EXPRESSION OF ERYTHROPOIETIN AND ITS RECEPTOR IN THE HUMAN RETINA. A comparative study of diabetic and non-diabetic subjects

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ABSTRACT

Objective: To evaluate Epo and Epo receptor (EpoR) expression in the retina and in vitreous fluid from diabetic and non-diabetic donors. In order to gain insight into the mechanisms responsible for the regulation of Epo production in the retina, we also assessed retinal expression of hypoxia inducible factors (HIF-1α and HIF-2α).

Research Design and Methods: Eighteen postmortem eyes from 9 diabetic patients without clinically detectable retinopathy were compared with 18 eyes from 9 non-diabetic donors. mRNA of Epo, HIF-1α and HIF-2α (Q-RT-PCR) were measured separately in neuroretina and retinal pigment epithelium (RPE). Epo and EpoR were assessed in the retina (immunofluorescence by confocal laser microscopy) and in the vitreous fluid (RIA and ELISA, respectively).

Results: Epo and EpoR mRNAs were significantly higher in RPE than in the neuroretina. A higher expression of Epo was detected in the retinas (both in the RPE and in the neuroretina) from diabetic donors. By contrast, EpoR expression was similar in both groups. We did not find any difference in HIF-1α and HIF-2α mRNA expression between diabetic and non-diabetic donors (both in RPE and neuroretina). Intravitreal Epo concentration was higher in diabetic donors than in non-diabetic controls. However, EpoR concentrations were similar in both groups.

Conclusions: Epo overexpression is an early event in the retina of diabetic patients and this is not associated with any change in EpoR. At this early stage, other factors apart from hypoxia seem to be more important in accounting for the Epo up-regulation that exists in the diabetic retina.
Erythropoietin (Epo) was first described as a glycoprotein produced exclusively in fetal liver and adult kidney that acts as a major regulator of erythropoiesis (1). However, Epo expression has been found in the human brain (2) and recently, we have demonstrated that Epo mRNA also exists in the adult human retina (3). In the brain as well as in the retina Epo has a potent neuroprotective effect (4, 5).

The retina is the most metabolically active tissue in the human body and, therefore, is highly sensitive to reduction in oxygen tension. Hypoxia inducible factor (HIF) is the primary hypoxic signalling protein in cells for regulating angiogenesis and is able to induce the transcription of more than 70 genes, such as Epo (6). Indeed, HIF was discovered during studies of the regulation of Epo (7). Hypoxia is a major stimulus for both systemic (1) and intraocular Epo production (8), and high intravitreous levels of Epo have been reported in ischemic retinal diseases such as proliferative diabetic retinopathy (PDR) (9-11). In addition, it has been reported that Epo has an angiogenic potential equivalent to VEGF (11, 12). However, we also found elevated intravitreal levels of Epo in patients with diabetic macular edema (DME), a condition in which neither hypoxia nor angiogenesis are predominant events (3). Therefore, other factors than ischemia must be involved in the primary regulation of Epo production in the human retina. Regarding the Epo receptor (EpoR), its expression has been detected in the mouse retina (8, 13) and in the human fetal retina (14) but until now it has not been investigated in the adult human retina.

In a previous study we found a higher expression of Epo mRNA in the retina from diabetic than from non-diabetic subjects (3). In the present study, we have evaluated not only Epo but also EpoR expression (mRNA and protein) in the early stages of diabetic retinopathy. In addition, intravitreal levels of Epo and EpoR have also been measured. Furthermore, to gain insight into the mechanisms responsible for the regulation of Epo production in the retina, we also assessed the retinal expression of hypoxia inducible factors (HIF-1α and HIF-2α).

