Erythropoietin Exerts a Neuroprotective Function Against Glutamate Neurotoxicity in Experimental Diabetic Retina

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PURPOSE. Retinal neuronal cell dysfunction and even cell death are associated with increased excitotoxic glutamate (Glu) level in the retina. Our aim was to study a causative mechanism of Glu on retinal cell death and explore the neuroprotective role of erythropoietin (EPO) against Glu neurotoxicity in the diabetic retina.

METHODS. Male Sprague-Dawley (SD) rats and R28 cell line were employed in this study. Diabetes was induced with intraperitoneal injection of streptozotocin (STZ) in SD rats. Two weeks after diabetes onset, the intravitreal injection was performed; 4 days later, the retinas were harvested for testing. R28 cells were treated with Glu, Glu + EPO, or Glu + EPO + soluble EPO receptor (sEPOR), respectively, for 24 hours, and then the cells were collected for the following studies. Glutamate level in the retina was measured with a glutamate assay kit. Cell death was determined with TUNEL staining. The changes in glutamine synthetase (GS), glutamate–aspartate transporter (GLAST), ionotropic glutamate receptors (iGluRs), apoptosis-inducing factor (AIF), and poly(ADP-ribose) (PAR) polymer were studied with RT-PCR, Western blot, and immunofluorescence.

RESULTS. In 2-week diabetic rat retinas, Glu concentration was approximately 1.21-fold that in normal control. TUNEL staining demonstrated that retinal cell death was increased. Retinal GS and GLAST expressions were decreased, while the iGluRs, for example, KA1 and NR1, and PAR polymer expression was increased. In R28 cells, 24 hours after Glu (10 mM) treatment, the cell viability was decreased by 52.7%; KA1, NR1, PAR polymer, and nuclear AIF all increased in expression. The above conditions could be largely reversed by EPO both in vivo and in vitro. The protective effect of EPO was abolished by sEPOR.

CONCLUSIONS. Erythropoietin showed a neuroprotective function against Glu-mediated neurotoxicity both in diabetic rat retina and in Glu-treated R28 cells. The neuroprotective mechanisms were largely through maintaining the normal expression of glutamate–glutamine cycle-related proteins and inhibiting AIF translocation and PAR polymer formation.

Keywords: diabetic retina, glutamate, erythropoietin, cell death

Glutamate (Glu) is the main excitatory neurotransmitter in the retina.1 However, it is neurotoxic in excessive amounts.2–5 Glutamate-induced cell death has recently been designated parthanatos to distinguish it from classical caspase-dependent apoptosis, autophagy, and necrosis.6–7 Parthanatos is prominently implicated in models of hypoxic-ischemic central nervous system disease, such as infarction and cerebral ischemia,8,9 but has been less studied in diabetic retinal cell death.
Several studies showed that Glu and gamma-aminobutyric acid (GABA) were elevated in the vitreous humor of individuals with proliferative diabetic retinopathy (PDR), while other amino acids remain unchanged. These findings suggested that a specific defect in Glu metabolism, rather than a general alteration in the metabolism of amino acids, was responsible for the observed changes. Thus, the regulation of retinal Glu deserves much attention.

Table 1. The Primers Used in This Study

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Tm, melting temperature.

Figure 1. Intravitreal injection of EPO protects retinal neurons from cell death (A) and reduces the retinal Glu level (B) in diabetic rat retinas. Compared with values in normal control, more TUNEL-positive cells and increased Glu levels were detected in the diabetic rat retinas, which were decreased after intravitreal injection of EPO (16 mU/eye). (A) Representative fluorescent microscopic images of TUNEL. The exposure time was 400 ms (TUNEL staining, green) and 30 ms (DAPI staining, blue); magnification is ×200. (B) EPO reduced retinal Glu level in diabetic rat retinas. Data are expressed as mean ± SE (n = 8). *P < 0.05 when compared with D2w group. NC, negative control; PC, positive control; N, normal retina; D2w, 2-week diabetic retina; E, EPO-treated retina.
FIGURE 2. Western blot and immunostaining of GS and GLAST in the retinas. Compared with values in normal control, the protein levels of GS and GLAST in diabetic rat retinas were decreased; they were increased after intravitreal injection of EPO (16 mU/eye). (A, B) The protein changes of GS and GLAST in the retinas. Data are expressed as mean ± SE (n = 10 [A], n = 14 [B]). β-actin was used as the internal control. *P < 0.05 when compared with D2w group. (C, D) Immunostaining of GS and GLAST in the retinas. The exposure time was 0.9 seconds (GS, red), 1.2 seconds (GLAST, green), and 30 ms (DAPI, blue), respectively; magnification is ×200. NC, negative control; N, normal retina; D2w, 2-week diabetic retina; E, EPO-treated retina.
more attention with regard to preventing vision impairment in DR.

