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MINI REVIEW

Erythropoietin produced by the retina: its role in physiology and diabetic retinopathy

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Abstract Erythropoietin (Epo) is the principal regulator of erythropoiesis by inhibiting apoptosis and by stimulating the proliferation and differentiation of erythroid precursor cells. However, Epo also performs extra-erythropoietic actions of which the neuroprotective effects are among the most relevant. Apart from kidney and liver, Epo is also produced by the brain and the retina. In addition, Epo receptor (Epo-R) expression has also been found in the brain and in the retina, thus suggesting an autocrine/paracrine action which seems essential for the physiological homeostasis of both brain and retina. In this review, we will give an overview of the current concepts of the physiology of Epo and will focus on its role in the retina in both normal conditions and in the setting of diabetic retinopathy. Finally, the reasons as to why Epo could be contemplated as a potential new treatment for the early stages of diabetic retinopathy will be given.

Keywords Diabetic retinopathy · Retina · Erythropoietin · Erythropoietin receptor · Neuroprotection · Neurodegeneration

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Introduction

It is more than a century since Carnot and Deflandre [1] postulated that a humoral factor, which they called "hemopoietine," regulates red blood cell production. Proofs establishing that (1) the kidney is the primary site of Epo production [2–5], (2) peritubular capillary intersticial cells in the kidney are the renal cells that produce Epo [6, 7], and (3) the liver is a secondary site of Epo production [8, 9] are illustrative of the major advances in Epo research. Another landmark in this field occurred when Miyake et al. [10] reported purification to homogeneity of human Epo. This paved the way for Lin et al. [11] and Jacobs et al. [12] to clone the gene for EPO and to develop a transfected cell line in Chinese hamster ovary cells that provided recombinant Epo for use in clinical anemias.

In this review, we will give an overview of the current concepts of the physiology of Epo and will focus on its role in the retina in both normal conditions and in the setting of diabetic retinopathy (DR).

Physiological actions of Epo

Epo, a 30.4 kDa glycoprotein hormone, is the principal regulator of erythropoiesis by inhibiting apoptosis and by stimulating the proliferation and differentiation of erythroid precursor cells [13]. However, Epo also has extra-erythropoietic actions. The clearest evidence of this is that both Epo and its specific receptor (Epo-R) are expressed in different tissues such as the nervous system. In the recent past, a wide variety of experimental studies have shown that Epo exerts a remarkable neuroprotective effect on both cell cultures and animal models of several nervous system disorders. Epo can prevent neuronal injury following

hypoxia and also after metabolic, neurotoxic, and excitotoxic stresses in the nervous system [14, 15]. Furthermore, both neuronal and glial cells have been shown to be immunohistochemically positive for Epo [16], suggesting that both these cell types are involved in the synthesis and production of Epo in the nevous system.

In addition, Epo inhibits apoptosis in adult rat cardiomyocytes exposed to hypoxia [17], and there is sufficient evidence to suggest a beneficial role of Epo in experimental models of both coronary ischemia [18, 19] and ischemia–reperfusion [17].

Epo-R is also expressed in endothelial cells, and it has been reported that Epo stimulates signaling and mitosis in endothelial cells [20–22]. In addition, it has been shown that Epo administration has a protective effect on endothelial cells [23] and confers vascular stability [24].

Sites of production, regulation, and signaling pathways

Epo is initially produced in the liver but shortly after birth, its production is shifted to the kidney [25]. In the kidney, Epo is produced mainly by peritubular capillary lining cells of the renal cortex, which are highly specialized epithelial-like cells.

Regulation mainly relies on a feed-back mechanism based on oxygen saturation through the activation of hypoxia-inducible factors (HIFs) [26]. HIFs act as cellular oxygen sensors conferring an adaptive response to decreased oxygen tension through the transcription and translation of Epo. Both HIF-1 and HIF-2 have been postulated as controlling Epo gene expression. However, it seems that HIF-2 (rather than HIF-1) is the main physiological transcription factor inducing Epo expression during hypoxia [27]. Interleukin-1 and tumor necrosis factor-alpha inhibit Epo production in vitro, and it has been suggested that these cytokines may contribute to the anemia of inflammatory diseases [28]. However, further study is necessary to determine whether these cytokines play a physiological role in a negative feedback system in the day-to-day control of Epo production.