**RESEARCH DESIGN AND METHODS**

**Human retinas.** Eighteen human postmortem eyes were obtained from 9 diabetic donors (age: 64.1±8.1 years) free of fundoscopic abnormalities in the ophthalmologic examinations performed during the preceding two years. Eighteen eye cups obtained from 9 non-diabetic donors matched by age (age: 66.9 ± 6.8 years) were used as the control group. The time elapsed from death to eye enucleation was 3.4±1.9 h. After enucleation, one eye of each donor was snap frozen in liquid nitrogen and stored at –80º until assayed for mRNA analysis. The other was fixed in 4% paraformaldehyde and embedded in paraffin for the immunohistochemical study. The clinical features of diabetic and non-diabetic donors included in the study are shown in table 1 (online appendix). Three patients who were under treatment with diet and/or oral agents were changed to insulin after admission, and the two patients who were under insulin plus metformin were maintained with insulin alone. All patients died within 15 days after admission.

All ocular tissues were used in accordance with applicable laws and with the Declaration of Helsinki for research involving human tissue. In addition this study was approved by the Ethics Committee of our hospital.

**RNA extraction and Epo mRNA quantification.** Human neuretina and retinal pigment epithelium (RPE) were harvested under the microscopic dissection of isolated eye cups from donors. Total RNA was extracted from isolated retinal tissues using the RNeasy®Mini Kit with DNAse digestion (Quiagen Distributors, IZASA, Barcelona, Spain) according to the manufacturer's instructions. Quantification and quality of total mRNA were determined with a Bioanalyzer in a LabChip (Agilent...
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Technologies, USA). One-microgram of total RNA was directly used for reverse transcription which was carried out with the TaqMan® Reverse Transcription Reagents (Applied Biosystems, Madrid, Spain) in a 50 µl reaction according to the manufacturer’s instructions. Quantitative Real-Time PCR was performed using 60 ng of the reverse transcription reaction as a template with the TaqMan Universal Mastermix (Applied Biosystems). All reactions were conducted as follows: 95º 10 min and 50 cycles of 15 s at 95º and 1 min at 60º in an Applied Biosystem 7000 equipment. Each sample was assayed in duplicate and control water samples were included in each experiment. Automatic Relative Quantification data was obtained in an ABI Prism 7000 SDS software (Applied Biosystem) using β-actin gene as the endogenous expression reference gene (Hs9999903_m1, Applied Biosystem). TaqMan® pre-made gene expression assays (Applied Biosystem) were used in order to amplify human Epo (Hs00171267_m1 GenBank accession number NM 000799.2) and the Epo-R (Hs00181092_m1 Exon Boundary 6-7, GenBank accession number NM 000121.2).

HIF mRNA expression. HIF-1α, Hif-2α, mRNA transcripts were studied in the same samples with the TaqMan® pre-made gene expression assays (Applied Biosystem) were used in order to amplify human Epo (Hs00171267_m1 GenBank accession number NM 000799.2) and the Epo-R (Hs00181092_m1 Exon Boundary 6-7, GenBank accession number NM 000121.2).

Glial fibrillar acidic protein (GFAP) mRNA expression. GFAP mRNA was assessed in neuroretinas using TaqMan® pre-made gene expression assays (Hs00157674_m1; Applied Biosystem, Madrid, Spain).

Epo and EpoR immunofluorescence. Paraffinized eyes were serially cut at 7-µm thickness. Sections were deparaffinized with xylene and rehydrated in ethanol. Sections were placed in antigen-retrieval solution (Dako A/S, Glostrup, Denmark) at 95°C. In order to eliminate the autofluorescence of RPE due to melanin and lipofuscin we used a method described elsewhere (15). Sections were then incubated for 1 h with 1% BSA in 0.3% Triton X-100 in PBS to block unspecific binding of the antibodies and then incubated overnight at 4 ºC with a specific primary antibody to human Epo (1:100, N-19, Santa Cruz Biotech, Germany) and Epo-R (1:200, M-20, Santa Cruz Biotech, Germany). After washing, sections were incubated with Alexa Fluor® 488 and 594, respectively (Molecular Probes; Eugene, OR, USA) secondary antibody at room temperature for 1 h. Slides were cover-slipped with a drop of mounting medium containing DAPI for visualization of cell nuclei (Vector laboratories, Burlingame, USA).