In normal retina, an appropriate clearance of synaptic Glu is required for the normal function of retinal excitatory synapses and for prevention of neurotoxicity. Müller cells are the principal glia cells of retina, playing a key role in retinal Glu clearance. Extracellular Glu is transported into Müller cells by glutamate-aspartate transporter (GLAST) and ammated to the nontoxic glutamine by a Glu-metabolizing enzyme, glutamine synthetase (GS). Glutamine is then released by the Müller cells and taken up by neurons, where it is hydrolyzed by glutaminase to form Glu again.12–15 Neuronal function deteriorates rapidly when there are deficiencies in either Glu uptake or the conversion of glutamate to glutamine.16–18

Glutamate elicits a neuronal response by binding to one or more Glu receptors. Glutamate receptors can be divided into two distinct groups: the fast-acting ligand-gated ion channels (iGluRs) and the slower-acting metabotropic receptors (mGluRs).19 Based on their physiological, pharmacologic, and molecular properties, the iGluRs are further subdivided into three groups: alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate (KA), and N-methyl-D-aspartate (NMDA) receptors. The ionotropic receptors are multimeric, with AMPA receptors being assembled from subunits designated GluR1-4; KA receptors from subunits KA1, KA2, and GluR5-7; and NMDA receptors from NR1, NR2A-D, and NR3A subunits.20

The neurotoxicity of Glu is mediated largely through influx of calcium through the NMDA receptor, leading to activation of poly(ADP-ribose) polymerase 1 (PARP-1) and generation of poly(ADP-ribose) polymer (PAR polymer), a newly described death signal that kills cells through apoptosis-inducing factor (AIF).21–23 Since broad-spectrum caspase inhibitors fail to prevent PAR polymer-induced cell death,24 this form of cell death is a caspase-independent process, and has recently been designated parthanatos to distinguish it from the other types of cell death.6,7 Previous studies reported that erythropoietin (EPO) administered intraperitoneally or intravitreally was protective for both retinal neurons and vascular cells in early diabetes;25,26 safety was proven even with high-dose EPO delivered into the eye in both experimental and clinical studies.27–29 As a matter of fact, a recent clinical series of intravitreal injections of EPO into
Western blot and immunostaining of KA1 and NR1 in the retinas. Compared with values in normal control, the protein levels of KA1 and NR1 in 2-week diabetic rat retinas were increased; they were decreased after intravitreal injection of EPO (16 mU/eye). (A, B) The protein changes of KA1 and NR1 in the retinas. Data are expressed as mean ± SE (n = 10). β-actin was used as the internal control. *P < 0.05 when compared with the D2w group. (C, D) Immunostaining of KA1 and NR1 in the retinas. The exposure time was 300 ms (KA1, green), 200 ms (NR1, green), and 30 ms (DAPI, blue), respectively; magnification is ×200. NC, negative control; N, normal retina; D2w, 2-week diabetic retina.
FIGURE 5. Glutamate and EPO effects on R28 cells by MTT assay and TUNEL staining. (A) Dose-dependent effect of Glu on R28 cell viability. R28 cells were treated with different concentrations of Glu for 24 hours, and then cell viability was measured with MTT assay. The data showed that Glu dose-dependently decreased the cell viability of R28 cells. Glu 10 mM was selected to treat R28 cells in a later study. (B) The protective effect of EPO on R28 cell viability. R28 cells, incubated with or without Glu (10 mM), were treated with different concentrations of EPO for 24 hours, and the cell viability was measured. A 0.2 U/mL dose was selected as the optimal concentration. (C) Soluble EPO receptor abolished the protective effect of EPO
eyes with severe diffuse diabetic macular edema produced short-term favorable visual outcomes and anatomic improvement.\textsuperscript{30} Although protective mechanisms of EPO have been extensively studied,\textsuperscript{26,51-54} whether EPO participates in regulation of Glu metabolism in diabetic rat retina has not been reported. The present study focused on the protective effects of EPO on Glu neurotoxicity in the diabetic retina both in vivo and in vitro.