Epo exerts its actions through Epo-R. Epo-R belongs to the cytokine receptor type I superfamily and is activated via homodimerization. When Epo binds Epo-R, it causes dimerization of the receptor, autophosphorylation, and activation of Janus kinase 2 (JAK2), which can initiate multiple signal transduction pathways associated with cell survival [7]. Of particular interest is the activation of the protein kinase B (PKB)/AKT pathway, which has been shown to stimulate anti-apoptotic signals that facilitate the inhibition of mitochondrial cytochrome *c* release and to help maintain mitochondrial membrane potential [23]. The Ras/MAP kinase pathway, which is involved with cell proliferation has also been found to be activated by Epo [29, 30]. Epo is also well known to activate phosphatidylinositol-3-kinase [PI(3)K], and STAT1, STAT3, STAT5A, and STAT 5B [31–36], especially in cytokine-induced signaling pathways. Finally, the stem cell factor (SCF) or c-kit is also known to interact with Epo-R [37].

Very recently, an additional receptor for Epo that mediates tissue protection has been identified. This is a heteromer composed of Epo-R and CD131—the β common receptor (βcR) [38]. Downstream signaling pathways activated by this receptor included Akt, ERK 1/2, PI(3)K, and STAT 3 [39]. It is worth mentioning that a 1–10 pM range is needed for the erythropoiesis mediated by Epo-R activation. By contrast, the tissue protective receptor exhibits a lower affinity for Epo (2–20 nM) and, therefore, does not respond to Epo at concentrations present within the circulation, but only to high levels of locally produced Epo [40].

The retina: a new site of Epo production

Apart from being found in kidney and liver, Epo expression has also been found in the human brain [41] and in the human retina [42]. In the recent past, we have demonstrated that besides Epo its receptor (Epo-R) also is expressed in the adult human retina [43], thus suggesting an autocrine/paracrine action. Epo and EpoR mRNAs are significantly higher in the retinal pigment epithelium (RPE) than in the neuroretina [43] (Fig. 1). In addition, intravitreal levels of Epo are $\sim$3.5-fold higher than those found in plasma [42]. The role of Epo in the retina remains to be elucidated, but it seems that it has a potent neuroprotective effect [44–47].

There are several reasons for pointing to Epo as a significant factor in the physiological homeostasis of the retina. First, it has been demonstrated using an in vitro model of the bovine blood–brain barrier (BBB) that Epo protects against vascular endothelial growth factor (VEGF)-induced permeability of the BBB and restores the tight junction proteins [48]. Epo treatment also prevents an increase in BBB permeability in a rat model of induced seizures [49]. Because the blood–retinal barrier (BRB) is structurally and functionally similar to the BBB, it is possible that Epo acts as an antipermeability factor in the retina. In fact, Epo was able to improve diabetic macular edema when it was administered for treatment of anemia in diabetic patients with renal failure [50]. Second, there is growing evidence that Epo is a neurotrophic factor not only in the brain but also in the retina [44, 45]. Third, Epo exerts an anti-inflammatory effect on the brain [51], and this action might also be extrapolated to the diabetic retina. Fourth, it has been reported that the retinal expressions of Epo and its

