effect of increased epo production may be to increase red blood cell mass and thereby increase oxygen delivery, a more immediate effect is the cytoprotective action that epo induces. This is supported by expression of epo in organs other than the kidney and liver, such as the lung, testis, uterus, and brain, where

Epo produced in extra-renal sites acts in an autocrine or paracrine manner. The realization that the brain, a privileged organ, was an extra-renal site of epo production confirmed an observation made a century ago that the brain also had hematopoietic activity[9]. More recent studies revealed that epo

production in the brain, as in the kidney, is regulated in an oxygen-dependent manner, although the level of induction is not as great as in the kidney[10]. Epo has protective effects *in vitro* for neuronal cultured cells[11] and *in vivo* against focal cerebral ischemia in adult[12] as well as newborn[13] rodents. The presence of epo receptors in the brain provided further evidence that epo signaling plays an important biological role in the brain[14,15].

Less than a decade ago, investigators provided evidence that epo plays a cytoprotective effect against ischemic insults to the brain using animal models and stroke induced by permanent middle artery occlusion (pMCAO)[16,17]. Further investigation revealed more widespread protective effects of epo against neurological insults, including that associated with autoimmune encephalitis, seizures, and traumatic brain injury[18]. Neurons and astrocytes are a source of epo under basal conditions[19]. Epo expression in the acute phase of stroke is enhanced in vascular endothelial cells[20]. The neuroprotective effects of epo extend to the peripheral nervous system as well. Schwann cells are the presumed cellular source for epo that prevents axonal injury in a rat model of peripheral neuropathy induced by oral acrylamide administration[21].

The success of these experiments in animal models prompted the examination of the utility of epo against stroke in humans[22]. Results from this recent Phase I/II clinical trial support use of epo as a treatment strategy in stroke[22] and other neurological disorders[23] and have prompted a larger clinical trial involving multiple centers. Identifying key regulators of epo regulation will provide additional opportunities for novel therapeutic strategies involving induction of epo in the brain.

Retinoprotection

The retina and brain share embryological features including the presence of neuronal cells as well as a vascular bed that develops within neural tissue. In addition, the retina is one of the most metabolically active tissues in the body and as such is a source as well as target for reactive oxygen species. Therefore, it is not surprising that the retina possesses high levels of antioxidant defenses as well as other cytoprotective factors. One of these protective factors is epo, produced locally in the retina. Similar to the blood-brain barrier, the blood-eye barrier likely precludes the transit of renally produced epo, making retinal epo production the sole source for the eye. Furthermore, the existence of epo receptors on photoreceptors completes an epo responsive circuit in the retina.

The retina is sensitive to ischemic injury. As with the brain, ischemic preconditioning induces protective measures against subsequent otherwise cytotoxic levels of ischemia. Ischemic preconditioning also is effective against light-induced photoreceptor degeneration. In a similar manner, systemic hypoxia likewise induces cytoprotection in the retina against a light-induced insult[24]. The protective effects of hypoxic preconditioning are evident at the morphological as well as functional levels in animal studies. As in the brain, epo is highly induced by hypoxia and its receptor is expressed on neuronal cells, photoreceptors[25].

Epo has proven effective as a mediator when administered exogenously for both ischemia[26] as well as light-induced injury[25]. However, other molecular mediators of ischemic preconditioning exist, as evident by the decreased protection afforded by exogenous epo or by increased retinal epo production by transgenic mice in comparison to protection afforded by systemic hypoxia pretreatment. Epo in the retina may have more limited therapeutic value as it is ineffective against retinal diseases induced by retinal degeneration models[27]. However, it nevertheless may offer unique opportunities for eye disease treatment[28], particularly for newer derivatives of epo or peptides that signal through the epo receptor that retain epo's neuroprotective abilities.

Cardioprotection

The effectiveness of epo treatment on cardiovascular disease has been recently reviewed[29]. Dialysis patients with significant coronary artery disease treated with epo have reduced myocardial ischemia as measured by exercise treadmill tests[30]. Whether the effectiveness of epo in this patient population stems from correction of the underlying anemia or to other actions of epo remains to be determined. In another study, higher serum epo levels at the time of myocardial infarction was a positive predictor of reduced infarct size whereas baseline hematocrits was not an independent predictor[31]. The authors reported that preinfarction angina was more prevalent in the higher epo group and may have afforded myocardial protection as a consequence of ischemic preconditioning, as previously noted[16,32]. This is consistent with the concept that ischemic preconditioning induces its effectiveness by induction of protective molecular mediators[33].