**Materials and Methods**

**Reagents and Antibodies**

Streptozotocin (STZ, 09350), recombinant human erythropoietin (r-HuEPO; 42364), and Thiazolyl Blue Tetrazolium Bromide (MTT, M2128) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soluble EPO receptor (sEPOR; 507-ER-050) was purchased from R&D (Shanghai, China). Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagents (78835) were purchased from Thermo Fisher (Shanghai, China). L-glutamate (Amresco 0421) was purchased from Amresco (Solon, OH, USA). Dulbecco’s modified Eagle’s medium (DMEM) Low Glucose Medium (SH30021.01B) was purchased from HyClone Laboratories (Logan, UT, USA). Penicillin-streptomycin (15140155) was purchased from Invitrogen (Carlsbad, CA, USA). The Glutamate Assay Kit (K629-100) was purchased from Biovision (Milpitas, CA, USA). In situ cell death detection kit (11684795910) was purchased from Roche (Shanghai, China). The primary antibodies against KA1 (ab67404, ab101011), NR1 (ab17345), GLAST (ab116), and GS (ab64613) were purchased from Abcam (Cambridge, UK). AIF antibody (No. 4642) was purchased from Cell Signaling Technology (Beverly, MA, USA). Poly(ADP-ribose) polymer antibody (ALX804-220) was purchased from Alex Biochemicals (Shanghai, China). Secondary antibodies, anti-mouse IgG (610-431-002) and anti-rabbit IgG (611-131-002), were purchased from Rockland Immunochemicals, Inc. (Limerick, PA, USA); FITC (fluorescein isothiocyanate) goat anti-mouse IgG (115-095-003) and FITC goat anti-rabbit IgG (115-095-003) were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA). Protein extraction radioimmunoprecipitation assay (RIPA) buffer (P0015B) was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

**Experimental Animals and Intravitreal EPO Treatment**

Male Sprague-Dawley (SD) rats, weight approximately 200 g, were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All rats were housed under a normal 12-hour light/dark cycle and with access to food and water ad libitum. The rats were divided into three groups: normal control (N), 2-week diabetic rats (D2w), and EPO-treated diabetic rats (E). Diabetes was induced by STZ (intraperitoneal injection, 60 mg/kg body weight freshly dissolved in citric buffer, pH 4.5) and was confirmed by blood glucose exceeding 300 mg/dL for 3 consecutive days. Two weeks after diabetes onset, intravitreal injection was performed.

Intravitreal injection was performed with a 30-gauge, 0.5-inch needle (BD Biosciences, Franklin Lakes, NJ, USA) on a microsyringe (Hamilton, Reno, NV, USA), using a temporal approach, 2 mm posterior to the limbus. For the EPO-treated group, the rats were injected intravitreally with EPO (16 mL/eye, dissolved in a volume of 2 mL); equivalent normal saline injections were performed on both the normal control rats and the untreated diabetic rats. Four days later, the rats were killed and the retinas were isolated and stored at −80°C until assay.

**Glutamate Assay**

Retinal Glu concentration was measured using the Glutamate Assay Kit according to the manufacturer’s instructions. Retinas were homogenized in 100 μL assay buffer and centrifuged for 10 minutes at 13,000 g at 4°C. The supernatant was collected, and the Reaction Mix (100 μL), containing assay buffer (90 μL), Glutamate Developer (8 μL), and Glutamate Enzyme (2 μL), was added. After incubation at 37°C for 30 minutes, the absorbance was measured at 450 nm.