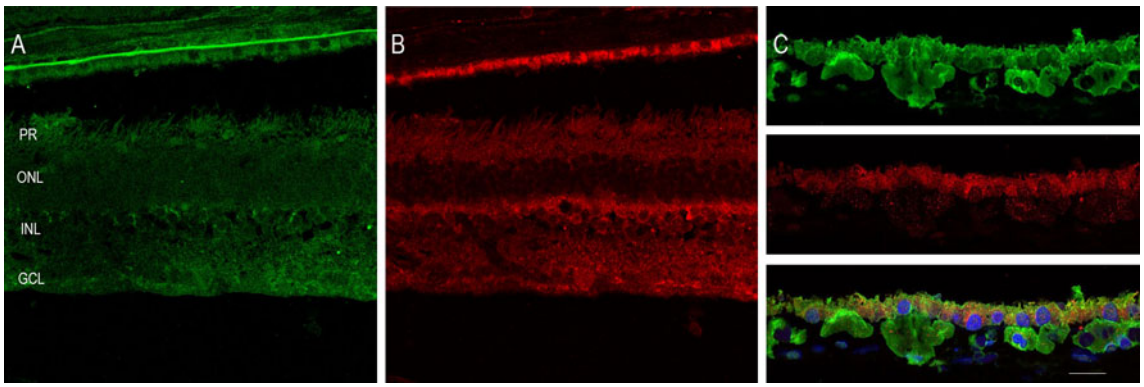

Fig. 1 Confocal laser scanning microscope images (FV 1000; Olympus, Hamburg, Germany) showing Epo and Epo-R immunohistochemical staining of human retina and retinal pigment epithelium (RPE) paraffinized sections. **a** Section incubated with an anti-Epo primary antibody (Santa Cruz Biotechnology, Heidelberg, Germany), followed by incubation with secondary antibody (ALEXA-488, Mol. Probes, OR). **b** Section incubated with anti Epo-R primary antibody (Santa Cruz Biotechnology, Heidelberg, Germany), followed by incubation with secondary antibody (ALEXA-568, Mol. Probes, OR). **c** RPE detail with the anti-Epo antibody (*upper*), anti-Epo-R (*middle*), and merged images with DAPI (to visualize nuclei) showing some degree of co-localization (*lower*). *PR* photoreceptors, *ONL* outer nuclear layer, *INL* Inner nuclear layer, *GCL* ganglion cell layer. The *bar* represents 20 μm

receptor (Epo-R), as well as Janus kinase 2 phosphorylation, are each tightly linked to a specific duration after oxidative stress in anticipation of daily light onset. This is consistent with physiological protection against daily light-induced, oxidatively mediated retinal apoptosis [52]. Finally, Epo is a potent physiological stimulus for the mobilization of endothelial progenitor cells [53], and, therefore, it could play a relevant role in regulating the traffic of circulating endothelial progenitor cells toward injured retinal sites.

Role of Epo in the development of diabetic retinopathy

Epo is upregulated in DR [42, 43, 54, 55]. Epo overexpression has been found in both the RPE and neuroretina of diabetic eyes [42, 43]. This is in agreement with the elevated concentrations of Epo found in the vitreous fluid of diabetic patients (~30-fold-higher than plasma and ~10-fold higher than in non-diabetic subjects) [42]. The retina is the most metabolically active tissue in the human body and, therefore, is highly sensitive to reductions in oxygen tension. Hypoxia is a major stimulus for both systemic and intraocular Epo production. In fact, high intravitreous levels of Epo have recently been reported in ischemic retinal diseases such as proliferative DR (PDR) [42, 54, 55]. In addition, it has been reported that Epo has an angiogenic potential equivalent to VEGF [55, 56]. Therefore, Epo could be an important factor involved in stimulating retinal angiogenesis in PDR. However, intravitreal levels of Epo have been found at a similar range in both PDR and DME (a condition in which hypoxia is not a predominant event). In addition, intravitreal Epo levels are not elevated in non-diabetic patients with macular edema secondary to retinal vein occlusion [57]. Finally, a higher expression of Epo has been detected in the retinas from diabetic donors at early stages of DR in comparison with non-diabetic donors, and this overexpression is unrelated to mRNA expression of hypoxic inducible factors (HIF-1 alpha and HIF-2 alpha) [43]. Therefore, stimulating agents other than hypoxia/ischemia are involved in the upregulation of Epo that exists in the diabetic eye.

Apart from hypoxia, other factors could regulate Epo expression. Watanabe et al. [56] observed an increase in the vitreous Epo levels in patients with inflammatory eye diseases. Given that inflammation has been involved in the pathogenesis of DR [58], this might be a contributing factor to the high levels of Epo observed in diabetic patients. Hyperglycemia could be another factor that induces Epo production. Although there are no studies evaluating the effect of glucose on Epo expression in retinal cells, a direct relationship has been shown between glucose and Epo concentrations in a Chinese hamster ovary cell line [59].

A reduction in Epo catabolism could also contribute to the higher Epo levels detected in the retina and the vitreous fluid from diabetic donors. In this regard, the glycosylation of Epo reduces its affinity for Epo-R [60]. Because Epo is degraded only by Epo-R-expressing cells and their receptor binding determines the rate of intracellular degradation [61], it is possible that a higher degree of Epo glycosylation is associated with lower clearance of Epo.

