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EPO reduces reactive gliosis and stimulates neurotrophin expression in Muller cells

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1. ABSTRACT

To characterize Müller cell-mediated neuroprotective and neurotrophic functions of the erythropoietin (EPO)/EPO receptor (EpoR) system in diabetic rat retina. A single intravitreal injection of EPO (8 mU/eye) was administered in rats 4 or 24 weeks after diabetes onset. The results showed that intravitreal EPO ameliorated the up-regulation of GFAP and vimentin in the diabetic retina evaluated by immunofluorescence and Western blotting; but up-regulated BDNF and CNTF expressions, quantified by real-time PCR and ELISA, in the 24-week diabetic rat retinas. *In vitro*, BDNF and CNTF expressions were stimulated by EPO through both extracellular signal-regulated kinase1/2 (ERK1/2) and Akt pathways. The neuro-regenerative function of EPO, as indicated by promotion of neurite outgrowth, was corroborated *in vitro*. BDNF was involved in EPO-induced neurite outgrowth of primary rat retinal neurons. Exogenous EPO exerts neuroprotective and neurotrophic functions by attenuating reactive gliosis and promoting neurotrophic factors in Müller cells in diabetic retina. Signaling pathways that are responsible for these Müller cell-mediated EPO/EpoR functions may be therapeutic targets for diabetic retinopathy.

2. INTRODUCTION

Diabetic retinopathy (DR), which results from neurodegeneration, microangiopathy, and glial dysfunction, is the leading cause of blindness in working adults (1). It has been recognized that changes in retinal neurons and glial cells precede the development of overt vascular pathology during diabetes (2-4). Therefore, the present study is focusing on glial dysfunction.

Müller cells, one of the principal macroglial cells of the retina, extend from the inner limiting membrane to the outer limiting membrane. Their somas locate within the inner nuclear layer (INL) and the processes envelop all neurons and synapses. Müller cells participate in maintenance of retinal homeostasis through synthesis of neurotrophic factors, uptake and metabolism of neurotransmitters, spatial buffering of ions during retinal activity, and are required for the blood-retinal barrier (BRB) (5). Thus, any malfunction of Müller cells could have deleterious effects on neurons and vascular cells in the diabetic retina. Reactive gliosis, which is a consistent feature of wound healing after retinal injury, is one of the characteristics of glial dysfunction in DR (1, 6, 7). Massive gliosis, accompanied by a long lasting, uncontrolled Müller

cell proliferation, is detrimental to the retina. Müller cells grow into the former lumen of occluded retinal vessels where they form a glial scar during development of DR (8). Aberrant proliferation of retinal glial cells is one major causative factor for the formation of fibroproliferative tissues, associated with proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR) (9). Therefore, a strategy for controlling excessive gliosis is critical.

Neurodegeneration is another early feature in DR (4), prompting investigations on neuroprotective agents in animal models of DR. Erythropoietin (EPO) as a neuroprotectant has drawn great attention recently (10). For instance, its effectiveness as a neuroprotective agent has been proved in central nervous system diseases (11, 12) and in retinal diseases (13). Recent work in our laboratory has demonstrated that a single intravitreal injection of EPO at the onset of diabetes in rats prevents retinal neuron death and protects the integrity of the BRB (14). The protective function is mediated by EPO/EPO receptor (EpoR) system. Binding of EPO to EpoR activates multiple down-stream signaling pathways in neurons (15-17). Among these possible signaling pathways, the mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinase1/2 (ERK1/2) pathway has been reported to play a critical role in EPO neuroprotection in early diabetic retinas (14). Recent studies have proposed that neurotrophic rescue by EPO may involve interactions between neurotrophic factors and glial cells (18, 19). EPO has been shown to promote neurogenesis in rat brain by inducing brain-derived neurotrophic factor (BDNF) expression by astrocytes (20). However, the possibility that EPO plays a neurotrophic role mediated through Müller cells in diabetic retina has not been explored. The present study is to explore 1) whether exogenous EPO is able to reverse diabetes-induced reactive gliosis and act indirectly to protect neurons by promoting production of neurotrophic factors in Müller cells; 2) whether the key players in EPO-mediated neurotrophic pathways can be used as potential therapeutic targets.

3. MATERIALS AND METHODS

3.1. Experimental animals and intravitreal injection

About 180 male Sprague-Dawley rats (160 g +/-; Slaccas, SIBS, Shanghai, China) were used in this study. The animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Diabetes induction has been described previously (14). The rats were divided into 3 groups: diabetic rats, EPO-treated diabetic rats, and age-matched normal controls. At two time points, 4 weeks and 24 weeks after diabetes onset, the data were analyzed. Intravitreal EPO treatment for DR was based on previous study (14). Briefly, intravitreal injections were performed into both eyes of the rats 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. A single injection of EPO (2 μL, 8 mU/eye; Sigma, St. Louis, MO, USA) was administered at above time points for EPO-treated diabetic rats. Sham injections (2 μL, normal saline) were performed on both normal control rats and the diabetic control rats. The rats recovered spontaneously from the anesthesia and then were sent back to the animal room with food and water ad libitum. Four days later, the rats were killed and both eyes were enucleated for the following experiments.

3.2. Cell culture of retinal Müller cells

Transformed retinal Müller cell line (rMC-1) was kindly provided by Dr. V. P. Sarthy (Northwestern University, Chicago, IL, USA) (21). For analysis of dose-dependence, the cells were treated with EPO (R&D Systems, Minneapolis, MN, USA) at concentrations ranging from 1 to 4 U/mL after 2-day incubation and synchronization, followed by 12 h serum starvation. 1 h later, the medium and the cells were harvested separately for ELISA and real-time PCR. For time-dependent assay, the cells were treated with EPO (1 U/mL), then the supernatant of medium and the cells were harvested separately at the following time points: 0, 0.5, 1, and 6 h for real-time PCR and ELISA.

3.3. Sample preparations and immunofluorescence study

Rats were killed after deep anesthesia (2% pentobarbital sodium). The eyes were enucleated and fixed in PBS (pH 7.2) buffered 4% paraformaldehyde for 24 h. The anterior segments of eyes were carefully dissected under microscope. The remaining eyecups were dehydrated and embedded in optimal cutting temperature (OCT) compound (Tissue Tek; Sakura Finetek, Tokyo, Japan) for sectioning. Serial sections (10 μm) were cut on a cryostat.

The cryosections were washed, permeabilized, and then incubated with the primary antibodies against EpoR (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), vimentin (1:100; Biovison, Mountain View, CA, USA), cellular retinaldehyde-binding protein (CRALBP, 1:5,000), glial fibrillary acidic protein (GFAP, 1:5,000) or BDNF (1:500), overnight at 4°C. Antibodies against GFAP, CRALBP, and BDNF were purchased from Abcam (Cambridge, MA, USA). The sections were rinsed in PBS, and incubated with the appropriate secondary antibodies (1:100, anti-rabbit-FITC or anti-mouse-Cy3; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), mounted and then observed by epifluorescence microscopy (magnification 200×, Nikon, Yokohama, Japan). Equivalent areas, about 1 mm from the optic nerve head, from 3 different sections per group were selected and viewed with equivalent brightness and contrast settings.

For immunocytochemistry, rMC-1 cells were fixed on cover-slides and processed as described above. The primary antibody was anti-phospho-cyclic AMP response element binding protein (phospho-CREB) (1:100; Cell signaling, Danvers, MA, USA), and the secondary antibody was anti-rabbit-Cy3 (1:100).

