Anti-VEGF effects of intravitreal erythropoietin in early diabetic retinopathy

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1. ABSTRACT

In the present study, a single intravitreal erythropoietin (EPO) to diabetic rats produced therapeutic effects on blood-retinal barrier (BRB) function and neuronal survival at different time courses of retinopathy. In parallel, the hypoxia-inducible factor 1 alpha (HIF-1 alpha) pathway has been quantitatively studied, including VEGF-A, endogenous EPO, EPO receptor (EpoR), prolyl hydroxylases (PHD1-3) and von Hippel-Lindau tumor suppressor (VHL). The mRNA levels of HIF-1 alpha, VEGF-A, endogenous EPO, PHD1-3 and VHL are all up-regulated in the diabetic retina, and suppressed by exogenous EPO. The increased protein levels of HIF-1 alpha, VEGF-A, and endogenous EPO found in diabetic retinas also have been down-regulated by exogenous EPO. The results demonstrate that the HIF-1 pathway is activated in the retina in early diabetes, but is negatively regulated by a feedback loop following the administration of exogenous EPO. Exogenous EPO at pharmacologic levels leads to suppression of VEGF and in turn, restoration of the normal functions of BRB in a time-dependent manner. In the diabetic retina, the same level of exogenous EPO that inhibits VEGF also exerted neuronal protection.

2. INTRODUCTION

Diabetic retinopathy (DR) is not only a microangiopathy but also is a neuronopathy (1-3). The early pathological processes of DR represent both blood-retinal barrier (BRB) breakdown and retinal neuron apoptosis (4, 5). Therefore, development of effective interventions for both processes, early as well as throughout their course, is imperative for treating diabetic retinopathy. Our previous report demonstrates that exogenous erythropoietin (EPO), when administered at the onset of diabetes, showed a cytoprotective effect on retinal neurons, and could maintain BRB integrity (4). Since the mechanisms of neuroprotection by EPO have been extensively studied (4, 6-8), the present study is focused on the underlying mechanism of BRB protection by EPO.

Increased vascular endothelial growth factor (VEGF) is found in the vitreous of patients with macular edema accompanying diabetic retinopathy (9, 10). In diabetic animal models, VEGF immunoreactivity was markedly increased and was observed in all layers of the retina, especially in the peri-vascular area, which is

associated with the breakdown of BRB (11). It has been well documented that VEGF is both a potent enhancer of vascular permeability and an essential inducer of angiogenesis (12). The regulatory mechanism for VEGF production functions through a HIF-1 alpha dependent pathway (13, 14), and a HIF-1 alpha independent pathway (15). Under hypoxic conditions, the HIF-1 pathway is activated to promote the transcription of target genes such as VEGF, EPO and inducible nitric oxide synthase (iNOS) (14, 16, 17). Inhibition of HIF-1 activity by iNOS has been demonstrated under hypoxia in C6 glioma cells transfected with an iNOS gene and a VEGF promoter-driven luciferase gene (18). Herein we hypothesize that EPO as a downstream product may negatively regulate the HIF-1 pathway. The present study demonstrates that exogenous EPO inhibits VEGF production through negative feedback on the HIF-1 alpha pathway, thereby regulating enzymes of the HIF-1 alpha pathway in the diabetic retina.

3. MATERIALS AND METHODS

3.1 Reagents

Evans blue (E2129-10G), streptozotocin (S0130-1G), anti-beta-actin antibody (A5441-2ML), and erythropoietin (E5627-10UN) are products of Sigma-Aldrich (Beijing Superior Chemicals and Instruments Co., Ltd., Beijing, China). Cell-viability assay kit (*In situ* Cell Death Detection Kit, Fluorescein) was purchased from Roche China, Ltd. (Shanghai, China). Anti-EPO antibody (H-162), anti-EPO receptor (EpoR) antibodies (M-20), and rabbit anti-VEGF (A-20) were purchased from Santa Cruz Biotechnology (Gene Company Ltd., Shanghai, China). Anti-HIF-1 alpha antibody (MAB 5382) was purchased from Chemicon International, Inc. (Genetimes Technology, Inc., Shanghai, China).

3.2 Experimental animals and intravitreal EPO treatment

Male Sprague-Dawley rats of about 150 g body weight (Slaccas, SIBS, Shanghai, China) were used. The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Diabetes induction and intravitreal injections have been described previously (4). Briefly, for diabetes induction, a single streptozotocin (60 mg/kg BW in citric buffer, pH 4.5) injection was performed after the rats had been fasted for 24 hours. The control rats received an equal volume of citric buffer. Animals receiving streptozotocin were declared diabetic when their blood glucose exceeded 250 mg/dL for three consecutive days. The rats were excluded from the experiment if they failed to develop diabetes. In addition to normal control and diabetic control, the EPO-treated diabetic rats were divided into 3 groups depending on the time point of intravitreal injection of EPO, i.e., early treatment group (intravitreal injection was performed at 1 week after diabetes onset); intermediate treatment group (intravitreal injection was performed at 4 weeks after diabetes onset); and late treatment group (intravitreal injection was performed at 4 to 5 months after diabetes onset). The intervention time was from 4 days to 5 weeks depending on the parameters measured.

Intravitreal injections were performed into both eyes with a 30-gauge, 0.5-in. needle (BD Biosciences, Franklin Lakes, NJ) on a microsyringe (Hamilton, Reno, NV), using a temporal approach, 2 mm behind limbus. EPO, ranging from 8 to 160 mU/eye, was administered in a volume of 2 µL. Sham injections (2 µL normal saline) were performed on both normal control rats and the untreated diabetic rats.

3.3 Examination of BRB permeability

BRB permeability was evaluated according to the published method of Zhang *et al* (4). Briefly, the rats were deeply anesthetized, and Evans blue solution (30 mg/mL) was injected intravenously. Then 0.1 mL blood was drawn at 15-minute intervals up to 2 hours to obtain the time-averaged Evans blue plasma concentration. After the dye had circulated for 2 hours, the chest cavity was opened, and rats were perfused via left ventricle with about 200 mL perfusion buffer (1% paraformaldehyde dissolved in 0.05 M citric acid, pH 3.5, 37 ^{o}C). Immediately after the perfusion, both eyes were enucleated. Two retinas from the same animal were pooled together and dried in a Speed-Vac (37 ^{o}C). After determining the dry weight, the retinas were incubated in 300 µL formamide for 18 hours at 70 ^{o}C. The extract was centrifuged through 30,000 Nominal Molecular Weight Limit (NMWL) centrifuge filter (Microcon, Millipore) at 3,000 g, 4 ^{o}C, for 45 minutes. Both blood samples and retinal extracts were measured with a spectrophotometer (Beckman DU800). Evans blue permeation (EBP), as a function of BRB permeability, was calculated and expressed in (µL plasma × g retinal dry wt^{-1}·hr^{-1}).

3.4. RNA isolation and gene expression determined by real-time PCR

Total RNA was extracted from neurosensory retina. The reverse transcription product was examined by real-time PCR. The specific primers were designed by software Primer Premier 5.0 and purchased from Shanghai DNA Biotechnology Corporation Ltd. (Shanghai, China). The primers for HIF-1 alpha, VEGF-A, EPO, PHD1, PHD2, PHD3, VHL, and beta-actin are shown in Table 1. Real-time PCR was performed in a 10 µL system containing SYBR green real-time PCR master mix (5 µL), ddH$_2$O (3.6 µL), primers (0.5 µM each, 0.2 µL for each primer), and cDNA (1 µL). All reactions were conducted using the PRISM 7900 analyzer (ABI, Foster city, CA, USA). The relative gene expression was represented by ratios of the normalized values of the experimental samples to that of the control. Four samples per group were used at each time point.