Epo is not produced locally in the heart. However, the heart has epo receptors and epo treatment provides significant benefits in animal models of cardiac ischemic injury. As with the brain, administration of exogenous epo results in a marked decrease in infarct volumes in experimental animal models[34,35]. The mechanism behind the protective effects of epo are due largely to its cytoprotective effects, although anti-oxidant effects of high-dose epo may also contribute to its effectiveness. Derivatives of epo lacking erythrogenic properties provide continued protection against injury accompanying myocardial infarction, comparable to that of native epo[36]. Clinical trials of epo for cardiac injury have lagged behind that for neurologic injury, although this is currently a focus of industry-sponsored trials using long-acting versions of epo. Although epo provides significant effectiveness as a cytoprotective agent, as with the brain and retina systemic hypoxia is more effective as a preconditioning agent implying that other molecular mediators are important for pre-conditioning.

A role for epo in congestive heart failure (CHF), both diastolic and systolic in origin, has been indirectly suggested. Patients with diastolic or systolic CHF have similar prevalence of anemia. Moreover, anemic patients with CHF do not appear to have worsened LV function compared to non-anemic patients with CHF. However, anemic CHF patients have worsened clinical symptoms and

lower exertional capacity as compared to non-anemic CHF patients. The pathogenesis of anemia in CHF patients may involve impaired renal perfusion and an associated decrease in epo secretion, although epo resistance may also be an etiologic factor. Other possible causes include systemic inflammatory effects, particularly that involving TNF-alpha, as well as iatrogenic effects from ace inhibitors or angiotensin receptor blockers causing impaired epo production.

Anemia in patients with diastolic dysfunction also may have detrimental effects on cardiovascular outcomes. Patients with chronic kidney disease (CKD) have increased mortality with coexistent left ventricular hypertrophy (LVH) or anemia, and even higher mortality rates when both LVH and anemia are present. This has prompted the initiation of a double-blinded clinical trial, The Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT), to assess the impact of treating anemia with darbepoetin alpha on cardiovascular events and mortality in patients with type II diabetes and CKD[37].

Renoprotection

Investigators initially thought that the kidney did not express epo receptors. This seemed reasonable since the adult kidney does not respond to epo nor does it change morphologically under conditions of chronic anemia or with chronic renal failure. However, once epo receptors were identified in multiple organs, investigators reexamined the kidney and confirmed the presence of epo receptors[38]. Since epo possesses mitogenic and cell survival properties, investigators hypothesized that epo would have protective effects against pathological insults[39]. Indeed, epo in experimental models has proven effective against acute ischemic renal injury[40], even if administered at the time of reperfusion[41,42]. In addition to acute ischemic injury, darbepo reduces progressive organ failure induced with the classic rat kidney remnant model[43].

Hepatoprotection

The use of epo in critical care was initially evaluated in terms of its effect upon outcome when used to increase red blood cell mass and was not effective in this regard[44]. However, both epo in animal models is effective at attenuating organ failure arising from hemorrhagic, but not septic shock[45]. Epo also is effective at alleviating ischemic liver injury arising from laparoscopic surgery, that principally affects the splanchnic blood supply of the liver and kidney[46]. However, as with other comparisons between preconditioning and epo treatment, preconditioning induced by mock laparoscopic surgery, a preconditioning induced b y mild oxidative stress, is more effective at prevention of organ injury than exogenous epo treatment [47].

III. Regulation of epo gene expression

Identification of the hypoxia responsive transcription factors

The induction of gene expression by ischemic or hypoxic preconditioning has been the subject of intense investigation for over a decade. Using epo as a paradigm, researchers sought to identify the molecular basis for induction of epo gene expression by hypoxia. Insight into the regulation of epo was facilitated by the delineation of the enhancer region of the epo gene conferring hypoxia inducibility. Hypoxia Inducible Factor 1 (HIF-1) was identified on the basis of its *in vitro* ability to bind to the hypoxic response element (HRE) in the epo enhancer region.

Hypoxia Inducible Factors (HIF) are transcriptional regulators of genes involved in hypoxic and other stress responses[48]. HIF members contain a PAS domain, a signaling module found in cellular factors that sense[49] and respond to environmental stress[50]. HIF-1, the prototype member of the HIF family, is comprised of two distinct polypeptides, HIF-1 alpha (HIF-1α) and HIF-1 beta (HIF-1β, also known as ARNT)[51]. HIF-1α confers biological specificity whereas HIF-1β is its obligate heterodimerization partner. Target genes identified for HIF-1α include factors involved in iron metabolism, angiogenesis, and glycolysis. There is a significant interest in development of HIF-1α agonists as well as antagonists since HIF-1 plays a vital role in allowing cancer cells to survive and multiply.