Image Acquisition. Images were acquired with a confocal laser scanning microscope (FV1000, Olympus. Hamburg, Germany), using a 488-nm laser line for Epo and a 594-nm laser for Epo-R. Each image was saved at a resolution of 1024 X 1024 pixel image size.

Image analysis. In order to quantify Epo and Epo-R immunofluorescence in RPE and the neuroretina the total fluorescence intensity values corresponding to ten field frame images (40X NA: 0.9) of each retina sample were measured. These results were then normalized taking into account the area analyzed. All these calculations were made using a specific software (Fluoview ASW 1.4).

Glial fibrillar acidic protein (GFAP) immunofluorescence. Tissue sections were incubated overnight at 4ºC with the primary antibody anti-human GFAP (1:200, SIGMA, Madrid, Spain). After washing, sections were incubated with Alexa Fluor® 488 (Molecular Probes; Eugene, OR, USA) secondary antibody for 1 h. GFAP immunofluorescence in the neuroretina was quantified using a laser confocal scanning microscope. The procedure was the same as mentioned above for Epo and Epo-R.

Epo and EpoR assessment in vitreous fluid. Epo was assessed by RIA (Incstar-Diasorin). The lowest limit of detection was 4.4 mU/ml. EpoR was measured by ELISA
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In the human retina, vitreal proteins were measured by a previously validated microturbidimetric method with an autoanalyzer (Hitachi 917; Boehringer, Mannheim). This method, based on the benzetonium chloride reaction, is a highly specific method for the detection of proteins and has a higher sensitivity and reproducibility than the classic method of Lowry. The lowest level of proteins detected was 0.02 mg/ml. Coefficients of variation intra and inter-assay were 2.9 and 3.7\%, respectively.

**STATISTICAL ANALYSIS**

Student’s t test was used to compare the variables. Correlations were examined by Spearman’s rank correlation. Levels of statistical significance were set at \( p<0.05 \).

**RESULTS**

_Epo and EpoR mRNAs in the human retina: comparison between diabetic and non-diabetic donors._ $\beta$-actin mRNA expression was similar in both the RPE and the neuroretina (\( p=\text{n.s} \)). In addition no differences were observed in $\beta$-actin mRNA expression between diabetic and non-diabetic retinas (\( p=\text{n.s} \)). Thus, we have calculated mRNA gene expression after normalizing with $\beta$-actin.

A significantly higher Epo mRNA expression was observed in RPE than in the neuroretina in the whole group (1.13±1.01 vs. 0.26±0.31; \( p=0.003 \)). A higher expression was also observed in RPE than in the neuroretina when separately analyzing diabetic donors and non-diabetic donors (1.62±1.16 vs. 0.33±0.24; \( p=0.01 \) and 1.35±1.36 vs. 0.41±0.55; \( p=0.07 \)). However, we did not find any difference between EpoR mRNA levels between diabetic and non diabetic donors in either RPE or the neuroretina (Fig. 1B).

In the neuroretina, but not in RPE, a direct correlation between Epo and EpoR mRNA was detected in diabetic (\( r=0.73; \ p=0.04 \)) and non-diabetic donors (\( r=0.90; \ p=0.001 \)).

HIF-1$\alpha$ and HIF-2$\alpha$ mRNAs expressions were similar for diabetic and non diabetic donors in both RPE (HIF-1$\alpha$: 0.44±0.26 vs. 0.42±0.37; \( p=0.89 \). HIF-2$\alpha$: 0.43±0.26 vs. 0.48±0.41; \( p=0.82 \)) and the neuroretina (HIF-1$\alpha$: 0.46±0.24 vs. 0.36±0.31; \( p=0.36 \). HIF-2$\alpha$: 0.29±0.16 vs. 0.24±0.21; \( p=0.58 \)). HIF-2$\alpha$ mRNA correlated with Epo mRNA in diabetic donors (\( r=0.62; \ p=0.03 \)) but not in non-diabetic donors (\( r=0.26; \ p=0.33 \)).