3.5. Western blotting analysis for HIF-1 alpha, VEGF, EPO and EPO receptor (EpoR)

Individual retinas from experimental and control rats were used in the study. The detailed methods have been described previously (4). The retinas were homogenized in ice-cold radioimmune precipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 1% sodium deoxycholate) for western blotting. RIPA buffer enables efficient retinal tissue lysis and protein

Table 1 Primer information for real-time PCR

Gene	Sequence (5'→3')		Base pair (bp)	Product size (bp)
beta-actin	Sense	GTAAAGACCTCTATGCCAACA	21	227
	anti-sense	GGACTCATCGTACTCCTGCT	20	
HIF-1 alpha	Sense	ACTATGTCGCTTTCTTGG	18	195
	anti-sense	GTTTCTGCTGCCTTGTAT	18	
VEGF-A	Sense	TTGAGACCCTGGTGGACATC	20	192
	anti-sense	CTCCTATGTGCTGGCTTTGG	20	
EPO	Sense	CTCCAATCTTTGTGGCATCT	20	134
	anti-sense	GGCTTCGTGACCCTCTGT	18	
PHD1	Sense	GCTGCTGCGTTGGTTAC	17	146
	anti-sense	GCCTCCTGGTTCTCTTG	17	
PHD2	Sense	CTGGGACGCCAAGGTGA	17	71
	anti-sense	CAATGTCAGCAAACTGG	17	
PHD3	Sense	GTTCAGCCCTCCTATGC	17	136
	anti-sense	ACCACCGTCAGTCTTTA	17	
VHL	Sense	CCCTACCCGACCTTACCA	18	313
	anti-sense	GCCGCTGTATGTCTTTCC	18	

solubilization while avoiding protein degradation and interference with immunoreactivity. This buffer was supplemented with a protease inhibitor PMSF (Shenergy Bicolor Bioscience Technology Company). Equal amounts of protein were resolved in SDS–polyacrylamide gels and transferred electrophoretically onto a nitrocellulose membrane (Bio-Rad). The membranes were cut into 4 blots according to the size of the detecting proteins, and then were blocked with 5% nonfat milk in TBST (50 mM Tris, pH 7.6; 0.9% NaCl, and 0.1% Tween-20) for 1 hour at room temperature. The blots, after blocking, were separately incubated overnight with anti-HIF-1 alpha antibody (1:1,000), anti-VEGF antibody (1:500), anti-EPO antibody (1:500), or anti-EpoR antibody (1:500) for detection. The blot, after VEGF detection, was stripped and re-probed with anti-beta-actin antibody (1:4,000) for actin detection. The optical density of each band was determined using Quantity One software (Bio-Rad). The densitometric values for the proteins of interest were normalized using beta-actin levels.

3.6. Sample preparation for morphologic studies, and measurement of the retinal thickness and cell counts

Cryosections of retinas were prepared according to the method published previously (4). The rats were killed after deep anesthesia. Both eyes were enucleated and fixed in PBS-buffered 4% paraformaldehyde for 24 hours and then were opened along the ora serrata, and the posterior eyecups were dehydrated through a gradient concentration of sucrose from 10% to 30%. After dehydration, the eyecups were embedded in OCT compound (Tissue Tek; Sakura) for sectioning. Serial sections (10 μm) were cut on a cryostat microtome for the measurements of retinal thickness and cell counts, and TUNEL assay.

The cryosectioned retinas were stained with hematoxylin and eosin. The thickness of the different retinal layers was measured under the 200x magnification, including the (1) outer limiting membrane to inner limiting membrane (OLM-ILM); (2) outer limiting membrane to ganglion cell layer (OLM-GCL); (3) outer nuclear layer and outer plexiform layer (ONL-OPL); (4) inner nuclear layer (INL); and (5) inner plexiform layer (IPL). Two measurements were taken on each section, at the two reference lines, which were 1 mm away from the optic nerve on both sides. The number of cells in the ONL and INL were counted in the same region as the thickness measured, under the 1,000x magnification. All the cell nuclei within a fixed 25-μm

column, centered with the 1 mm reference lines, were counted. The cell density was then expressed as the cell count per millimeter width of retina in the different layers.

3.7. *In situ* detection of cell death in retina by TUNEL assay

A TUNEL assay was performed with a kit (*In situ* Cell Death Detection Kit) according to the manufacturer's instructions. Positive controls were retinal sections that had been treated with grade I DNase-I for 10 minutes at room temperature, before the labeling procedure. Negative controls were the retinal sections treated with 10 μL label solution but incubated in the absence of terminal transferase. The sections (10 μm) were analyzed by fluorescence microscopy (Nikon, Yokohama, Japan), with an excitation wavelength at 450-490 nm.

3.8. Electron microscopy (EM)

The enucleated eyes were opened along the equators and immersed in 2.5% glutaraldehyde with 0.1 M phosphate buffer (pH 7.4) for 24 hours. The eyes were then fixed in 1% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in Epon 618 (TAAB Laboratories Equipment, Berks, UK). Ultrathin sections of the posterior region of the retina were stained with lead citrate and uranyl acetate, and then examined by transmission electron microscopy at 100 kV (CM-120; Philips, Eindhoven, the Netherlands).

3.9. Statistics

Data are expressed as mean +/- S.E.. The statistical analyses were carried out using the Student's *t*-test. A *P*-value of 0.05 or less was considered statistically significant.

4. RESULTS

4.1. EPO protection of retinal neurons from cell death in early diabetes

With the progression of the diabetes from 6 to 9 weeks in the untreated diabetic eyes, TUNEL-positive cells were significantly increased in the ONL (Figure 1A). TUNEL-positive cells were also detected in the INL 6 weeks after the onset of diabetes. No TUNEL-positive cells were detected in either the ONL or INL 5 weeks following
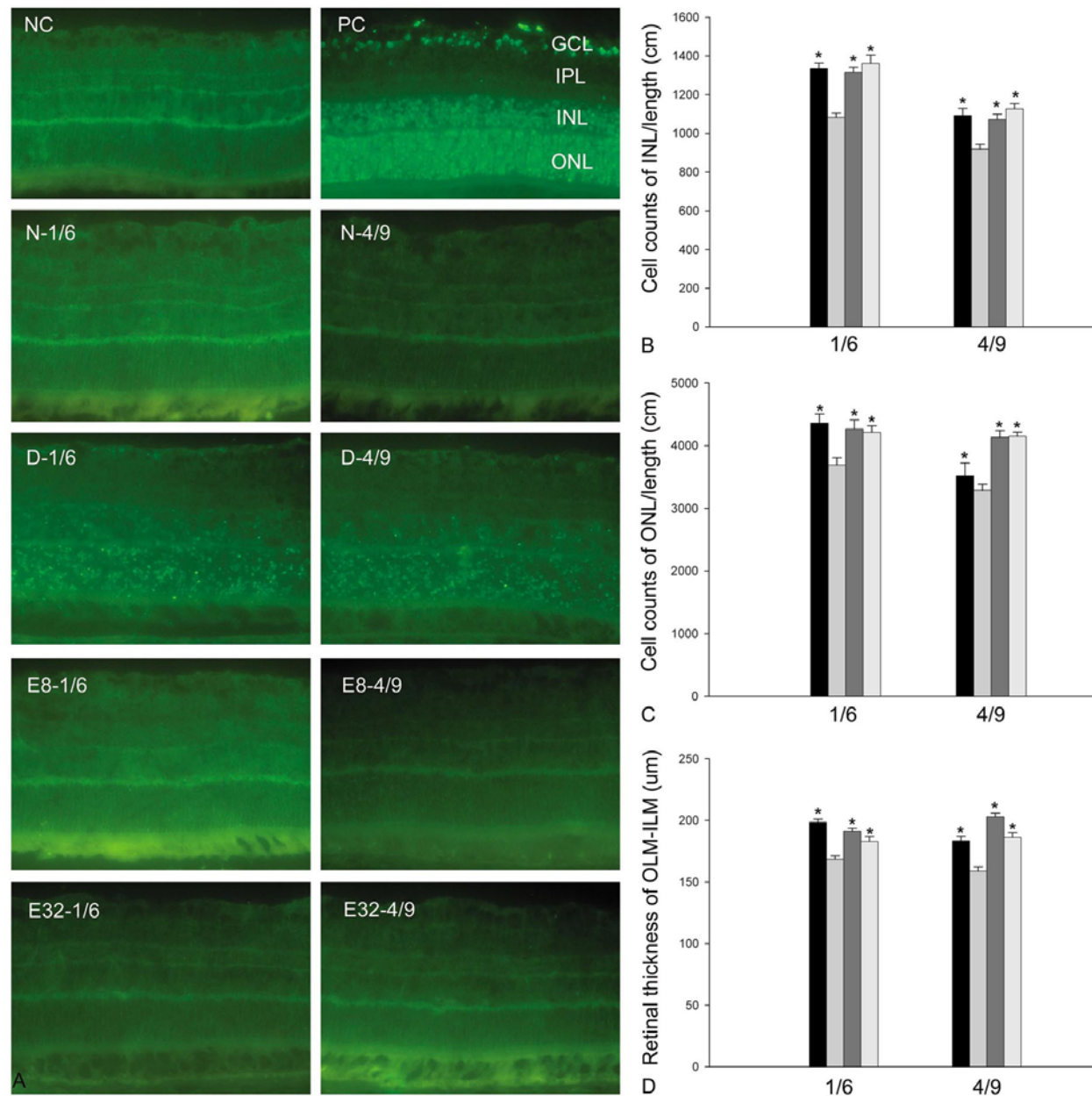
Figure 1. Treatment effects of intravitreal EPO in protecting retina in streptozotocin-diabetic rats. (A): TUNEL staining; (B): retinal cell counts in the INL; (C): retinal cell counts in the ONL. (D): retinal thickness (OLM-ILM). NC: negative control; PC: positive control; N: normal control; D: diabetes; E8 and E32: the dose of intravitreal EPO was 8 mU/eye and 32 mU/eye, respectively. The number 1/6 means the intravitreal EPO injection was performed 1 week after diabetes onset (early treatment) and the rats were killed 6 weeks after diabetes onset. Similarly, 4/9 indicates the rats were treated with EPO in week 4 (intermediate treatment) and killed in week 9. There is no difference in cell counts and retinal thickness measurement between 2 doses of EPO. * means $P<0.05$ when compared with diabetic group.