**RNA Isolation and Determination of Gene Expression**

Each neurosensory retina was subjected to extraction of total RNA from retina using Trizol (Invitrogen, Shanghai, China). The RT product (0.8 μL) was then amplified with PCR. The specific primers, designed by the software (Primer Premier Version 5.0), were purchased from Shanghai DNA Biotechnology Corp., Ltd. (Shanghai, China). The Table shows the primer information for iGluRs, GLAST, and GS. β-Actin was used as an internal standard. Following RT-PCR, the products were fractionated electrophoretically in 1.5% agarose gel. The optical densities were determined with Quantity One software (Bio-Rad, Hercules, CA, USA) and normalized with β-actin.

**R28 Cell Culture**

R28 cells, an E1A-immortalized model of retinal neurons, were a generous gift from Gail M. Seigel (State University of New York, Buffalo, NY, USA). The cells were cultured in low-glucose DMEM containing 10% fetal bovine serum (FBS; HyClone Laboratories) and 1% penicillin-streptomycin at 37°C with 5% CO\textsubscript{2} in a humidified incubator.

**Cell Viability Assay**

R28 cells were seeded in 96-well plates at a density of 1.6 × 10\textsuperscript{4} cells/well. Cells were treated with Glu, Glu+EPO, and Glu+EPO+ePO, respectively, for 24 hours. Then the medium was discarded, and the cells were washed with PBS. After addition of fresh medium containing MTT (5 mg/mL) and 4-h incubation, the MTT medium was discarded and 100 μL 10% SDS (pH 4.5) was added following another 4 hours incubation. The optical density (OD) value was measured. The results are expressed relative to the controls specified in each
experiment. They are expressed as the mean of six determinations within the same experiment ± SE; each experiment was repeated at least three times with similar results.

In Situ Detection of Cell Death in the Retina and R28 Cells

Cell DNA strand breaks were determined with TUNEL assay according to the manufacturer's instruction. Positive controls were treated with grade I DNase I for 10 minutes at room temperature before the labeling procedure. Negative controls were treated with 10 μL label solution but incubated in the absence of terminal transferase. The retinal sections and R28 coverslips were rinsed three times with PBS after incubation and analyzed under a fluorescence microscope (DMi3000; Leica, Wetzlar, Germany) with an excitation wavelength in the range of 450 to 490 nm.

Protein Extraction and Western Blotting

Total retina and R28 cells were broken with ultrasonic wave and protein was extracted with RIPA buffer. Nuclear protein was extracted with the Thermo Nuclear and Cytoplasmic Extraction kit, and protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA). Proteins were resolved on SDS-polyacrylamide gels (10%) and transferred electrophoretically onto a nitrocellulose membrane (Bio-Rad). The membranes were cut into several blots according to the size of the proteins being detected, and then blocked with 5% nonfat milk in TBST (50 mM Tris, pH 7.6, 0.9% NaCl, and 0.1% Tween 20) for 30 minutes at room temperature. The blots were then separately incubated overnight with the primary antibodies: anti-KA1 (1:500), anti-NR1 (1:500), anti-GLAST (1:500), anti-GS (1:1000), anti-AIF (1:500), anti-PAR polymer (1:500), and β-actin antibody (1:5000). After washing three times, the blots were incubated with their respective secondary antibodies for 1 hour: anti-mouse IgG (1:5000) or anti-rabbit IgG (<100), at 4°C overnight. Slides without primary antibody served as negative control. After washing for 15 minutes in PBS, the slides were incubated with their appropriate secondary antibodies (1:100; anti-rabbit FITC, anti-rabbit CY3, or anti-mouse CY3) for 1 hour at room temperature in the dark. After incubation with 4′,6-diamidino-2-phenylindole (DAPI) for 2 minutes to stain nuclei at room temperature, the slides were extensively washed four times with PBS and then mounted with coverslips. Retina slides were visualized with the Leica DMI3000 microscope. R28 cells slides were visualized with a Carl Zeiss Microsystems confocal microscope (488 nm excitation, 495–532 nm emission band; LSM 710, Königsallee, Germany). Exposure conditions in the same channel for different groups in each experiment were consistent.