With the use of bioinformatics, additional members of the HIF alpha family were identified (Figure 4). Endothelial PAS domain protein 1 (*EPAS1*)[52], also known as HIF-2α/HLF/HRF[52-55], is closely related to HIF-1α in composition and regulation. Initial studies evaluated whether biological actions of HIF-2α mirrored that of HIF-1α. For example, HIF-1α and HIF-2α protein levels are both regulated in an oxygen-dependent manner by a Von Hippel Lindau-dependent ubiquitin-mediated degradation pathway[56]. More recent investigations have highlighted unique aspects for HIF-2α or HIF-1α[57-67]. In this regard, HIF-2α, as compared to HIF-1α, is expressed in a more restricted range of tissues during development and in the adult, including in human[68] and mouse[52,63] vascular endothelial cells.

Role of HIF factors in regulation of epo gene expression

The close similarity of HIF-2α to HIF-1α raises the question as to which HIF alpha member is the key regulator for epo gene expression *in vivo*. Insight into this question has come from identification of expression sites for HIF family members and from studies of loss-of-function mouse models. Studies of the regulation of epo in the kidney and brain are particularly informative as HIF-1α and HIF-2α are expressed in mutually exclusive cell populations in the kidney and brain[52,63].

In the kidney, HIF-2α is expressed in vascular endothelial cells and in interstitial fibroblast-like cells, the latter being the site of epo synthesis. Mice completely lacking HIF-2α have marked reduction of epo in the kidney under

ambient oxygen conditions and an inability to induce epo after exposure to hypoxia. Mice partially lacking HIF-2α, i.e. lacking one HIF-2α allele, when exposed to hypoxia have impaired renal epo expression relative to wildtype mice[69]. These data provide convincing evidence that HIF-2α plays an extremely important role for regulation of epo gene expression in the kidney.

As in the kidney, HIF-1α and HIF-2α are expressed in mutually exclusive cell populations in the brain. Mice completely lacking HIF-2α mice have marked reduction of epo in the brain under both ambient as well as reduced oxygen conditions. An impaired hypoxic induction of epo in the brains of mice partially lacking HIF-2α is seen, similar to that observed in the kidneys of these mice. Thus, HIF-2 is an essential regulator of epo in both the kidney and brain, under both ambient as well as hypoxic conditions.

Role of HIF factors in neuroprotection

HIF members are prime candidates for orchestrating cytoprotective mechanisms, given their role in regulation of epo gene expression. The most knowledge we currently have on this topic is with respect to the role of HIF factors in neuroprotection. Stroke leads to induction of HIF-1α and HIF-2α in the peri-infarct or penumbral zone[70], the anatomical region where cell life is precariously balanced. Stabilization of HIF-1α correlates with hypoxia-induced neuroprotection in animal[71] as well as neuronal cell culture models. However, a mechanistic role of HIF-1α in hypoxic neuroprotection is less obvious from recent *in vitro* cell culture and *in vivo* mouse knockout models.

Enhanced activity of HIF-1α in a mouse hippocampal cells resulted in increased sensitivity to oxidative stress-induced cell death whereas decreased expression of HIF-1α by RNA interference prevented this oxidative stress-induced cell death[72]. HIF-1α deficient mice were evaluated in terms of response after cerebral infarction[73]. Surprisingly, mice completely lacking HIF-1α in neurons had decreased, rather than increased, susceptibility to stroke-induced damage. Similar results were observed for mice partially deficient for HIF-1α. These observations suggest that HIF members other than HIF-1α may be the primary mediators of hypoxic and ischemic preconditioning in the brain.

HIF-2α remains an excellent candidate as the principal HIF member responsible for neuroprotection. As stated earlier, HIF-1α and HIF-2α are expressed in mutually exclusive cell populations in the brain. These differences are maintained during stroke. In the penumbra, HIF-1α is expressed in neurons whereas HIF-2α expression is limited to vascular endothelial cells in the brain[52]. Cerebral microvascular endothelial cells are critical for neuroprotection[74-76] and are the site of erythropoietin[20] and antioxidant enzyme (AOE) production in acute stroke[77]. Endothelial-derived factors[78] also mediate neovascularization[79,80] and local tissue remodeling[81-83] in the penumbra following ischemia or infarction. Finally, over-expression of HIF-1α and HIF-2α in neurons revealed novel hypoxic

target genes regulated in a HIF-2α selective manner, including several known to be important in cell survival[84].