EPO (8 and 32 mU/eye) treatment when the intravitreal injections were performed after 1 (early treatment) or 4 (intermediate treatment) weeks of diabetes onset (Figure 1A).

In all diabetic groups, and at all time points assessed, there were significantly lower cell numbers in both the INL (Figure 1B) and ONL (Figure 1C) as compared with non-diabetic control eyes. Morphometric examination of the diabetic retinas demonstrated a significant reduction in the thickness of the total retina (Figure 1D) as well as in individual layers (data not shown). For eyes treated with EPO (8 and 32 mU/eye), the total retinal thickness and cell numbers in both the INL and ONL remained similar to those of the non-diabetic controls (Figure 1B-1D). The increased apoptosis in both INL and
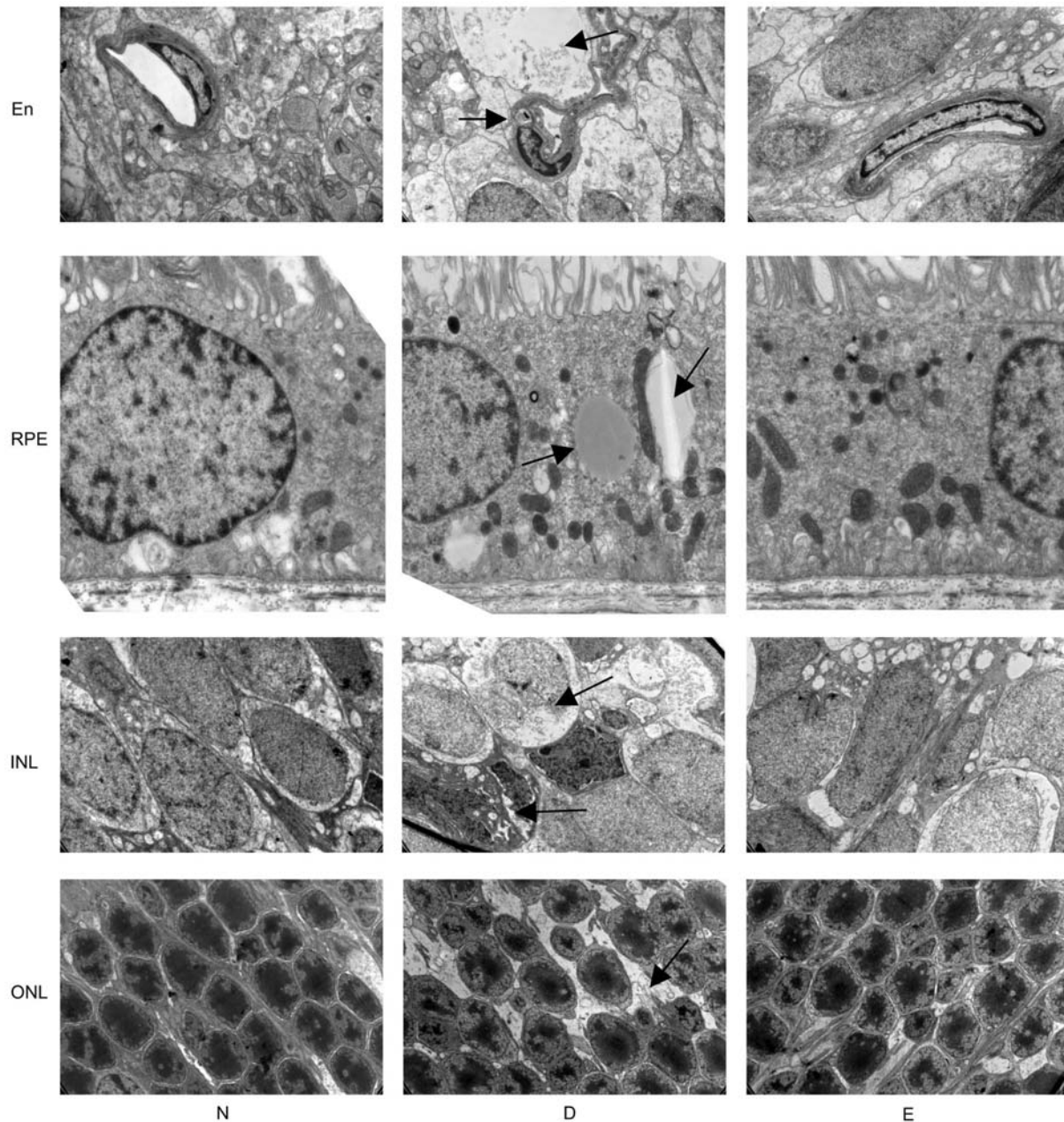
Figure 2. Representative EM of retina from diabetic rats with or without EPO teatment. N: normal control; D: diabetes; E: EPO treated rats. The intravitreal injection was performed 1 week after diabetes onset with 8 mU/eye of EPO (early treatment); one week later, the rats were killed for EM examiniation. En: endothelial cells (9,700x); RPE: retinal pigment epithelium (5,800x); INL: inner nuclear layer (5,800x); ONL: outer nuclear layer (5,800x). Arrows show the changes induced by diabetes, including vacuolar degeneration and peri-vascular edema and exudates (En-D); accumulation of lipid-like structures (RPE-D); cell degeneration and apoptosis (INL-D) and the wider spaces between cell bodies (ONL-D).

ONL of diabetic retinas was correlated with the reduced retinal thickness and cell counts in these both layers (Figure 1).