Statistical Analysis

The results were expressed as mean ± SE from at least three independent experiments. Statistical analysis was carried out with the SPSS software, version 22.0 (IBM Company, Armonk, NY, USA), and one-way ANOVA with Dunnett's post test was confirmed with immunofluorescence. Glutamine synthetase

RESULTS

EPO Protects Retinal Neurons From Death and Decreases Retinal Glutamate Levels in Diabetic Rats

The TUNEL assay is a method to detect DNA damage. To confirm the protective effect of EPO on retinal neurons in diabetic rats, retinal cell death was examined with TUNEL assay. In 2-week diabetic retina, TUNEL-positive cells were obviously increased in all three nuclear layers, especially in the outer nuclear layer (ONL). With diabetes progression, more TUNEL-positive cells were detected in 6-week diabetic rat retinas (Supplementary Fig. S1). In the retina of EPO (16 mU/eye)-treated diabetic rats, few or no TUNEL-positive cells were detected (Fig. 1A). At the same time point, the Glu concentration in 2-week diabetic rat retinas was increased by 20.9% in comparison with that in the normal control (n = 8, P < 0.05). In the EPO-treated group, the Glu level was decreased significantly by 13.8% in comparison with that in the 2-week diabetic rats (n = 8, P < 0.05, Fig. 1B). These data suggested that increased Glu level in the diabetic retina might be related to the death of retinal neurons, which could be reversed by EPO through downregulation of Glu level.

EPO Prevents the Decreased Expression of GS and GLAST in Diabetic Retina

Since the expression of GLAST and GS was decreased in DR, which could contribute to Glu accumulation, we examined if EPO could prevent the decrease in GS and GLAST. In the retinas of 2-week diabetic rats, GLAST and GS protein levels were decreased significantly but were increased after EPO treatment. The level of GS protein was decreased by 24.0% (n = 10, P < 0.05) in diabetic rat retinas compared to that in normal control, and it was increased by 13.7% (n = 10, P < 0.05) in the EPO-treated group (Fig. 2A) in comparison with the diabetic group. For GLAST, similar effects and changes were observed. The GLAST protein decreased by 23.8% (n = 14, P < 0.05) in diabetic retinas compared to normal control and recovered to near-normal control level (n = 14, P < 0.05) in the EPO-treated group (Fig. 2B). The protein changes of GS and GLAST were further confirmed with immunofluorescence. Glutamine synthetase
FIGURE 6. Western blot and immunostaining of EPO and sEPOR effects on KA1 and NR1 expression in Glu-treated R28 cells. Compared with those in normal control, the protein levels of KA1 and NR1 in Glu-treated R28 cells were increased; expression was decreased by EPO and increased again by sEPOR. (A, B) Western blot results of KA1 and NR1 in R28 cells. Data are expressed as mean ± SE from at least three independent experiments. β-actin was used as the internal control. *P < 0.05 when compared with the Glu group; #P < 0.05 when compared with the Glu+EPO+sEPOR group. (C, D) Confocal images of KA1 and NR1 in R28 cells (magnification, ×650). The doses used were 10 mM (Glu), 0.2 U/mL (EPO), and 100 ng/mL (sEPOR). NC, negative control; Cont, cells treated with culture medium only.
FIGURE 7.  EPO reduced AIF level in the nucleus and prevented AIF translocation to the nucleus. (A) Western blot results on increased AIF level in nuclear fraction in R28 cells treated with Glu. R28 cells were treated with Glu (10 mM) for 12 and 24 hours. Data are expressed as mean ± SE from three independent experiments. β-actin was used as the internal control. *P < 0.05 when compared with untreated group. (B) Confocal evidence of AIF translocation to nucleus in Glu-treated R28 cells. After 24-hour treatment, control cells (Cont) and Glu-treated R28 cells (10 mM Glu) were stained for AIF (red) and DAPI (blue). Note that most AIF proteins remained in the cytoplasm of untreated R28 cells (Cont) and were translocated to
was expressed in Müller cells, which extend from the inner limiting membrane (ILM) to the outer limiting membrane (OLM), spanning all the layers of the retina. In normal retina, the GS staining was primarily in Müller cell end-feet in the ganglion cell layer (GCL), nerve fiber layer (NFL), and cell bodies in the inner nuclear layer (INL). In the 2-week diabetic retinas, the intensity of GS was decreased in the whole retina, mainly in GCL, NFL, and INL. In the EPO-treated group, GS expression appeared to be maintained at the normal level (Fig. 2C). The same finding was also observed with GLAST. Glutamate–aspartate transporter was extensively expressed in normal neural retina and was dramatically decreased in 2-week diabetic rat retinas. In EPO-treated rat retinas, GLAST expression was nearly at the same level as in the normal control (Fig. 2D). Hence, decreased GS and GLAST in diabetic retina may contribute to Glu accumulation, and prevention of GS and GLAST from a decrease should account for one of EPO’s protective actions in diabetic retina.