The consequences of Epo overexpression in DR remain to be elucidated but the bulk of the available information points to a protective effect rather than a pathogenic effect, at least in the early stages of DR. Retinal neurodegeneration is an early event in DR, and therefore, it is possible that higher production of Epo is needed as a neuroprotective factor. In fact, there are a lot of experimental studies

demonstrating the beneficial effects of Epo in front of diverse neurologic insults such as ischemia/hypoxia [62], photo-oxidation [63], and increase of intraocular pressure [64]. Interestingly, Layton et al. [65] demonstrated in primary retinal cultures that the neurotrophic function of Epo is attenuated at glucose concentrations similar to those which occur in diabetes. Recently, it has been demonstrated that exogenous Epo administration by intravitreal [66] or intraperitoneal injection [67] in early diabetes may prevent structural vascular and neural damage in STZ-DM rats. In addition, intravitreal Epo administration is able to upregulate BDNF and CNTF expression, two well-recognized neurotrophic factors [68].

Epo also protects RPE cells against the increase of permeability induced by diabetic conditions, and this effect is mainly mediated by the downstream signaling of JAK2 and PI3/AKT pathways. Moreover, Epo treatment leads to an increase of intracellular free Ca^{2+} in RPE cells by inducing the influx from the extracellular space. This effect could contribute to the protective effects of Epo on the barrier function of RPE cells and is also mediated by the JAK2 and PI3/AKT pathways [69].

Apart from its antipermeability, anti-inflammatory, and neuroprotective actions, Epo protects against high-glucose-induced apoptosis as well as the deleterious effect of free radicals [70, 71].

Finally, as previously mentioned, Epo is a potent physiological stimulus for the mobilization of endothelial progenitor cells (EPCs) and, therefore, it could play a significant role in regulating the traffic of circulating EPCs toward injured retinal sites [24]. For all these reasons, the increase of intraocular synthesis of Epo that occurs in DR can be contemplated as a compensatory mechanism to restore the damage induced by the diabetic milieu rather than a pathogenic contributor.

Nevertheless, in advanced stages, when retinal hypoxia is a predominant event and high levels of VEGF exist, Epo could enhance the effects of VEGF, thus contributing to neovascularization and, in consequence, worsening PDR [24–72]. In addition, in these advanced stages, the recruitment of EPCs might aggravate retinal neovascularization [73].

Therefore, endogenous Epo might act as a double-edged sword in the pathogenesis of DR. To circumvent these undesirable effects, the Epo molecule has been successfully altered to selectively eliminate erythropoietic and prothrombotic potencies, while preserving tissue-protective activities [40]. In this regard, it has been demonstrated that intraperitoneal administration of a peptide based on the Epo helix-B domain inhibits diabetes-related retinal edema in rats with diabetes induced by streptozotocin [74]. Furthermore, McVicar et al. [75] demonstrated that this treatment (helix B peptide of Epo) significantly protects against neuroglial and vascular degenerative pathology in rats with diabetes induced by streptozotocin. Notably, this treatment had no effect on retinal neovascularization.

Concluding remarks and future insights

The neuroprotective properties of Epo, its effects in preventing microvascular damage in the diabetic retina, as well as its capacity to protect the barrier function of RPE cells, are solid reasons for proposing Epo or Epo-R agonists as new therapeutic agents in the treatment of the early stages of DR. When the early stages of DR are the therapeutic target, it would be inconceivable to recommend an aggressive treatment such as intravitreous injections. The use of eye drops has not been considered an appropriate route for the administration of drugs aimed at preventing or arresting DR because of the general assumption that they do not reach the posterior chamber of the eye (i.e., the vitreous and the retina). However, there is emerging evidence to show that a lot of drugs are able to reach the retina in pharmacological concentrations, at least in animal models [76–79]. In addition, topical administration of drugs limits their action to the eye and minimizes the associated systemic effects [80]. However, to the best of our knowledge, there are no experimental or clinical studies using eye drops of Epo for treating DR. Alternatively, the systemic administration of Epo-derived peptides without capacity to increase hematocrit or to exacerbate neovascularization but retaining tissue-protective properties against neuroglial and vascular degenerative pathology could be a new approach for preventing or arresting this devastating complication of diabetes [75].

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