We did not find any significant differences in Epo and EpoR mRNA levels between patients who were changed to insulin treatment after admission and patients who remained under oral treatment.

_Epo content in the human retina: comparison between diabetic and non-diabetic donors._ Laser scanning confocal images of Epo and EpoR immunofluorescence are displayed in Fig. 2 and 3. Epo immunofluorescence intensity was higher in diabetic donors than in non-diabetic donors (5770±1491 vs. 3933±1530; \( p=0.05 \)) and in the neuroretina (5644±3671 vs. 3671±1401; \( p=0.06 \) (Fig. 2).

Epo immunofluorescence was higher in RPE than in the neuroretina in both diabetic donors (8883±2171 vs. 4734±2474; \( p<0.001 \)) and non-diabetic donors (9079±6260 vs. 4763±3680; \( p=0.07 \)). However, no differences in EpoR were detected between diabetic and non diabetic donors either in the RPE or in the neuroretina (Fig. 3).

A direct correlation was observed between Epo and EpoR in diabetic donors
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(RPE: \( r=0.87; \ p=0.002 \). Neuroretina: \( r=0.86; \ p=0.01 \)) and non-diabetic donors (RPE: \( r=0.94; \ p=0.005 \). Neuroretina: \( r=0.24; \ p=0.5 \)).

**Neuroglial activation.** GFAP mRNA expression was higher in the neuroretina from diabetic donors than non-diabetic donors (0.40 ±0.21 vs. 0.13±0.14; \( p=0.04 \)). Confocal microscopy revealed a higher immunofluorescence intensity in the neuroretinas from diabetic donors (10148±3909 vs. 6798±1544; \( p= 0.04 \)) (Fig. 4), thus confirming that neuroglial activation does exist.

Epo and EpoR assessment in vitreous fluid. Comparison between diabetic and non-diabetic retinas. Intravitreal Epo concentration was higher in diabetic donors than in non-diabetic controls (124 mU/ml ± 92 vs. 36 mU/ml ± 13; \( p=0.03 \)). However, no differences in intravitreal EpoR concentration were detected between diabetic donors and non-diabetic controls (99 pg/ml±58 vs. 96 pg/ml ± 86; \( p= 0.95 \)). Since intravitreal proteins were similar in diabetic and in non diabetic donors, the results were not influenced by this confounding factor.

**CONCLUSIONS**

In the present study we have found a higher expression of Epo (both mRNA and protein) in the retinas from diabetic donors without clinically detectable diabetic retinopathy in comparison with the retinas from non-diabetic donors. In addition, intravitreal Epo concentrations were significantly higher in diabetic donors than in non diabetic donors. Therefore, our results suggest that Epo overexpression is an early event in the retina of diabetic patients. It is important to note that in the retinas from diabetic donors reactive changes in Müller cells such as up-regulation of GFAP were present. Therefore, although the retinas from diabetic donors were without overt vascular abnormalities, they were actually already being damaged by the diabetic milieu. In fact, glial activation and neurodegeneration have been described as early events in the pathogenesis of diabetic retinopathy (16).

The complete mechanisms that regulate Epo expression in the human retina remain to be elucidated. The hypoxia-induced up-regulation of Epo expression is modulated at the transcriptional level by HIF and in particular by HIF-2α (17). In the present study, no differences between HIF-1α and HIF-2α were detected between diabetic and non-diabetic donors. These findings suggest not only that Epo overexpression is an early event in the retina of diabetic patients but also that at this stage it is unrelated to an hypoxic-ischemic stimulus. Supporting this concept, it should be stressed that EpoR expression, which is up-regulated by hypoxia in rat brains (18) and in rat retinas (8), was similar in diabetic and in non-diabetic retinas. It should be noted that EpoR expression has not been previously described in human retina.