**EPO Downregulates the Expression of KA1 and NR1 in Diabetic Retina**

Glutamate elicits neuronal responses through binding to its receptors. Changes of Glu receptors in diabetic retina treated with or without EPO were detected. Our data showed that 2 weeks after diabetes onset, the levels of mRNA for retinal KA1, KA2, and NR1 were all increased significantly but were decreased in EPO-treated diabetic rats. The mRNA levels were increased by 21.0% (KA1), 76.5% (KA2), and 29.2% (NR1) in diabetic rats, respectively, in comparison with normal control.

With EPO treatment, the mRNA levels were decreased by 9.4% (KA1), 74.6% (KA2), and 24.1% (NR1) in comparison with those in the diabetic retinas (n = 6, P < 0.05). No significant difference was found for GluR1-4 and NR2A-C among the three groups. The mRNA level of NR2D was significantly upregulated in diabetic retina, but EPO had no effect on it (Fig. 3D).

Considering that the proteins KA1 and KA2 have high homology in structure and function, the KA1 protein was studied in the following experiments. The levels for KA1 and NR1 protein were increased by 25.3% and 16.3%, respectively, in diabetic rats, and were decreased by 29.0% and 15.2% in the EPO-treated diabetic group (n = 10, P < 0.05). The results demonstrated that KA1 and NR1 expression was increased in diabetic retinas, and that EPO exerts its neuroprotective function at least partly through decreased KA1 and NR1 expression.

**EPO Rescues R28 Cells From Glutamate-Induced Toxicity**

To confirm the causal effect of Glu on neuronal damage in retina, R28 cells were used as an in vitro model. Figure 5A shows that the relative cell viability was reduced by Glu in a concentration-dependent manner. When Glu concentration reached 10 mM, cell viability was significantly reduced to approximately 47.5%. Beyond this concentration, the cells were nearly all dead, indicating the toxic effect of Glu. Furthermore, when cells were cultured with 10 mM Glu, exogenous EPO addition increased cell viability dose-dependently, with the optimal dose of 0.2 U/mL (Fig. 5B). The protection by EPO of Glu-treated R28 cells was abolished by sEPOR (Fig. 5C). The cell viability in the sEPOR (100 ng/mL)-treated group was approximately 60.1% of that in the control. Erythropoietin or sEPOR alone had no effect on cell viability in untreated R28 cells (Figs. 5B, 5C). Each experiment was repeated at least three times, yielding similar results.

Figure 5D shows the characteristic morphological changes of R28 cells under different treatments. When treated with Glu (10 mM) for 24 hours, some cells underwent shrinkage; the cell morphology appeared to be normal with coadministration of EPO (0.2 U/mL). However, this protection by EPO was abolished by sEPOR (100 ng/mL). TUNEL staining showed more TUNEL-positive cells in the Glu-treated group, and less after EPO treatment; addition of sEPOR largely abolished EPO’s protection (Fig. 5E). Therefore, the in vitro results verified the damaging effect of Glu in diabetic retinas and in R28 cells, while EPO could protect them from cell death.