3.4. Western blotting analysis for GFAP, phospho- and total-CREB

Individual retinas from experimental and control rats (four single retinas from four rats selected randomly

per group) were isolated and homogenized in ice-cold radioimmune precipitation assay (RIPA) buffer. Retinal protein extracts were prepared and kept on ice for Western blotting as previously described (14, 22). rMC-1 cells treated as described above were harvested with RIPA supplemented with protease inhibitors, 1% PMSF and the cocktail (Sigma), and kept on ice for Western blotting. Equal amounts of protein were resolved in SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane (Bio-Rad Laboratories, Munich, Germany). The membranes were blocked, and then incubated overnight with primary antibodies against GFAP (1:4,000), phospho-CREB (1:1,000) or beta-actin (1:4,000; Invitrogen, Carlsbad, CA, USA). Following three rinses with TBST, membranes were incubated with appropriate secondary antibodies (IRDye800cw (Infrared-dye) labeled goat anti-rabbit or mouse IgG, 1:5,000, Li-Cor, Lincoln, NE, USA). After extensive wash with TBST, the blots were scanned with the Li-Cor odyssey (Li-Cor). After phospho-CREB detection, the membrane was stripped and re-probed with the antibody against total-CREB (1:1,000; Cell signaling). The optical density of each band was determined using Quantity One software (Bio-Rad).

3.5. ELISA

The retinal protein extracts or the supernatants of conditioned medium of rMC-1 cells were prepared for ELISA to detect the protein levels of BDNF and CNTF. The ELISA was performed according to the manufacturer's instruction (BDNF, Chemicon International, Temecula, CA; CNTF, R&D Systems).

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

Total RNA was extracted from neurosensory retinas or cultured rMC-1 cells. The reverse transcription product was then examined by real-time PCR. The specific primers were purchased from Shanghai DNA Biotechnology Corporation (Shanghai, China). Primers were as follows: for rat CNTF, 5'-ATGGCTTTCGCAGAGCAAA-3' (sense), 5'-TCAGTGCTTGCCACTGGTACA-3' (antisense); for rat BDNF, 5'-CTGTGCGGACCCATGGGACTCTG-3' (sense), 5'-GTGGCGCCGAACCCTCATAGACAT-3' (antisense); for rat beta-actin, 5'-GTAAAGACCTCTATGCCAACA-3' (sense), 5'-GGACTCATCGTACTCCTGCT-3' (antisense). All reactions were conducted using the PRISM 7900 analyzer (ABI, Foster city, CA, USA). A tube containing the same amount of cDNA and primers for beta-actin was included in all runs to provide normalization. The number of cycles required to detect the fluorescence signal (termed threshold or Ct) for a gene was recorded and compared with Ct for beta-actin in the same sample. The relative gene expression was represented by the ratio of the normalized value for the experimental samples to that of the control. Four samples per group were used at each time point with four replicates.

3.7. Primary retinal neuronal cell culture and neurite outgrowth assay

Neurosensory retinas from 1-day-old SD rats were dissociated enzymatically to make a single-cell suspension, as previously described (22). The dissociated cells were seeded at a density of approximately 1-1.5 × 10^6/mL on 6-well culture plates pre-coated with poly-D-lysine (Chemicon International, Billerica, MA, USA). On day 7, more than 90% of the cells had differentiated into neurons.

One day later, fresh medium containing EPO (1 U/mL), with or without TrkB/Fc, an inhibitor of BDNF (2 μg/mL; R&D), or IgG/Fc (2 μg/mL; R&D) was added for an additional 24 h incubation, then the cells were observed by light microscope. One hundred cells in each sample were randomly photographed under the microscope, and neurite outgrowth was assessed by measuring the longest neurite length (LNL) for each cell with image analysis software (Image-Pro plus 5.1.; Media cybernetics).

3.8. Statistic analysis

Data are expressed as mean +/- SE. The statistical analysis was performed by using LSD test after One-way ANOVA; A *P*-value of 0.05 or less was considered statistically significant.

4. RESULTS

4.1. Müller cells become reactive with the progression of diabetes

In diabetic retina, GFAP protein was used as a key marker of gliosis. The GFAP protein levels in both 4- and 24-week diabetic retinas were about 2-fold higher than those in age-matched normal control rats (Figure 1A and 1B). Immunofluorescence confirmed the increased expression of GFAP in diabetic retinas (Figure 1C and 1D). In normal control retinas, GFAP immunostaining was mainly confined to the ganglion cell layer (GCL) and nerve fiber layer (NFL) where astrocytes and endfeet of Müller cells are located. In 4-week diabetic retinas, GFAP expression increased in GCL and NFL, and a few GFAP-positive processes of Müller cells were observed in the inner plexiform layer (IPL) (Figure 1C). In the 24-week diabetic rat retinas, dramatic up-regulation of GFAP was evident, with most GFAP-positive processes vertically spanning the entire retina (Figure 1D). A similar pattern was also observed for vimentin immunostaining, another marker of Müller cells, but for CRALBP, expression was most intensive in GCL and NFL (Figure 1C and 1D).

4.2. Glial localization of EPO receptor

To detect changes in EpoR in diabetic retinas, immunostaining was performed on 4- and 24-week diabetic rat retinas. Figure 2A shows that EpoR was expressed in the normal control retina, and up-regulated in diabetes, while exogenous EPO mildly down-regulated EpoR expression in 24-week diabetic retina. However, there are no obvious differences in 4-week groups. By overlay of EpoR and CRALBP immunostainings, we found that EpoR was mainly expressed by the retinal Müller cells, specifically in the somas and endfeet. The expression of EpoR in Müller cells was confirmed *in vitro* using the rMC-1 cell line (Figure 2C).