4.2. EM evidence of the protective effect of EPO on retinal cells in diabetes

The representative ultrastructural changes in the rat retinas are illustrated in Figure 2. The cell and tissue alterations that were detected in the different retinal layers in comparison with non-diabetic retinas were observed at 2 weeks after the onset of diabetes. Prominent changes of microvascular endothelial cells were discerned including vacuolar degeneration accompanied by peri-vascular edema and exudates (Figure 2 En-D). In the retinal pigment epithelium (RPE) of the diabetic eyes, accumulation of lipid-like structures was observed (Figure 2 RPE-D). Cell
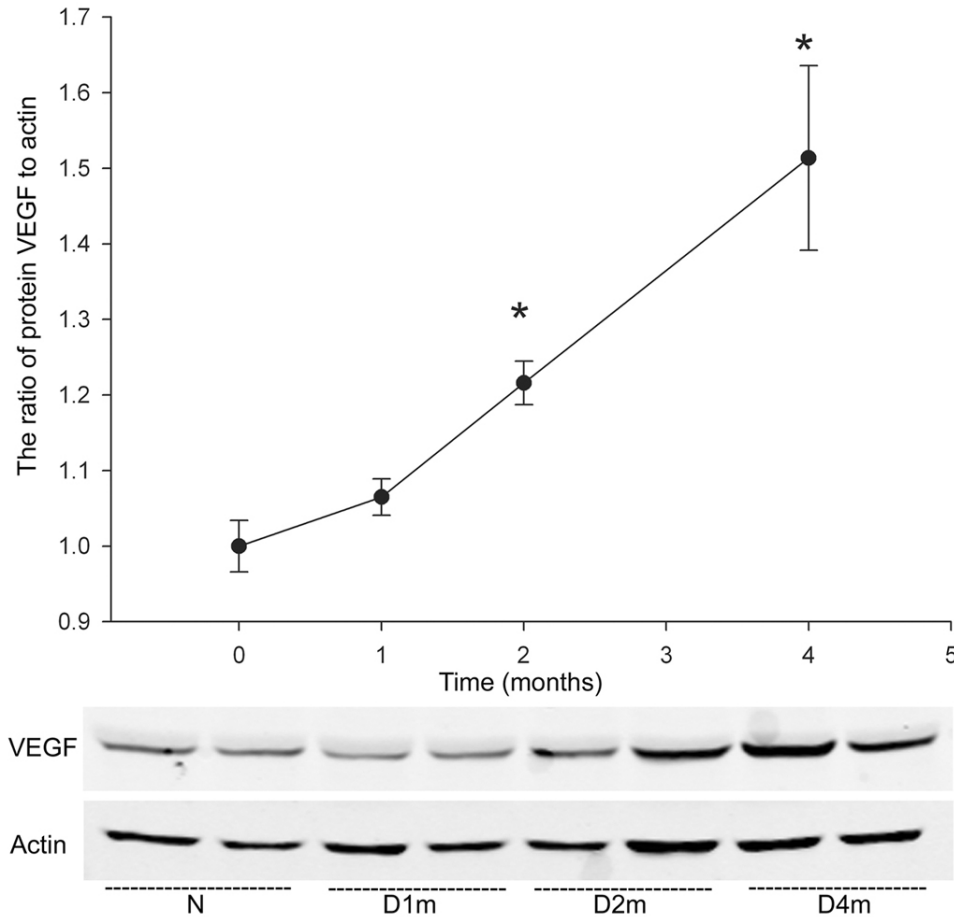
Figure 3. Retinal VEGF was up-regulated with the progression of diabetes. Bands for two representative retinas per group are shown. The graph presents data from 4 retinas at each timepoint. N: normal control; D1m, D2m and D4m: diabetes of 1, 2 and 4 months. * means $P<0.05$ when compared with normal control.

degeneration and apoptosis were evident in the INL (Figure 2 INL-D) with wider spaces between cell bodies and a decreased number of cells in the ONL (Figure 2 ONL-D), that correlated with the cell loss (Figure 1B and 1C) and increased TUNEL-positive cells (Figure 1A) in both the INL and ONL. At the same time point, the diabetic rats treated with EPO (8 mU/eye, early treatment) 1 week after diabetes onset showed either no change or minimal changes in individual layers of retina, in comparison with the non-diabetic control group (Figure 2).

4.3. Retinal VEGF was up-regulated with the progression of diabetes, and down-regulated by time-dependent EPO treatment

The expression of VEGF protein was up-regulated in rats with 1- to 4-months' diabetes (Figure 3). The retinal VEGF expression in the diabetic group was about 1.2 (2-month diabetes) and 1.5 (4-months diabetes) fold that in the normal control animals (n=4, p<0.05). The up-regulated VEGF expression was decreased by 23.2% (32 mU/eye, intermediate treatment, n=12, p<0.05) in 1-month diabetes, and 18.1% (16 mU/eye, late treatment, n=6, p<0.05) in 4-month diabetes with exogenous EPO (4 days after treatment, Figure 4). This anti-VEGF effect by

EPO is time-dependent (Figure 5). In Figure 5, VEGF protein expression in 4-month diabetes was down-regulated with intravitreal EPO (16 mU/eye) by 29.1% (6 hours), 38.3% (12 hours), 27.6% (1 day), 32.0% (2 days), 36.9% (4 days) and 14.6% (7 days), respectively. After seven days with the single EPO injection, the anti-VEGF effect began fading.

The real-time PCR data confirmed that the mRNA levels for VEGF-A were up-regulated in diabetes and down-regulated 4 days after EPO injection at two conditions, i.e., 4 weeks (Figure 6A, intermediate treatment) and 5 months (Figure 6B, late treatment) after diabetes onset. At 4 weeks of diabetes, the retinal mRNA expression of VEGF-A was 1.7 fold that in the normal control. This expression was decreased by lower doses of EPO, 17.3% (8 mU/eye) and 29.8% (32 mU/eye) reduction as compared with the untreated diabetic retina after 4 days intervention (Figure 6A). The same trend was also found in 5 months of diabetes (Figure 6B). The inhibition of VEGF mRNA by EPO is dose-dependent (Figure 7A). In Figure 7A, intravitreal injection of EPO was performed 1 week after diabetes onset; the retinal VEGF mRNA was examined by real-time PCR 1 week later. The results
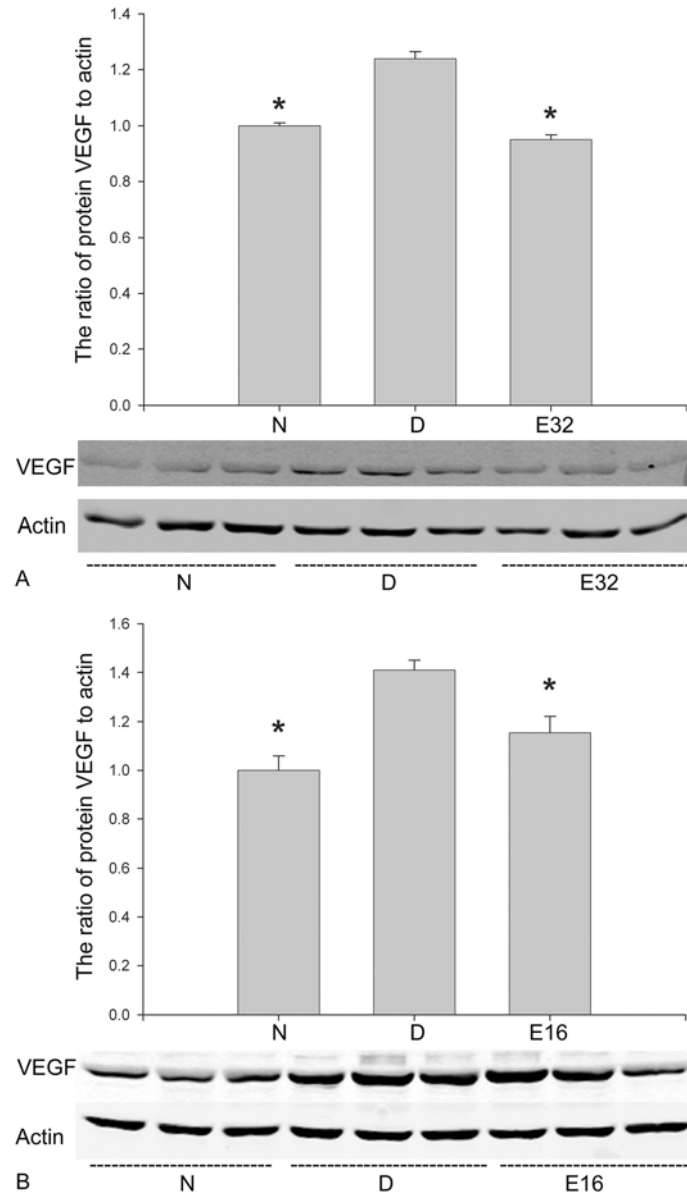
Figure 4. Retinal VEGF was up-regulated in 1-month (A), and 4-month (B) diabetes, but was down-regulated by exogenous EPO 4 days after intervention. Representative data from 3 different retinas per group are shown (n=12 in A; and n=6 in B). N: normal control; D: 1- or 4-month diabetes; E: EPO (16 or 32 mU/eye)-treated group. Data are expressed as mean ± S.E. * means $P<0.05$ when compared with diabetic group.