**EPO Downregulates the Expression of KA1 and NR1 in Glutamate-Treated R28 Cells**

To verify the in vivo results, Glu and EPO effects on retinal R28 cells were studied. Our in vitro studies confirmed the changes in KA1 and NR1 seen in vivo. The levels of KA1 and NR1 protein in R28 cells increased by 34.0% and 52.2% (n = 8, P < 0.05) in the Glu-treated group when compared to the control. Erythropoietin treatment reduced their levels by 29.4% and 41.9% (n = 8, P < 0.05) in comparison with the Glu-treated group. With coaddition of sEPOR (100 ng/mL), the levels of KA1 and NR1 protein in R28 cells were increased by 29.4% and 40.0%, respectively (n = 8, P < 0.05). The immunofluorescence results for KA1 and NR1 in R28 were consistent with the Western blot data. Compared with that in normal control, the staining intensity of both KA1 and NR1 was much stronger in R28 cells treated with Glu (10 mM). After EPO (0.2 U/mL) treatment, decreased expression and redistribution of both KA1 and NR1 were observed (Figs. 6C, 6D). Again, the EPO effect on KA1 and NR1 was abolished by sEPOR (100 ng/mL). Collectively, upregulated iGluRs may partially explain the toxicity of high Glu to retinal neurons, and EPO protects the cells through downregulating these receptors.

**EPO Inhibits AIF Translocation to Nucleus in Glutamate-Treated R28 Cells**

Apoptosis-inducing factor translocation from mitochondria to the nucleus is a crucial step in the Glu-induced parthanatos pathway. Translocation of AIF was studied with Western blot analysis of the nuclear fraction in R28 cells and with immunostaining. The data showed that the nuclear AIF level was increased after Glu treatment. At 12 and 24 hours after Glu
FIGURE 8. Western blot and immunostaining of PAR polymer in the retina and R28 cells. EPO reduced PAR polymer levels in diabetic rat retina and Glu-treated R28 cells. PAR polymer expression was increased in 2-week diabetic rat retinas and Glu-treated R28 cells, which was decreased by EPO. The effect of EPO was abolished by sEPOR in R28 cells. (A, B) Western blot results of PAR polymer. β-actin was used as the internal control. Data are expressed as mean ± SE. *P < 0.05 when compared with the D2w group in rats or compared with Glu in R28 cells; #P < 0.05 when compared with the Glu+EPO+sEPOR group in R28 cells (n = 10 [A], n = 3 [B]). (C) Immunostaining of PAR polymer in normal control (N), 2-week diabetic rats
(10 mM) treatment, the AIF level was increased by 44.7% and 53.9%, respectively (Fig. 7A), and AIF translocation to the nucleus was observed with immunostaining (Fig. 7B). We also found that EPO (0.2 U/mL) administration significantly decreased the nuclear AIF level by 19.7%, and sEPOR (100 ng/mL) increased its level by 24.0% (Fig. 7C).

**EPO Inhibits PAR Polymer Expression in Diabetic Retina and Glutamate-Treated R28 Cells**

Poly(ADP-ribose) polymer is a marker in the parthanatos pathway. The level of PAR polymer protein was increased by 82.2% in 2-week diabetic rat retinas, and was decreased by 61.5% after intravitreal injection of EPO (n = 10, P < 0.05, Fig. 8A). The immunostaining of PAR polymer was much stronger in 2-week diabetic rat retinas, mainly in GCL, INL and ONL, and was decreased after EPO treatment (Fig. 8C). With diabetes progression, immunostaining of PAR polymer was much stronger in 4- and 6-week diabetic rat retinas (see Supplementary Fig. S2).