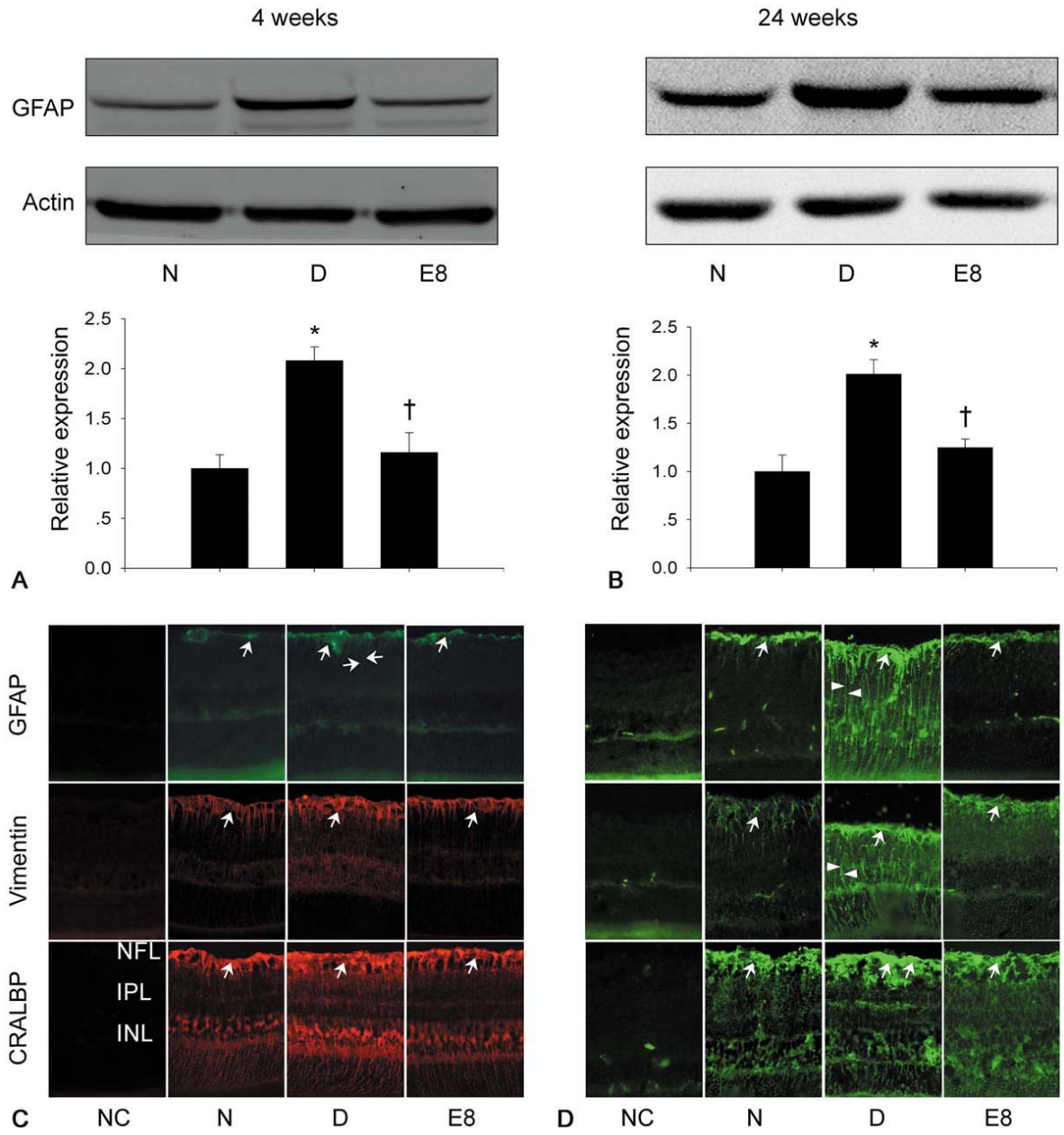

Figure 1. EPO down-regulated GFAP and vimentin expressions in diabetic rat retinas. (A and B) Western blotting for GFAP in 4- and 24-week diabetic rat retinas with or without EPO treatment (8 mU/eye). Densitometric analysis of GFAP protein was normalized using beta-actin level. LSD test after One-way ANOVA gave **P*<0.05 *vs* normal control; †*P*<0.05 *vs* diabetic control. Samples were from 4 animals each group, and repeated in triplicate. (C and D) Representative images of immunofluorescence for GFAP, vimentin and CRALBP in 4- and 24-week diabetic rat retinas, respectively, with or without EPO treatment (8 mU/eye). Arrows indicate inner limiting membrane. Arrowheads indicate processes of Müller cells. NC: negative control (without primary antibody); N: normal control; D: diabetic control; E8: diabetes treated with EPO (8 mU/eye). NFL: nerve fiber layer; IPL: inner plexiform layer; INL: inner nuclear layer. Original magnification: ×200.

4.3. EPO down-regulates GFAP and vimentin expressions in diabetic rat retinas

To analyze the regulatory effects of EPO on retinal glial cells, intravitreal injections of EPO were performed on 4- and 24-week diabetic rats. Retinal GFAP protein levels were significantly decreased in these two EPO-treated groups by 50% as compared with the diabetic control (*P*<0.05; Figure 1A and 1B). The effect of exogenous EPO on retinal GFAP expression was also confirmed by immunofluorescence (Figure 1C and 1D). Vimentin, expressed by astrocytes and Müller cells in normal retinas (middle panels in Figure 1C and 1D),

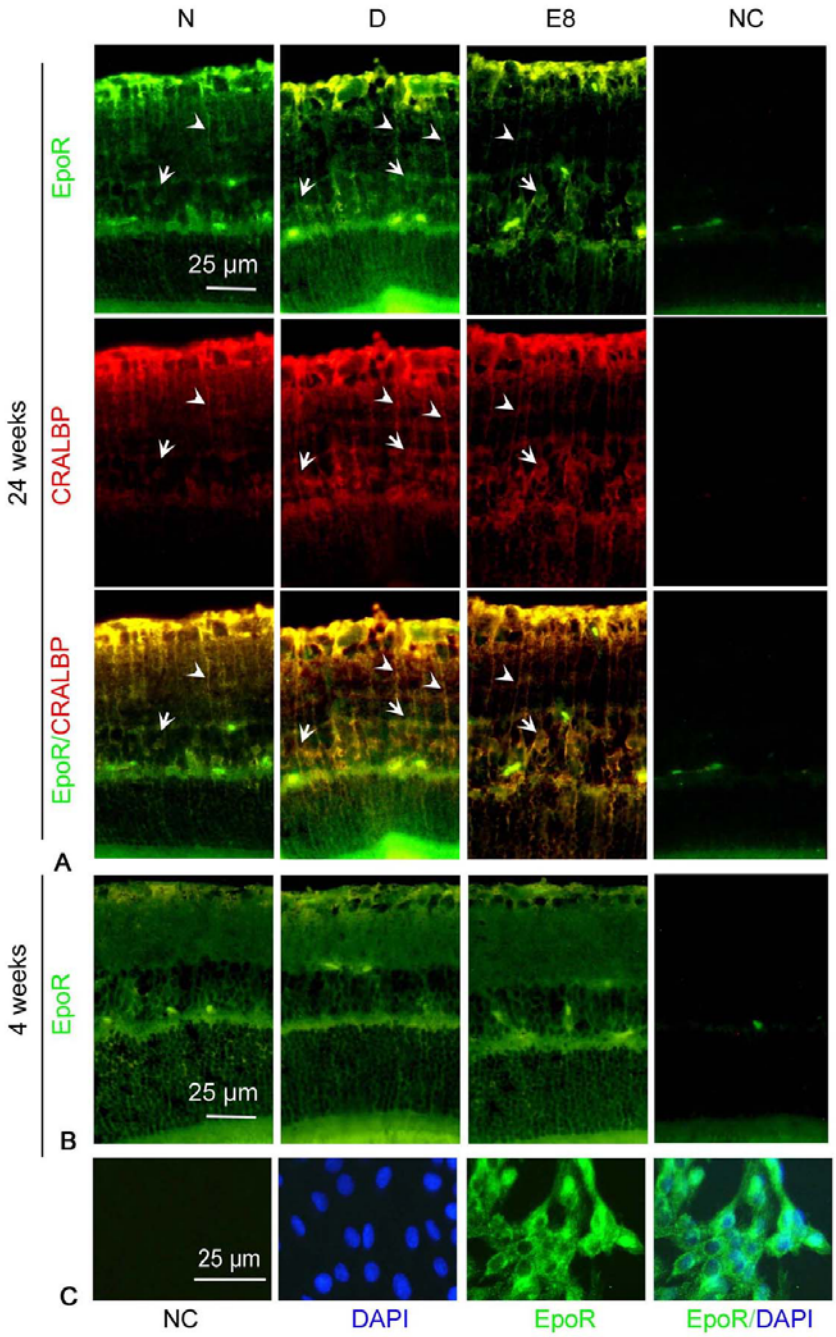

Figure 2. EpoR expression by Müller cells in retinas and rMC-1 cells *in vitro*. (A) Immunostaining of EpoR (green) and CRALBP (red) were co-localized in rat retinas; EpoR was down-regulated by exogenous EPO in Müller cells of 24-week diabetic rat. (B) EpoR was expressed diffusely in 4-week diabetic retina, with no obvious differences among groups. (C) The presence of EpoR (green) was demonstrated in rMC-1 cells. Cell nuclei were stained with DAPI (blue; 4', 6-diamidino-2-phenylindole). Arrows indicated somas of Müller cells. Arrowheads indicated processes of Müller cells. NC: negative control; N: normal control; D: diabetic control; E8: diabetes treated with EPO (8 mU/eye).

intensified along Müller cell processes in 4-week diabetic retinas and was further up-regulated by 24 weeks. However, its staining intensity was significantly reduced in EPO-treated 24-week diabetic retinas. These results were concordant with the changes of GFAP in EPO-treated diabetic retinas and controls. Staining for the Müller cell-specific protein, CRALBP, was not obviously affected in the 4-week group, but was increased in 24-week diabetic rats (lower panels in Figure 1C and 1D). This increase was also attenuated by EPO (lower panels in Figure 1D).