Apart from hypoxia, other factors could regulate Epo expression. Watanabe et al (11) observed an increase in the vitreous Epo levels in patients with inflammatory eye diseases. Given that inflammation has been involved in the pathogenesis of diabetic retinopathy (19), it 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 (CHO) cell line (20).

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 EpoR (21). Since Epo is degraded only by EpoR-expressing cells, and their receptor binding determines the rate of intracellular degradation (22), it is possible that the higher the degree of Epo glycosylation the lower the clearance of Epo.
The mechanisms why Epo is increased in the diabetic retina can not be answered in the present study. However there are many reasons for thinking that in these early stages of diabetic retinopathy Epo might have beneficial rather than pathogenic actions. First, it has been demonstrated using an in vitro model of the bovine blood-brain barrier (BBB), that Epo protects against the VEGF-induced permeability of the BBB and restores the tight junction proteins (23). Epo treatment also prevents an increase in the BBB permeability in a rat model of induced seizures (24). Since the blood-retinal barrier (BRB) is structurally and functionally similar to the BBB, it is possible that Epo could act as an anti-permeability factor in the retina. In fact, Epo was able to improve DME when administered for treatment of anemia in diabetic patients with renal failure (25). Second, there is growing evidence that Epo is a neurotrophic factor not only in the brain but also in the retina (4, 5). Third, Epo exerts an anti-inflammatory effect on the brain (26) and this action might also be extrapolated to the diabetic retina. Finally, Epo is a potent physiologic stimulus for the mobilization of endothelial progenitor cells (EPCs) (27) and, therefore, it could play a relevant role in regulating the traffic of circulating EPCs towards injured retinal sites.

We conclude that Epo overexpression is an early event in the retina of diabetic patients and that at this stage it is not mediated by HIF. This finding suggests that other factors besides hypoxia might be crucial in the upregulation of Epo in the initial stages of diabetic retinopathy. EpoR is also expressed in the retina from diabetic patients but it is similar in non-diabetic subjects. Further studies are needed to investigate not only the precise regulation of Epo/EpoR in the retina in physiological and pathological conditions, but also its potential role in the therapeutic armamentarium.

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FIGURE 1. A) Real-time quantitative RT-PCR analysis of Epo mRNA in human retinas. Bars represent the mean±SD of the values obtained in the nine diabetic and the nine non-diabetic donors studied. Epo mRNA gene expression was calculated after normalizing with β-actin. B) Real-time quantitative RT-PCR analysis of EpoR mRNA in human retinas.
FIGURE 2. A) Comparison of Epo immunofluorescence (green) in the human retina between representative samples from a diabetic donor (a) and non-diabetic donor (b). RPE, retinal pigment epithelium; PR, photoreceptors; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. The bar represents 20 µm. B) Quantification of Epo immunofluorescence in non-diabetic and diabetic retinas.
FIGURE 3. A) Comparison of EpoR immunofluorescence (red) in the human retina between representative samples from a diabetic donor (a) and non-diabetic donor (b). RPE, retinal pigment epithelium; PR, photoreceptors; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. The bar represents 20 µm. B) Quantification of EpoR immunofluorescence in non-diabetic and diabetic retinas.
FIGURE 4. A) Comparison of GFAP immunofluorescence (green) in the human retina between representative samples from a non-diabetic donor (a) and a diabetic donor (b). PR, photoreceptors; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. In the diabetic retina, the Müller cells’ endfeet show abundant GFAP immunofluorescence, and the radial processes stain intensely throughout both the inner and outer retina. The bar represents 20 µm. B) Quantification of GFAP immunofluorescence in non-diabetic and diabetic retinas.