showed that the inhibition of VEGF mRNA was more significant between the doses of 32-160 mU/eye after 7 days intervention. At this time point, EPO at 16 mU/eye lost the inhibitory effect. This finding is consistent with the data in Figure 5, in which anti-VEGF effect by EPO (16 mU/eye) began fading beyond 7 days after intravitreal injection.

4.4. Down-regulation of HIF-1 alpha and VEGF by intravitreal EPO was correlated with BRB restoration in a time- and dose-dependent manner

Real-time PCR showed the mRNA changes of HIF-1 alpha and EPO (Figure 6). The mRNA levels of

HIF-1 alpha and EPO were both up-regulated in diabetes and down-regulated 4 days after EPO injection at both the intermediate (Figure 6A) and the late treatment (Figure 6B). After 4 weeks of diabetes, the retinal mRNA expression of HIF-1 alpha and endogenous EPO were 4.7 and 1.6 fold, respectively, that in the normal control. These levels were decreased 4 days after injection of EPO (8 mU/eye) by 7.6% and 27.3%, respectively, in comparison with the untreated diabetic retina. They were decreased by a higher dose of EPO (32 mU/eye) by 41.4% and 36.6% relative to the untreated diabetic retina (Figure 6A). At 5 months of diabetes, the retinal mRNA levels of HIF-1 alpha and endogenous EPO were increased 1.8 and 1.1 fold of the
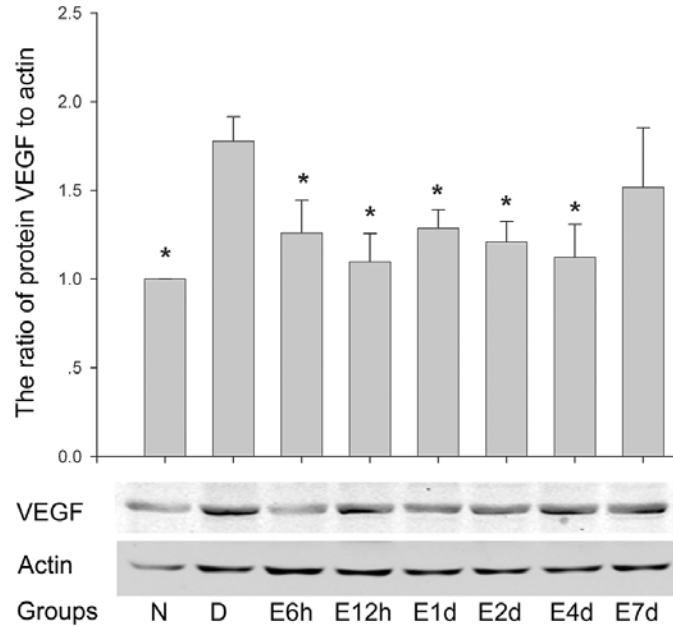
Figure 5. Anti-VEGF effects following intravitreal injection of EPO are time-dependent. N: normal control; D: diabetic control; E: EPO-treated rats. Exx: time (h for hours and d for days) after EPO (16 mU/eye) intravitreal administration. N=5-6, * means $P<0.05$ when compared with diabetic control.
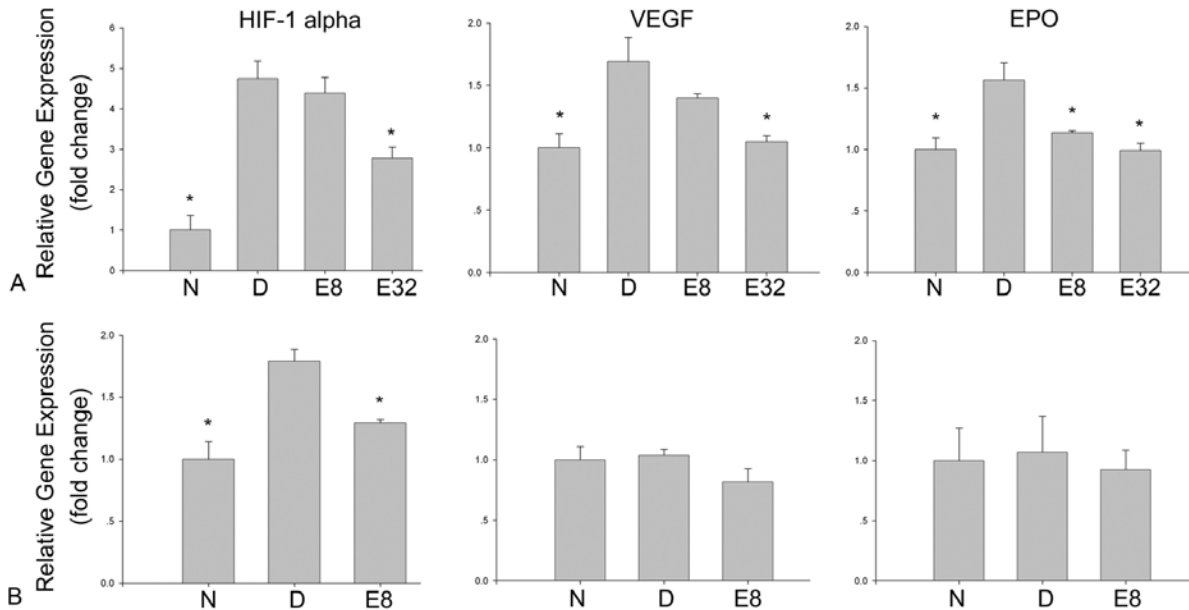

Figure 6. The relative mRNA changes for HIF-1 alpha, VEGF, and endogenous EPO in diabetic retina after exogenous EPO administration. The EPO treatment was performed at both intermediate (4 weeks, A) and late stage (5 months, B), for 4-day. Each column stands for N (normal control), D (diabetes), E8 (EPO 8 mU/eye) or E32 (EPO 32 mU/eye), separately, from the left to the right of each panel (A and B). * means $P<0.05$ when compared with diabetes.

normal control, respectively. These levels were decreased with exogenous EPO (8 mU/eye) by 24.8% and 13.7%, respectively, when measured 4 days after the injection (Figure 6B). The dose-dependent inhibition of HIF-1 alpha was also observed in 2-week diabetic rats with 1 week intervention (Figure 7B).