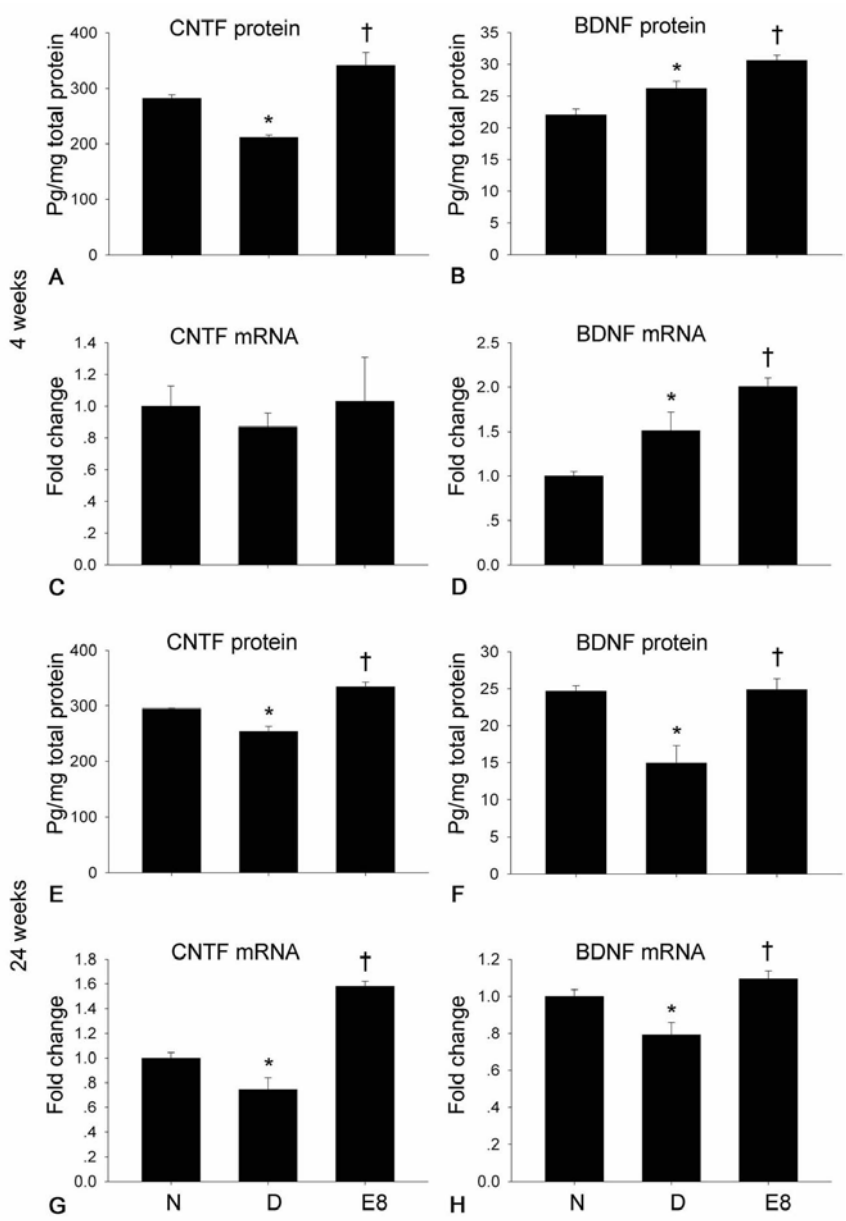

Figure 3. EPO promoted production of BDNF and CNTF in diabetic rat retinas. Effects of EPO on CNTF (A, C, E and G) and BDNF (B, D, F and H) expressions in 4- and 24-week diabetic rat retinas. Retinal protein levels of CNTF (A and E) and BDNF (B and F) were quantified by ELISA; mRNA levels of CNTF (C and G) and BDNF (D and H) were determined by real-time PCR. N: normal control; D: diabetic control; E8: diabetes treated with EPO (8 mU/eye). LSD test after One-way ANOVA gave **P*<0.05 *vs* normal control; †*P*<0.05 *vs* diabetic control. Samples were from 4 animals each group, and each experiment was repeated in triplicate.

4.4. EPO enhances the expressions of BDNF and CNTF by Müller cells in diabetic rat retinas

In early diabetes (4-week), as shown in Figure 3A, retinal CNTF protein was decreased by 30% compared with that in normal control (*P*<0.05). In contrast, a slight increase of BDNF protein level was observed in diabetic retinas as compared with the normal control (*P*<0.05; Figure 3B). These protein changes were also confirmed by real-time PCR (Figure 3C and 3D). However, with the progression of diabetes (24-week), retinal BDNF and CNTF were significantly down-regulated at both mRNA and protein levels (*P*<0.05; Figure 3E-H).

After a single EPO intravitreal injection, the mRNA and protein levels of both BDNF and CNTF were up-regulated in both 4- and 24-week diabetic rats. All of these increases were statistically significant (*P*<0.05; Figure 3) except for CNTF mRNA in the EPO-treated 4-week diabetic retina. The increase in BDNF was further validated by immunofluorescence studies on the 4-week