The level of PAR polymer in Glu-treated R28 cells was increased by 78.7% (n = 8, P < 0.05) when compared with the control. After EPO treatment, the level of PAR polymer was reduced by 70.5% (n = 8, P < 0.05), and this effect was partially abolished by sEPOR (100 ng/mL). The level of PAR polymer was increased by 50.4% after sEPOR treatment (n = 8, P < 0.05, Fig. 8B). The immunofluorescence results for PAR polymer also confirmed above results in R28 cells (Fig. 8D).

**DISCUSSION**

Our serial studies have demonstrated the protective effects and the mechanisms of EPO action on retinal neuronal cells and the blood–retinal barrier (BRB), as well as vision improvement in both experimental DR and “phase 0” clinical studies. In this study, we report that EPO protects against Glu neurotoxicity in the parthanatos pathway.

Glutamate is the main retinal excitatory transmitter, necessary for retinal functions. It is also a potent death inducer of retinal neurons when present at elevated concentrations. In the present study, we detected significantly elevated Glu levels in 2-week diabetic retinas (Fig. 1B). In fact, retinal Glu increased with progression in diabetic rats, while expression of GS and GLAST decreased in a time-dependent manner (see Supplementary Fig. S3). Although we did not measure the Glu metabolism, for example, upregulation of GS and GLAST, showed that diabetes had a greater effect on gene expression of iGluR subunits than on AMPA receptor subunits in rat retinas. And in human diabetic retinas, the intensity of NR1 subunit immunoreactivity was increased in all retinal layers, particularly in the GCL. In our study, when exogenous EPO was administered, Glu receptors, such as KA1 and NR1 subunits, were downregulated at both the mRNA and protein levels. However, EPO showed no effect on other types of iGluR subunits (Fig. 4). Although we did not measure the changes in calcium ion and PARP-1 activity in R28 cells, we did observe decreased levels of PAR polymer and nuclear AIF after EPO treatment in Glu-treated R28 cells. These results demonstrated that one of EPO's functions might be through the inhibition of the parthanatos pathway.

Diabetic retinopathy is a very complex disease; multiple pathological processes and cytokines are involved, and Glu is one of them. In this study, we found that in glutamate induced caspase-independent apoptosis pathway, such as PAR polymer and AIF was involved, and that EPO played a role in inhibition of this pathway. Although caspase-3 and cleaved caspase-3 proteins remained unchanged among the 3 groups of rats, we cannot exclude the involvement of caspase-dependent pathways in diabetic rat retinas because of the limitations of the current method (see Supplementary Fig. S6). In addition, EPO could increase the expression of GS and GLAST (Fig. 2) and decrease Glu receptors such as KA1 and NR1 (Fig. 6), thereby promoting the metabolism of Glu and decreasing its toxic effect on the retina. Reports have shown NR1 expression in the nucleus and even NR1 translocation to the nucleus. From this point of view and consistent with our immunofluorescence results, EPO might also promote the translocation of KA1 and NR1 from the cell membrane to the nucleus. The underlying mechanisms for this redistribution need further study.

In conclusion, we demonstrated that an elevated Glu level is one of the causal factors in the pathogenesis of neuronal death in diabetes. erythropoietin, as a neuroprotective reagent, exerted its protective functions partially through regulating Glu metabolism, for example, upregulation of GS and GLAST, thus maintaining the normal glutamate–glutamine cycle, and downregulation of Glu receptors, thus ameliorating neuronal overexcitation and neuronal toxicity. Moreover, EPO could also
inhibit the formation of PAR polymer and reduced the nuclear AIF level, thus protecting the diabetic retina from parthanatos cell death.

This is a new mechanism for EPO protection in the diabetic retina and expands our understanding of its protective mechanisms. In the last 20 years, a large number of Glu antagonists have been developed, but most failed due to serious adverse effects from suppression of Glu receptor function. For instance, a reversible NR1 gene knockout technique damaged learning and memory functions in a mouse model.48 In the present study, EPO normalized these receptors. This could make EPO more attractive for developing a new drug against Glu toxicity in central nervous system diseases. Due to the prominent role of Glu in many neurologic diseases,49 therapies aimed at blocking Glu-induced cell death by EPO could represent novel therapeutic targets for prevention of the toxic effects of Glu.

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