The expressions of HIF-1 alpha, EPO and EpoR protein in the retina were all up-regulated in diabetes and down-regulated after exogenous EPO (8 and 32 mU/eye) administration (Figure 8). EpoR expression in the 5-week diabetic retinas was increased by 18.0% (p>0.05), as compared to the normal retinas. This increase in EpoR was
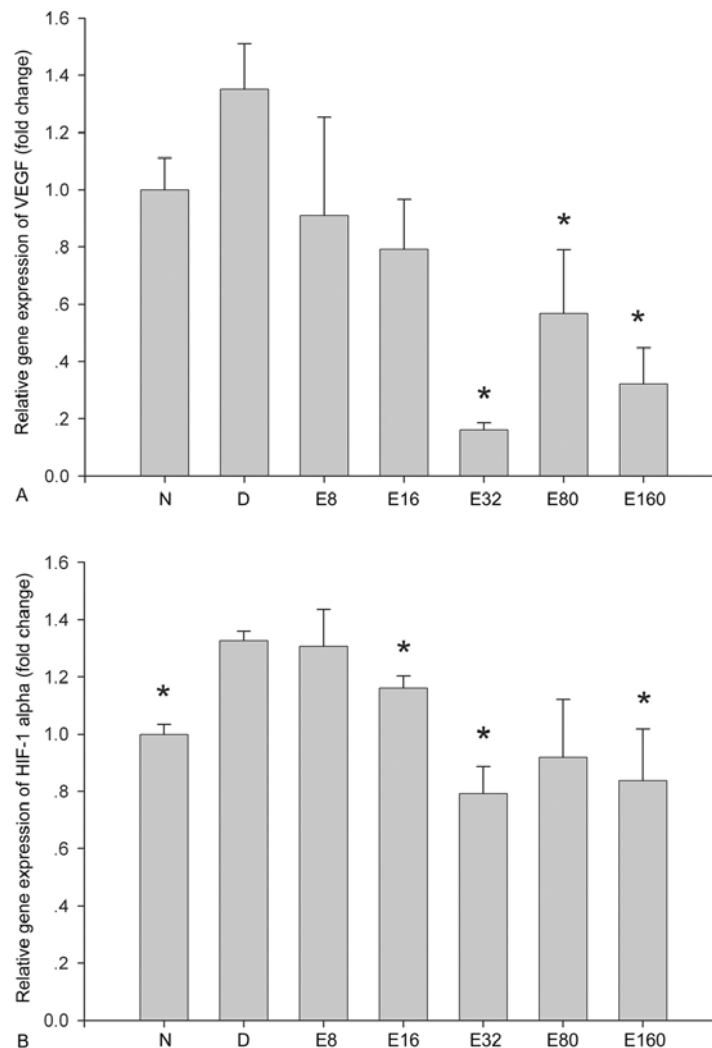
Figure 7. Dose-dependent inhibition of both VEGF (A), and HIF-1 apha (B) by EPO. The intravitreal injection was performed 1 week after diabetes onset; 1 week later, the retinas were examined for the mRNA changes of VEGF (A) and HIF-1 alpha (B) by real-time PCR. N=4, and * means *P*<0.05 when compared with diabetes.

slightly reduced 7 days after EPO injection by 5.1% (8 mU/eye, p>0.05) and 5.4% (32 mU/eye, p>0.05), respectively (Figure 8A). The retinal expression of HIF-1 alpha and endogenous EPO after 4 weeks of diabetes onset was increased by 18.8% and 11.7% (p<0.05), respectively, as compared with the normal control (Figure 8B). These elevated expressions returned to the baseline after EPO treatment (Figure 8B). Similar results were also observed 1- and 7-days after the injections when performed at 4 weeks after diabetes onset (data not shown).

In order to study the links between the BRB breakdown and the activation of the HIF-1 alpha pathway, Evans blue permeation (EBP) was assayed in 2-week diabetic rats. The intravitreal injection was performed 1 week after diabetes onset with 16 mU/eye of EPO (early treatment); one week later, the rats were killed for EBP test. Figure 9 demonstrates that EBP in diabetic rats was about 2 fold that in the normal retinas. Intravitreal injections of

EPO (16 mU/eye) at 1 week after diabetes onset, exerted its protection to the BRB and led to 44.2% decreased permeation relative to the untreated diabetic retina (n=5, p<0.05). This result shows that the protective effect of EPO on BRB correlated with the inhibitory effect of EPO on the HIF-1 alpha pathway, specifically the down-regulation of VEGF. However, it is worth noting that the inhibition of VEGF by EPO began fading beyond 7 days, while the maintenance of BRB integrity lasted for at least 4 weeks, indicating other mechanism(s) may be involved (4).

4.5. Down-regulation of PHDs and VHL expressions after intravitreal injection of EPO as post-translational regulation to HIF-1 pathway

The mRNA levels of PHD1-3 and VHL were increased in diabetes and down-regulated after EPO treatment (Figure 10). After 4 weeks of diabetes (intermediate treatment), the retinal mRNA expressions of PHD1, PHD2, PHD3 and VHL were 2.1, 1.2, 1.5, and 1.5
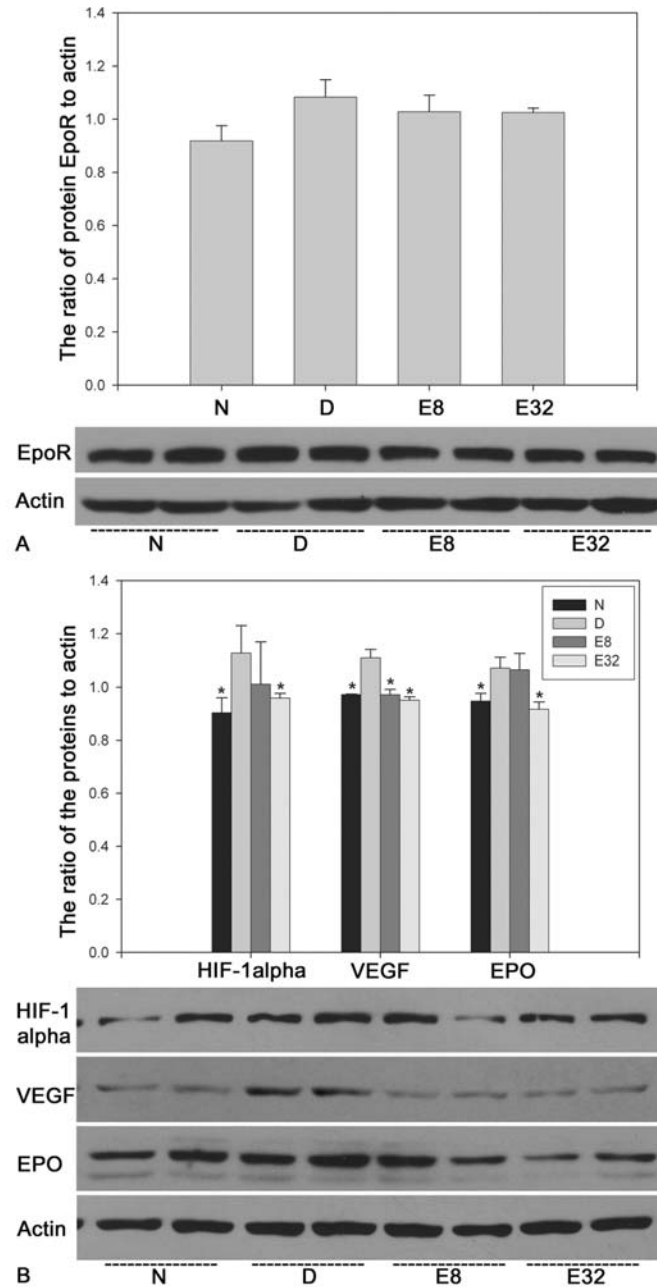
Figure 8. The expression changes of the proteins in HIF-1 alpha pathway after EPO treatment (intermediate treatment). (A) EpoR expression in retina from diabetic rats with EPO treatment (intermediate treatment, 4 weeks diabetes plus 1 week EPO); (B) HIF-1 alpha, VEGF and EPO expressions in retina from diabetic rats treated with 4-day EPO treatment following 4 weeks diabetes (intermediate treatment). Data from 2 different retinas per group (4 independent experiments) are represented. N: normal control; D: Diabetes; E8 and E32: the dose of intravitreal EPO was 8 mU/eye and 32 mU/eye, respectively. * means $P<0.05$ when compared with diabetes.