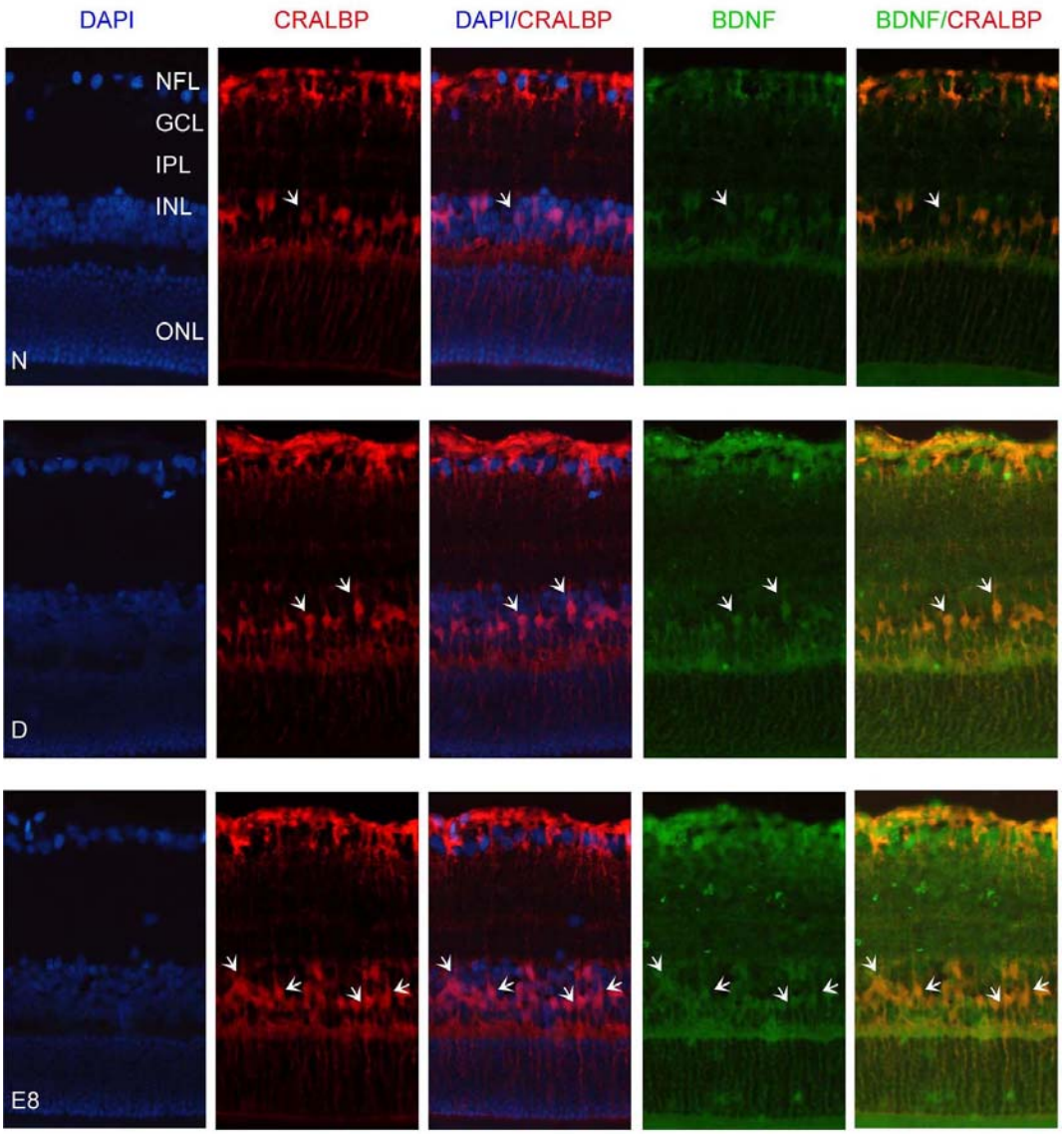

Figure 4. EPO promoted expression of BDNF by Müller cells in diabetic rat retinas. Immunostaining of BDNF was performed in 4-week diabetic rat retinas. BDNF (green) and CRALBP (red) were co-localized in retina as shown in merged images. Cell nuclei were stained with DAPI (blue). Arrows indicated somas of Müller cells. N: normal control; D: diabetic control; E8: diabetes treated with EPO (8 mU/eye). NFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer. Original magnification: ×200.

diabetic group (Figure 4). By co-labeling BDNF and CRALBP, Müller cells were proved to be the primary source of neurotrophins in rat retina.

4.5. EPO up-regulates the production of BDNF and CNTF by rMC-1 cells

As shown in Figure 5, EPO up-regulated BDNF mRNA expression dose-dependently from 1 U/mL to 4 U/mL, about 2.5-fold (4 U/mL) of that in control (Figure 5A). BDNF mRNA expression increased significantly 1 h after EPO treatment (1 U/mL; Figure 5C). BDNF protein expression also increased rapidly after 6 h EPO treatment (Figure 5E). In contrast, CNTF mRNA expression was increased 4-fold by EPO at 1 U/mL, but was decreased with higher doses of EPO (Figure 5B). CNTF mRNA showed a transitory increase at 1 h followed by a reduction to the control level by 6 h (Figure 5D). CNTF protein level slowly responded to EPO treatment (1 U/mL) and reached a plateau after 6 h (Figure 5F). It is noteworthy that BDNF and CNTF had different patterns of response to EPO treatment at both mRNA and protein levels (Figure 5).

4.6. EPO promotes neurite outgrowth in part via BDNF

To demonstrate whether or not BDNF is involved in EPO's neurotrophic effect, neurite outgrowth by primary retinal neurons was measured after treatment with EPO (1

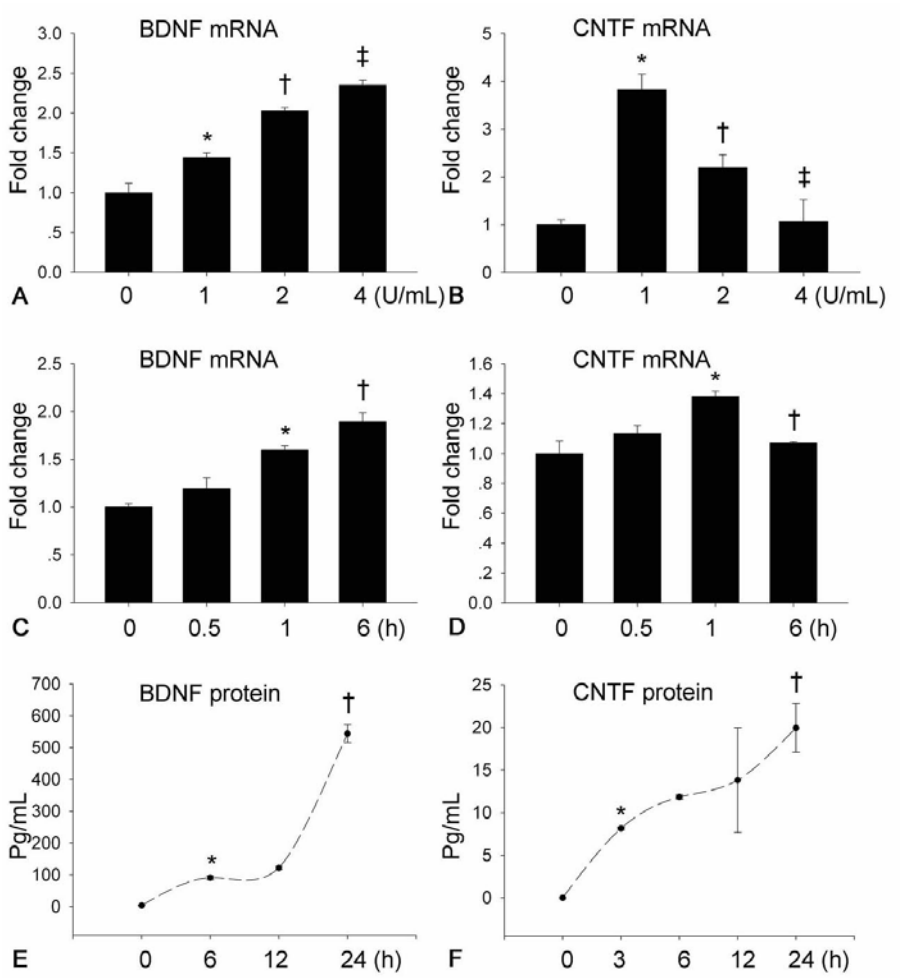

Figure 5. EPO promoted production of BDNF and CNTF in rMC-1 cells in a dose- and time-dependent manner. The mRNA levels of BDNF were shown in A and C; and CNTF in B and D. EPO (1 U/mL) was used for time-dependent mRNA analysis (C and D). The protein levels of BDNF (E) and CNTF (F) were also time-dependently up-regulated by EPO (1 U/mL). LSD test after One-way ANOVA gave **P*<0.05 in comparison with control (0); †*P*<0.05 in comparison with column *; ‡ *P*<0.05 in comparison with EPO (2 U/mL). Each experiment was performed in triplicate.

U/mL), in the presence or absence of TrkB/Fc, a known inhibitor of BDNF. The data showed that the mean LNL for the cells was greater in the EPO-treated group compared with control, but was greatly reduced after blockade of BDNF with TrkB/Fc (2 mg/mL) (Figure 6), suggesting that EPO's potentiation of neurite outgrowth is mediated at least in part by BDNF.