Rationale for stimulation of HIF signaling for more effective cytoprotection

Despite its potent cytoprotective effects, exogenous epo is only 50% to 75% as effective as hypoxic preconditioning in alleviating stroke outcome[85] or myocardial damage[86]. Other biological mediators controlled in a HIF-dependent manner likely contribute to the neuro- and cardio-protective effects induced by preconditioning. Oxidative stress is another cause of pathology in stroke[87]. Antioxidant enzymes (AOE) in the brain[88] and endothelium[89] are induced in response to hypoxic and oxidative stress and alleviate the effects of oxidative stress[90].

Regulation of AOE gene expression by HIF-2α represents another potentially important defense against stroke-induced damage[91]. SOD2 encodes the mitochondrial-localized major AOE manganese superoxide dismutase. In mice completely lacking HIF-2α, SOD2 gene expression was reduced in multiple sites including the liver[91] and brain, even more significant given that the increased oxidative stress present in these HIF-2α knockout mice normally leads to induction of SOD2 gene expression. In addition to SOD2, other major AOE genes are likewise depressed in HIF-2α knockout mice indicating the likelihood that major AOE genes are a functionally related HIF-2α target gene cluster[91].

The vascular endothelium is a prominent site of HIF-2α expression. After the dynamic aspects of vascular development have ceased, most vascular beds in the adult mammal are quiescent with the notable exception of the uterus. However, changes in adult vascular beds do occur in response to pathological stimuli. Collateral vessels form in heart and skeletal muscle subjected to chronic ischemia. Neovascularization is a prominent feature of several eye disease states and is induced in the penumbra after stroke, noteworthy since the cerebral and retinal vascular beds share specialized vascular features distinguishing them from other vascular beds.

HIF-2α deficient mice have defects in retinal angiogenesis that vary depending upon HIF-2α gene dosage and the environment. HIF-2α knockdown mice, with HIF-2α levels approximately 20% of wildtype mice, have normal retinal artery development, but impaired neovascularization after exposure to hyperoxygen/reoxygenation, an experimental paradigm of neovascularization induced in infants with retinopathy of prematurity[92]. HIF-2α knockout mice have overt retinal arterial abnormalities[93]. The impaired development and function of retinal vasculature in HIF-2α deficient mice has important implications for neoangiogenesis in the cerebral vasculature as both vascular beds have common embryonic and structural aspects[94].

IV. Conclusion

Circulating epo is made in the adult kidney. The cellular basis for the pro-erythrogenic effect of epo includes prevention of apoptosis of erythroid progenitors. However, epo receptors are present on a variety of cell types. The more widespread significance of the cytoprotective action of epo is just now being appreciated from studies in a variety of animal and cell culture models. Epo is a major contributor to the protective effect induced by hypoxic or ischemic preconditioning, the cytoprotective effect induced when an organism or cell is exposed to a sub-lethal dose of hypoxia or ischemia and then challenged with a dose that would otherwise be lethal.

Preconditioning is seen in multiple organs including the brain, heart, kidney, liver, and eye. Locally produced or paracrine epo is the major contributor to the neuroprotective and retinoprotective effect seen with preconditioning induced by sub-lethal doses of hypoxia or ischemia. Endocrine (i.e. renal produced) epo provides a major component of cytoprotection seen in the heart after hypoxic preconditioning using systemic hypoxia in whole animal experiments. The beneficial effects of epo upon liver ischemia may involve both circulating as well as local epo, since the liver is the site of epo production during the early neonatal period, but also serves as a site of extra-renal epo production during in severe anemic states.

The clinical implications for epo were readily apparent and resulted in recombinant epo becoming one of the earliest biotechnology-derived blockbuster products for the treatment of anemia. A genetically engineered form of epo has recently been developed that markedly increases the half-life of epo and results in reduced dosing frequency. Other derivatives that lack the erythrogenic properties, but retain the cytoprotective properties are also being tested extensively in animal models.

Although epo holds tremendous promise as a single agent for cytoprotection, epo is only about half as effective as hypoxic preconditioning. Regulation of epo levels occurs primarily at the level of transcription. Epo gene expression is increased in response to hypoxia and is elevated in clinical states associated with reduced blood oxygen levels or with impaired oxygen sensing by the kidney. Control of epo gene expression is by members of the Hypoxia Inducible Factor (HIF) family, a set of transcription factors whose activity is regulated in an oxygen-dependent manner. There are therefore likely additional factors regulated in a HIF-dependent manner that are important for cytoprotection. There is currently interest in develop of HIF agonists that may prove even more potent than epo at preventing injury in disease states affected by ischemia or other conditions positively affected by epo.

V. References

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