fold that in the normal control retina, respectively (Figure 10A). These levels were decreased 4 days after EPO injection (low dose, 8mU/eye) by 16.5%, 16.0%, 24.1% and 20.1%, respectively relative to the untreated diabetic retina. Four days after injection of a higher dose of exogenous EPO (32 mU/eye), these levels of expression were decreased 21.0%, 22.9%, 19.9% and 17.0%, respectively, relative to the untreated diabetic retina (Figure 10A). After 5-months of diabetes, the retinal mRNA expressions of PHD1, PHD2, PHD3 and VHL were 1.4, 1.2, 1.5, and 1.1 folds, higher than that in the normal control. These elevated levels of expression were decreased by 30.2%, 27.4%, 40.2% and 24.7%, respectively, in comparison with the untreated diabetic retina, when
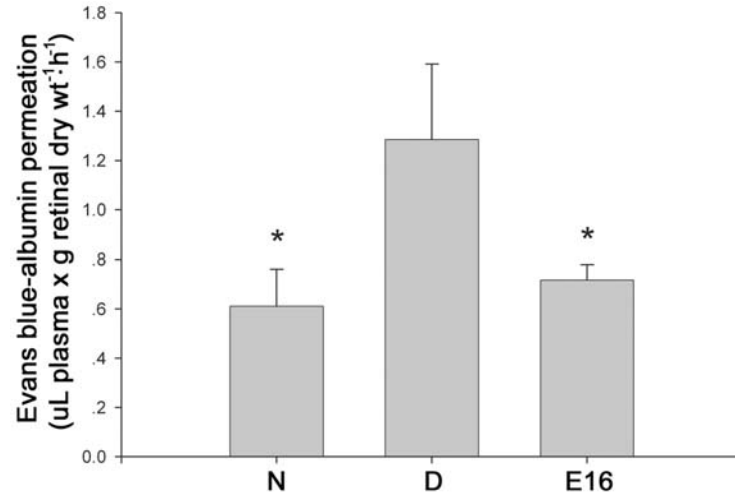
Figure 9. Evans blue-albumin permeation into the diabetic retina after treatment with or without intravitreal EPO for 1 week (16 mU/eye, early treatment). The intravitreal injection was performed 1 week after diabetes onset (early treatment); one week later, the rats were killed for EBP test. N=5, * means *P*<0.05 when compared with diabetes.

measured 4 days after EPO injection (8 mU/eye, Figure 10B). Similar results were also observed 1- and 7-days after the injections with 2 or 4 weeks diabetic duration (data not shown).

5. DISCUSSION

Retinal neurodegeneration and breakdown of the BRB are thought to be the most important components of early diabetic retinopathy (DR) (2-4, 19). Retinal neuronal apoptosis occurs early both in human subjects with diabetes (20, 21) and in streptozotocin-induced diabetic animal models (4, 22, 23). The present study demonstrated a progressive retinal neuron apoptosis and breakdown of BRB during the course of experimental diabetes. By using intravitreal EPO, the restoration of BRB integrity and prevention of neuron losses were observed at different time points after diabetes onset.

In terms of breakdown of BRB, several mechanisms related to VEGF functions have been proposed (24). VEGF expression is enhanced by hypoxia (25), which is a major stimulus for retinal hyperpermeability and neovascularization (26). The increased levels of VEGF in both vitreous fluid and aqueous humor of patients with DR indicate that VEGF plays a major pathogenic role in the development of DR (9, 27-29). Experimental data also support this notion. Retinal VEGF is demonstrated to be up-regulated in diabetes in rodents and humans coinciding with BRB breakdown (11, 30). Intravitreal injections of VEGF cause the breakdown of BRB (31, 32), while intravitreal injections of anti-VEGF therapies demonstrate a beneficial effect on diabetic macular edema as measured by central retinal thickness reduction and visual acuity improvement (33-36).

In studying EPO's protective function on retinal neurons and BRB, we have found both time- and dose-dependent effects. Exogenous EPO inhibits VEGF production throughout the course of diabetic retinopathy (Figure 4), but its efficacy starts fading beyond 7 days after treatment (Figure 5). In contrast, the EPO anti-apoptotic effect that leads to maintaining the normal retinal thickness in diabetic rats lasts at least 5 weeks (Figure 1); thus demonstrating a HIF/VEGF independent pathway of the EPO neuroprotection. On the other hand, a time- and dose-correlation between VEGF-inhibition and BRB protection by EPO was observed within 1 week after EPO treatment (Figure 5, Figure 7A, and Figure 9). After 1 week, the integrity of BRB was maintained in the EPO treatment group for at least 4 weeks (4), although the inhibition of VEGF diminished (Figure 5). This time-dependent EPO protection on BRB may consist of two phases. At the early phase, the pharmacologic levels of EPO directly target HIF-1 alpha pathway at both transcriptional and translational levels, leading to a reduction of VEGF. The suppressed VEGF might be responsible for the initial improvement of BRB. At the late phase (after 7 days), the EPO concentrations of retina after a single intravitreal injection reached to a low level, 0.06 mU/mg protein (37). At that time, the negative feedback function by EPO may not be able to continue. Whereas, the persistent protective effect of EPO on BRB could be achieved by up-regulation or preservation of molecules that are responsible for the capillary tight junctions (38-40) or indirect inhibition of inflammatory factors (41). It has been reported that EPO is able to attenuate blood-brain barrier (BBB) disruption in focal cerebral ischemia (40, 42), and to restore VEGF-induced BBB breakdown *in vitro* through EpoR signaling pathway and with re-establishment of the cell junctions (38). Meanwhile, the inhibition of VEGF by EPO shows a dose-dependent fashion. One week after EPO injection, the inhibitory effect on VEGF was more prominent with higher doses of EPO (Figure 7A), indicating the concentration-dependent effect of EPO is critical for VEGF inhibition.

Interestingly, both VEGF and EPO are up-regulated in the retinas of early diabetics (Figure 3-8), and
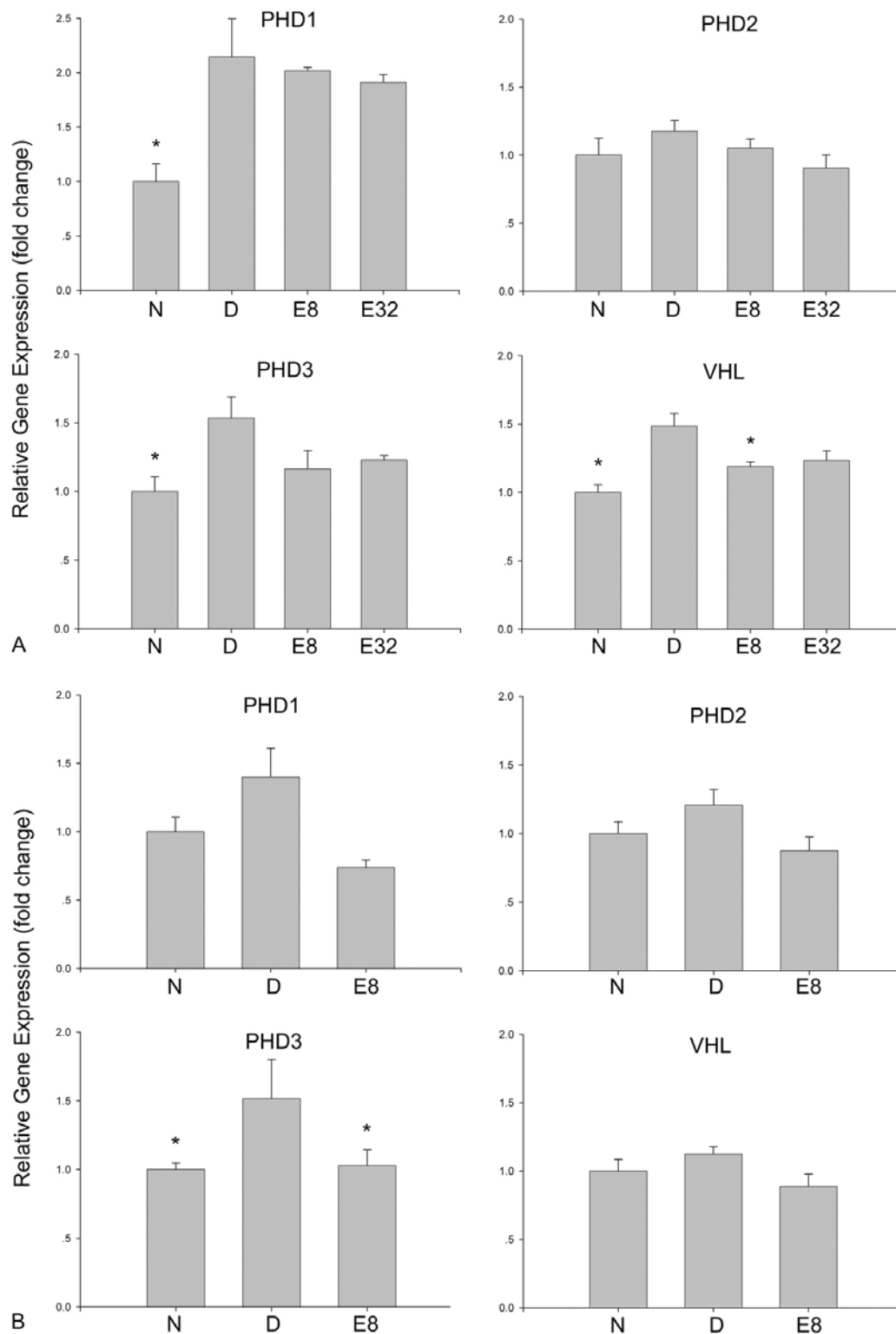
Figure 10. The relative mRNA changes of PHD1-3 and VHL (A and B) in diabetic retina after exogenous EPO administration. The EPO treatment was performed in both intermediate (4 weeks, A) or late stage (5 months, B), 4 days later, the rats were killed for real-time PCR examination. Each column stands for N (normal control), D (diabetes), E8 (EPO 8 mU/eye) or E32 (EPO 32 mU/eye), separately, from the left to the right of each panel (A and B). * means $P<0.05$ when compared with diabetes.