4.7. ERK1/2 and PI3K pathways are involved in EPO's neurotrophic effect

To identify pathway(s) involved in neurotrophin regulation, the MAPK/ERK1/2 and PI3K/Akt pathways were studied in rMC-1 cells by using specific inhibitors of ERK1/2 (U0126, Cell signaling; 10 μM; 1 h prior to EPO) or PI3K (Wortmannin, Cell signaling; 0.5 μM; 1 h prior to EPO). As shown in Figure 7, EPO-induced release of BDNF was abolished by pre-incubation with U0126 (Figure 7A), while Wortmannin had no effect (Figure 7B). In contrast, EPO-induced up-regulation of CNTF was suppressed by Wortmannin (Figure 7D), but not by U0126 (Figure 7C). These results indicated that EPO's neurotrophic effect may be mediated through multiple pathways. MAPK/ERK1/2 and PI3K/Akt pathways are upstream of CREB, which is responsible for BDNF regulation in brain (23, 24). Our present study showed that expression of phospho-CREB was increased with EPO administration (1 and 4 U/mL) (Figure 8A), reaching plateau 3 h after EPO supplementation (1 U/mL) (Figure 8B). However, the EPO-induced phospho-CREB expression was abolished by both U0126 and Wortmannin (Figure 8C).

5. DISCUSSION

Although lots of research has focused on microangiopathy and neuronopathy in DR (25), a role for dysfunction of the glial cells that closely interact with vessels and retinal neurons is emerging (4). The present

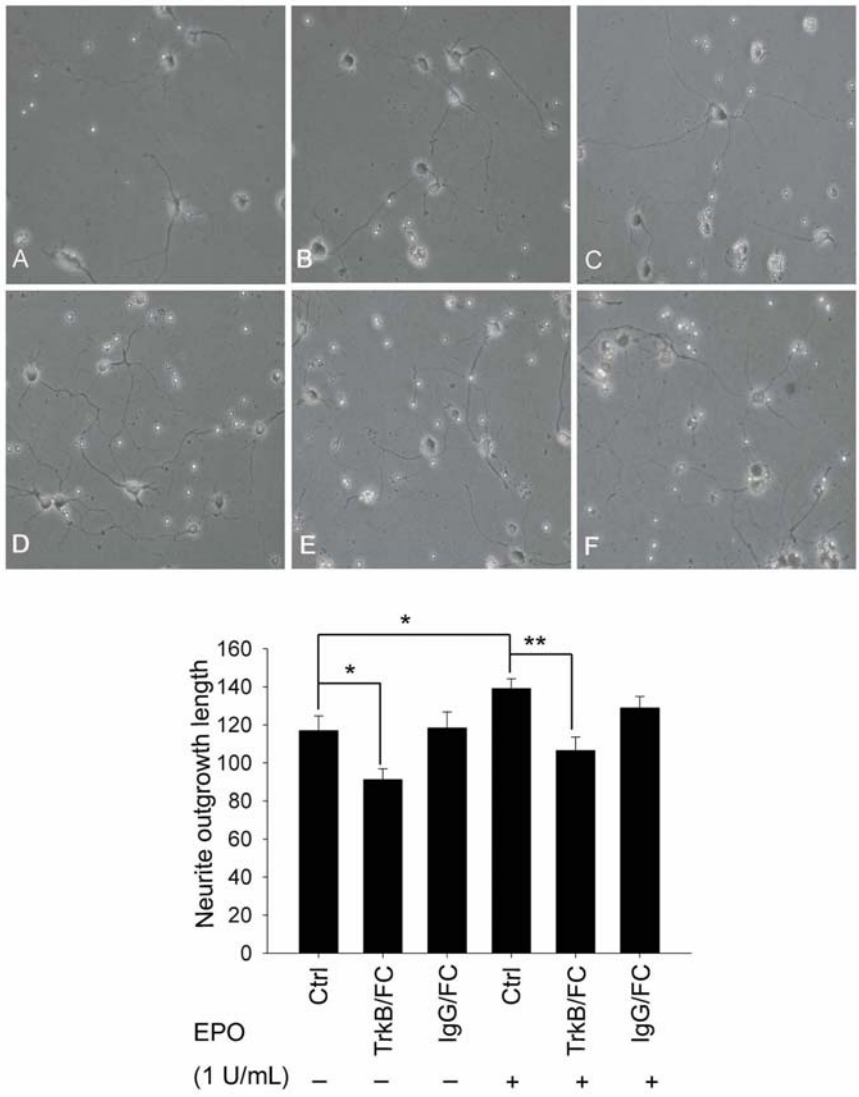

Figure 6. BDNF stimulated EPO-induced neurite outgrowth by primary retinal neurons. (A) control (ctrl); (B) TrkB/Fc; (C) IgG/Fc; (D) EPO; (E) EPO + TrkB/Fc; (F) EPO + IgG/Fc. The neurite outgrowth was determined by measuring longest neurite length in micrographs using image analysis software. Data were expressed as mean +/- SE (n=100), **P*<0.05 and ***P*<0.01. Original magnification: ×200.

study demonstrates that reactive gliosis parallels up-regulation of EpoR expression by Müller cells in early diabetic retinas. Moreover, exogenous EPO is able to abrogate the reactive gliosis and exert indirect neurotrophic function via Müller cells through MAPK/ERK1/2 and PI3K/Akt pathways.

In the present study, GFAP-positive staining in early diabetic retinas extended into the IPL where Müller cell processes are rich in distribution. Furthermore, a marked up-regulation of GFAP was confirmed by Western blotting in diabetic retinas. With progression of DR, GFAP immunostaining in 24-week diabetic retinas extended throughout the entire neurosensory retina and exhibited stronger staining. Nevertheless, ratios of total GFAP protein between diabetic and normal control retinas were unchanged in 4- and 24-week groups. Since the whole retinal preparation contains all GFAP-positive cells including Müller cells and astrocytes, the increased glial reactivity of astrocytes in aging normal retina (26) and the progressive decrease of astrocytes in early diabetic retina (27) may blunt the dramatic increase in GFAP in Müller cells of the diabetic retina as demonstrated by immunostaining in Figure 1.

The EpoR is widely expressed in mouse (28), human (29), and rat (30) retinas. In this study, we have shown co-localization of immunoreactivity of EpoR and CRALBP in Müller cells (Figure 2). Furthermore, EpoR expression by Müller cells was significantly increased in 24-week, but not in 4-week diabetic rat retinas (Figure 2B). These findings indicate that in diabetes, Müller cells

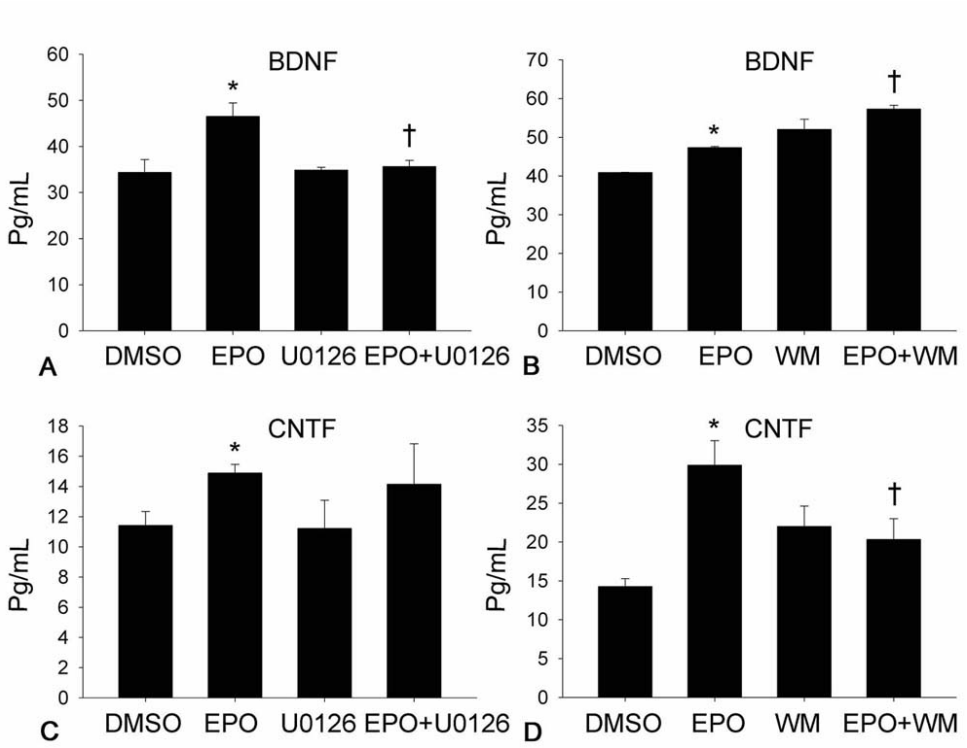

Figure 7. MAPK/ERK1/2 and PI3K/Akt pathways were involved in EPO-induced secretion of BDNF and CNTF by rMC-1 cells. ERK1/2 inhibitor (U0126; 10 μM; 1 h prior to EPO) or PI3-kinase inhibitor (Wortmannin, WM; 0.5. μM; 1 h prior to EPO) were used to treat rMC-1 cells for 12 h with or without EPO (1 U/mL). Following treatment, conditioned medium was harvested for ELISA. Roles of ERK1/2 and PI3K pathways were assessed by measuring BDNF (A and B) and CNTF (C and D) following exposure to U0126 or Wortmannin, separately. DMSO served as a vehicle to dissolve these inhibitors. LSD test after One-way ANOVA gave **P*<0.05 *vs* DMSO; and †*P*<0.05 *vs* EPO (1 U/mL). Each experiment was repeated in triplicate.

respond to the stress of the disease by increasing their ability to benefit from the protective effects of EPO (14). When exogenous EPO was administered, down-regulation of EpoR was noted in the retina in 24-week diabetic rats; in contrast, the levels of EpoR remained no change after EPO treatment in 4-week diabetic rats (Figure 2B). The mechanism whereby exogenous EPO regulates the reactivity of Müller cells has not been defined. It was reported that EPO is neuroprotective in a model of experimental closed head injury by reducing activation of glial cells (GFAP-positive astrocytes) and inflammatory damage (31). Furthermore, the protective effect of EPO in experimental autoimmune encephalomyelitis (EAE), or white matter damage, is also paralleled by a marked decrease in inflammatory infiltrate and glial activation (32, 33). In diabetic rat retina, EPO strongly down-regulates inflammatory cytokines such as TNF-alpha and IL-6 (34). These data support our hypothesis that EPO is an anti-inflammatory cytokine, thereby reducing damage to retinal neurons and Müller cells. EPO should, therefore, reduce the expression of GFAP, a biomarker of reactive Müller cells and an indicator of gliosis and inflammatory activity.

Gliotic Müller cells may display a dysregulation of various neuronal-supportive functions (8). For instance, neurotrophin deficiency has been reported in diabetic retina (35, 36). The present results also demonstrated decreased expressions of BDNF and CNTF in the retinas of streptozotocin (STZ)-induced diabetic rats. To our knowledge, the primary source of these neurotrophic factors in retina is Müller cell (Figure 4), although other retinal cell types (e.g., amacrine cells) cannot be excluded (35). Both BDNF and CNTF, responsible for differentiation, neurite outgrowth, neurogenesis, and synaptic modulation (37), decreased significantly at both mRNA and protein levels in 24-week diabetic retinas (Figure 3E-H). However, in 4-week diabetic rat retinas, BDNF expression was detected a transitory increase (Figure 3A-D). It has been reported that BDNF was up-regulated in the brain under dietary restriction associated with mild hypoinsulinemia (38), but it was shown to be decreased in STZ-induced diabetic rat retinas due to hypoinsulinemia (35). Although the reason for BDNF up-regulation in early diabetic retina remains unknown, BDNF expression may be sensitive to retinal metabolism. It is also possible that BDNF, as an acute response protein, responds to diabetic stimuli very rapidly to protect retina from injury (39). In contrast to the transitory up-regulation of BDNF in the diabetic retina, CNTF remained no change. This might indicate that these two factors are subject to different transcriptional regulations (40). At 24 weeks after diabetes onset, both BDNF and CNTF were significantly reduced, suggesting diminished Müller cell function, which could lead to a depleted capacity for neuronal regeneration (41). With the progression of diabetes, the deterioration of

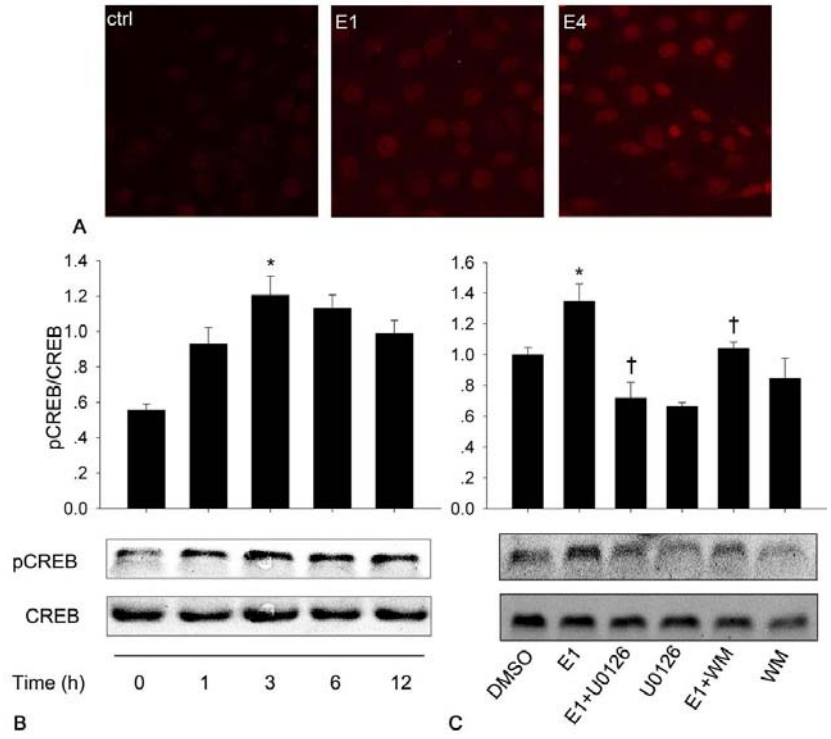

Figure 8. EPO increased phosphorylation of CREB through MAPK/ERK1/2 and PI3K/Akt pathways. (A) Immunocytochemistry for phospho-CREB, E1: EPO (1 U/mL, 12 h); E4: EPO (4 U/mL, 12 h). Original magnification: ×200. (B) Müller cells were exposed to 1 U/mL EPO for 0, 1, 3, 6 or 12 h. Quantitative analysis was shown above. (C) EPO-induced phosphorylation of CREB was blocked by U0126 and WM. ERK1/2 inhibitor (U0126; 10 μM; 1 h prior to EPO) or PI3-kinase inhibitor (WM; 0.5 μM; 1 h prior to EPO) was used to treat Müller cells for 12 h with or without EPO (1 U/mL). Proteins were extracted for Western blotting. Relative phosphorylation levels were calculated by the ratio of densitometric values of phospho-CREB to total-CREB and normalized by control group as shown above. LSD test after One-way ANOVA gave **P*<0.05 *vs* control and †*P*<0.05 *vs* EPO (1 U/mL). Each experiment was repeated in triplicate.