have been found significantly elevated in the vitreous of patients with proliferative diabetic retinopathy (PDR) (43). Both VEGF and EPO are downstream products of HIF-1 alpha, and are both neuroprotective and angiogenic (44, 45). Nevertheless, a fundamental difference between VEGF and EPO is that VEGF increases permeability of BRB (30), while EPO maintains the integrity of BRB (Figure 9) (4).

The specific mechanisms responsible for increased EPO in diabetic retinas are not completely understood. In PDR, the high level of EPO in vitreous was believed to be induced by hypoxia (43). However, similarly high vitreous EPO level has also been observed in patients with diabetic macular edema, in which ischemia was not a predominant event (46). Examinations of the epiretinal membranes of PDR revealed a significant correlation between the number of blood vessels expressing the pan-endothelial marker CD34 and the numbers of blood vessels expressing HIF-1 alpha and VEGF, while no immunoreactivity has been found for EPO, suggesting that HIF-1 alpha and VEGF play the major angiogenic roles in PDR, while EPO does not (47). Other conditions, such as hypoglycemia and oxidative stress (48, 49), have been shown to induce the expression of HIF-1 alpha, which induces both VEGF and EPO production. In early DR, the data from our laboratory have documented that the endogenous EPO and EpoR were up-regulated (Figure 6 and 8) (4). These findings support our previous hypothesis that the EPO/EpoR system acts as a maintenance/survival mechanism in BRB and retinal neurons, responding to early diabetic insults (4). In addition, one report shows that VEGF, but not EPO, is predominantly regulated by HIF-1 alpha in human RPE cells under hypoxic conditions. This observation suggests that EPO may also be regulated by a HIF-1 alpha independent pathway (50).

It is noted that the up-regulation of EPO in early DR is only modest (Figure 6 and 8). The level of EPO shown to protect BRB and to suppress VEGF is proximately 20-70 times higher than that in patients with macular hole (30 mU/mL) (46). In our previous report, the dose-dependent protection by EPO on BRB began to lessen when EPO was below 10 mU/mL (4). Therefore, the therapeutic level of EPO must be high enough to exert its function. In fact, the therapeutic levels of EPO used (ranging 500-2,000 mU/mL) are even higher than the median level of endogenous EPO reported in vitreous of patients with PDR (367 mU/mL) (51). The mechanism for the requirement of high therapeutic level of EPO on top of the already high endogenous EPO in diabetic retina is currently unknown. We proposed that EPO/EpoR system may not function at all in the neovascular stage of DR (4). For instance, EpoR could be dissociated from the EPO/EpoR system (52). Under such condition, a pharmacologic level of exogenous EPO may rejuvenate EPO/EpoR system to exert its protective functions. As a matter of fact, a recent clinical series of intravitreal injections of EPO at a high dose (1,000 mU/mL) into eyes with severe diffuse diabetic macular edema produced short-term favorable visual outcomes and anatomic improvement (53).

Evidence exists that the HIF-1 pathway is under precise regulation at transcriptional, translational, and post-translational levels. Some of the downstream products of HIF-1 alpha, such as EPO, iNOS, and Hey proteins, are able to down-regulate HIF-1 alpha under a number of conditions. For instance, Hey factors are capable of repressing HIF-1 alpha induced gene expression under hypoxic conditions, suggesting the existence of a negative feedback loop to prevent excessive hypoxic gene induction (54). EPO's effect of decreasing hypoxia-induced HIF-1 alpha protein in a dose-dependent fashion has been demonstrated at the posttranslational level in ovarian cancer cells and MCF-7 breast cancer cells (55). In the study reported here, we have, for the first time, demonstrated that the HIF-1 alpha pathway, activated in the diabetic retina, could be down-regulated at both transcriptional and translational levels by exogenous EPO (Figure 6-8).

It is interesting to note that exogenous EPO may also be involved in the posttranslational regulation of HIF-1 alpha pathway in early diabetic retina but not through a negative regulation. It has been known that VHL, PHD2, and PHD3 all destabilize the HIF-1 alpha protein (50, 56, 57). In the present work, increased mRNA expression of all three PHDs and VHL were detected in the diabetic rat retinas 2 weeks after diabetes onset (data not shown), and continued to 5 months after diabetes onset (Figure 10). This increase in mRNA levels of PHDs and VHL is presumed to be a regulatory step toward limiting the overshoot of HIF-1 alpha level in diabetic retina, while the exogenous EPO may modulate HIF-1 alpha pathway by reducing the mRNA levels of these factors (Figure 10). The precise posttranslational regulatory mechanism of EPO on the HIF-1 alpha pathway merits further study.

Taken together, the inhibition of HIF-1 alpha, leading to a suppressed level of VEGF in early diabetic retina, is a direct effect of the negative feedback regulation of HIF-1 alpha by exogenous EPO. This negative regulation is at both transcriptional and translational levels. By this mechanism, the inhibition of exogenous EPO on HIF-1 alpha-induced VEGF production in early diabetic retina may explain in part, at least in the initial phase, the protective function of EPO toward restoration of BRB integrity. The exogenous concentrations of EPO which inhibited VEGF, also demonstrated protective functions for neurons, microvascular endothelium and RPE layer (4), in the diabetic retina. Therefore, intravitreal EPO appears to be a multi-functional therapy for early DR.

6. ACKNOWLEDGEMENT

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Abbreviations: BRB: blood-retinal barrier; BBB: blood-brain barrier; DR: diabetic retinopathy; EPO: erythropoietin; EpoR: erythropoietin receptor; EBP: Evans blue permeation; EM: electron microscopy; HIF-1: hypoxia-inducible factor 1; INL: inner nuclear layer; iNOS: inducible nitric oxide synthase; IPL: inner plexiform layer; ONL: outer nuclear layer; OLM-ILM: outer limiting membrane to inner limiting membrane; OPL: outer plexiform layer; PDR: proliferative diabetic retinopathy; PHD: prolyl hydroxylases; RPE: retinal pigment epithelium; VEGF: vascular endothelial growth factor; VHL: von Hippel-Lindau tumor suppressor

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