Müller cell function may therefore accelerate diabetic microangiopathy and neuronopathy.

Intraocular administration of BDNF has been reported to rescue dopaminergic amacrine cells from neurodegeneration in diabetic retina (35). Similarly, exogenous CNTF was reported to improve nerve conduction, ameliorated regenerative deficits, and prevented retinal degeneration in STZ-induced diabetic rats (36, 42). EPO has been reported to promote release of beta-nerve growth factor (beta-NGF) and bone morphogenetic protein 2 (BMP-2) in rat brain (43). In view of these reports, we decided to explore whether EPO could promote the production of neurotrophins in diabetic retina. As shown above, EPO up-regulated BDNF and CNTF expressions differently in 4- and 24-week diabetic retinas. These *in vivo* findings were confirmed by *in vitro* studies, demonstrating different dose- and time-dependent effects of EPO on BDNF and CNTF expressions. It appears that increased BDNF mRNA expression is more sustainable (Figure 5A-D) and that increased BDNF protein is at least 25-fold higher than that of CNTF (Figure 5E and F). This suggested a critical role for BDNF in EPO's neurotrophic function. EPO-induced increase in BDNF was previously reported in correlation with increased neurogenesis following stroke (20) and with a neuroprotective effect on primary hippocampal neurons exposed to the prototypic neurotoxicant, trimethyltin (TMT) (19). Our studies showed that EPO induced neurite outgrowth of primary retinal neurons, and this regenerative effect was diminished by TrkB/Fc, an inhibitor of BDNF, indicating that BDNF is involved in the neurotrophic function of EPO.

With regard to the specific signaling pathways that mediated EPO-induced production of BDNF and CNTF, we studied classical EPO/EpoR downstream pathways, including MAPK/ERK1/2 and PI3K/Akt. In our study, the inhibition of ERK1/2 by U0126 blocked EPO-induced expression of BDNF, but not CNTF. The inhibition of PI3-kinase by Wortmannin blocked EPO-induced CNTF expression, but enhanced BDNF expression (*P*<0.05; Figure 7B). These phenomena suggested that MAPK/ERK1/2 is a positive regulator, whereas PI3K/Akt is a negative regulator in EPO-induced BDNF expression; while, PI3K/Akt positively regulates EPO-induced CNTF expression. Other signaling pathways and regulators may also be included in the induction of neurotrophic factors by EPO, such as, STAT5 (44), Ca^{2+}, or CREB (19). CREB is known to be activated by phosphorylation on serine 133 and reported to be phosphorylated by many signaling

pathways, such as MAPK/ERK1/2 and PI3K/Akt (45). Once CREB is activated, it binds to the CREB binding protein and thus activates target genes such as BDNF (46). In the present study, EPO enhanced phosphorylation of CREB in a dose- and time-dependent manner. Interestingly, the EPO-induced increase in phospho-CREB was abolished by either U0126 or Wortmannin. Thus, we speculated that EPO-induced expression of BDNF may result from stimulation of CREB via activation of both MAPK and PI3K/Akt pathways (Figure 8). On the other hand, the PI3K/Akt pathway itself may exert dual functions, i.e., a CREB-dependent (Figure 8) and a CREB-independent function to BDNF expression as shown in Figure 7B, in which the Wortmannin treatment did not suppress but rather stimulated, EPO-induced BDNF

In summary, gliosis of Müller cells may have both cytoprotective and cytotoxic effects on retinal neurons (7). Protective responses of Müller cells include production of neurotrophic factors and cytokines (7, 47). However, activated Müller cells also possess significant cytotoxic effects by releasing pro-inflammatory factors such as TNF-alpha (48), monocyte chemoattractant protein-1 (MCP-1) (49), and nitric oxide (NO) (50), and by down-regulating neurotrophic factors (7). Thus, control of excessive gliosis may be a useful therapeutic approach for protecting retinal neurons. EPO may be an ideal agent in this regard, since it is also able to promote the production of neurotrophic factors by Müller cells. The neurotrophic function of Müller cells was mediated by MAPK/ERK1/2 and PI3K/Akt pathways which were also involved in EPO neuroprotection (22). Other reports demonstrated that EPO exerts its protective effects on diabetic retinas through anti-apoptotic (22), anti-oxidant (51) and anti-inflammatory effects (34), maybe by reducing the loss of pericytes in early DR as well (52). However, Tong et al. (53) showed T allele of SNP rs1617640 in the promoter of the EPO gene is significantly associated with proliferative diabetic retinopathy (PDR) and end stage renal disease, and the EPO concentration in human vitreous was 7.5-fold higher in normal subjects with the TT (T allele) risk genotype than in those with the GG (G allele) genotype. Therefore, EPO may act as an angiogenic factor potentially aggravating PDR. It has been known that individual genetic polymorphisms are risk factors that may be not causative factors of specific diseases. Thus, the association of this polymorphism with PDR requires extensively studies to reduce both false-positive and false-negative results. In fact, the examinations of epiretinal membranes of eyes with PDR revealed significant correlations between the number of blood vessels expressing the panendothelial marker CD34 and those expressing hypoxia-inducible factor-1alpha (HIF-1alpha) and vascular endothelial growth factor (VEGF), but no correlation with immunoreactivity for EPO, suggesting that HIF-1alpha and VEGF play the major angiogenic roles in PDR, while EPO does not (54). Recent studies in rat (55), rabbit (56, 57), and human (58, 59) showed that intravitreal injection of EPO is a safe procedure. It is still unresolved whether intraocular injection of EPO could induce neovascularization, especially repeated injections and prolonged expressions of EPO in the eye. Further studies need to be carried out to test the potential risks of EPO on retinal neovascularization. It should be noted that this study has examined only Müller cells; other glial cells, e.g. astrocytes, may also contribute to the neurotrophic effect. It must also be realized that experimental studies in rodents have potential shortcomings, for instance, animals which lack an inner retinal circulation won't develop real human DR changes (60). Notwithstanding its limitations, this study does provide a better understanding of multiple potential protective mechanisms of EPO in the diabetic retina, and suggest that Müller cells, as primary mediators of these effects, may be important targets in the therapy of DR, and perhaps other neurodegenerative diseases of the retina.

6. ACKNOWLEDGEMENTS

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Abbreviations: BDNF: Brain-derived neurotrophic factor; BMP-2: Bone morphogenetic protein 2; BRB: Blood-retinal barrier; Beta-NGF: Beta-nerve growth factor; CNTF: Ciliary neurotrophic factor; CRALBP: Cellular retinaldehyde-binding protein: CREB: Cyclic AMP response element binding protein; DR: Diabetic retinopathy; EAE: Experimental autoimmune encephalomyelitis; EPO: Erythropoietin; EpoR: Erythropoietin receptor; ERK1/2: Extracellular signal-regulated kinase; GCL: Ganglion cell layer; GFAP: Glial fibrillary acidic protein; HIF-1alpha: Hypoxia-inducible factor-1alpha; INL: Inner nuclear layer; IPL: Inner plexiform layer: LNL: Longest neurite length; MAPK: Mitogen-activated protein kinase; MCP-1: Monocyte chemoattractant protein-1; NO: Nitric oxide; OCT: Optimal cutting temperature; PDR: Proliferative diabetic retinopathy; PVR: Proliferative vitreoretinopathy; RIPA: Radioimmune precipitation assay; rMC-1: Retinal Müller cell line; STZ: Streptozotocin; TMT:Trimethyltin; VEGF: Vascular endothelial growth factor.

Key Words Diabetic Retinopathy, Erythropoietin, Müller cells, Neurotrophin, Gliosis, MAPK/ERK, PI3K

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