ERK- and Akt-Dependent Neuroprotection by Erythropoietin (EPO) against Glyoxal-AGEs via Modulation of Bcl-xL, Bax, and BAD

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PURPOSE. To characterize the neuroprotective mechanisms of erythropoietin (EPO) against the stress of glyoxal-advanced glycation end products (AGEs) in retinal neuronal cells.

METHODS. Rat retinal organ culture, primary retinal neuron culture, and retinal cell line (R28 cell) culture under glyoxal-AGEs insult were used as in vitro models. Exogenous EPO was applied to these models. Retinal neuronal cell death was assessed by TUNEL, ethidium bromide/acridine orange staining, and cell viability assay. R28 cell proliferation was evaluated by BrDU incorporation and propidium iodide staining. Real-time RT-PCR and Western blot analysis were used to detect Bcl-xL, Bcl-2, Bax, BAD, and products of extracellular signal regulated kinase (ERK) and Akt pathways. Specific inhibitors and plasmids were used to pinpoint the roles of ERK and Akt pathways.

RESULTS. EPO protected the retinal cells from glyoxal-AGE-induced injury in a time- and dose-dependent fashion. The protective function of EPO was proved to be antiapoptotic, not pro-cell proliferative. Glyoxal upregulated Bax expression but suppressed Bcl-xL expression and BAD phosphorylation. In contrast, EPO enhanced BAD phosphorylation and Bcl-xL expression but downregulated Bax. The regulation of these apoptosis-related proteins by EPO was through ERK and Akt pathways.

CONCLUSIONS. These data demonstrate that exogenous EPO significantly attenuates the retinal neuronal cell death induced by glyoxal-AGEs by promoting antiapoptotic and suppressing apoptotic proteins. EPO/EPO receptor signaling through ERK and Akt pathways is pivotal in EPO neuroprotective mechanisms.

ADVANCED GYLATION END PRODUCTS (AGEs) LEAD TO PROGRESSIVE AND IRREVERSIBLE CROSS-LINKING OF PROTEINS, THEREBY CAUSING DAMAGE TO CELLS.1 Several previous studies have shown that the enhanced formation and accumulation of AGEs is one of the major pathogenic factors in diabetic retinopathy (DR) and other neuronal diseases2,3 because AGEs cause abnormalities and apoptosis to neuronal/glial cells.4,5 Increasing in vivo and in vitro evidence indicates that erythropoietin (EPO) exerts several nonerythropoietic functions, including neuroprotective functions in various central nervous system and retinal injury models.6–8 However, the underlying mechanism of EPO neuroprotection in diabetes is not fully understood.

To test the possibility that EPO may protect retinal neurons from the toxicity of AGEs, we have established in vitro models of AGE stress. In particular, glyoxal, a defined reactive intermediate that triggers and perpetuates AGE formation, has been used as an inciting factor of neuronal injury by which AGE accumulation in diabetic condition is simulated.9 For better biological relevance, the protective effects of EPO have been demonstrated in retinal organ culture, in primary retinal neuronal cells, and in retinal neuronal line R28 cells. To explore the protective mechanisms of EPO in detail, we examined its role in cell proliferation and antiapoptosis in our models and focused on the study of antiapoptotic signaling pathways. Oxidative damage and mitochondrial malfunction are known to play pivotal roles in AGE-induced cell death.10 Therefore, we looked at the effect of EPO on the mitochondrial protein-controlled intrinsic death pathway. Particularly, the responses of Bcl-2 family proteins11,12 including Bcl-xL, Bcl-2, and Bax, to exogenous glyoxal and EPO have been explored. In addition, the phosphorylation of BAD has been studied because of its regulatory effects on Bcl-xL/Bcl-2, and Bax function.13 Further- more, caspase activity has also been assessed because of its role in cell proliferation and antiapoptosis.14 These data demonstrate that exogenous EPO significantly attenuates the retinal neuronal cell death induced by glyoxal-AGEs by promoting antiapoptotic and suppressing apoptotic proteins. EPO/EPO receptor signaling through ERK and Akt pathways is pivotal in EPO neuroprotective mechanisms.
neuroprotection and in the regulation of Bcl-2 family proteins were also studied.

This study aims to determine the neuroprotective role of EPO against the toxicity of glyoxal/AGEs, a diabetes-like condition, in retinal neurons and to explore the underlying mechanism, in particular, regarding the regulation on Bcl-2 family proteins and to study the signaling pathways downstream of EPO/EPOR.

**Materials and Methods**

**Reagents and Antibodies**

All biochemicals and enzymes were of analytical grade and were purchased from commercial suppliers. r-Hu-EPO, glyoxal, MTT [3-(4,5)-dimethylthiazol-2-yl]-5,5,5-triphenyltetrazoliumbromide], medium (Ames), and BrdU (5-bromo-2'-deoxyuridine) were purchased from Sigma-Aldrich (St. Louis, MO). Erythropoietin (EPO) (Sigma) was added to the culture medium with or without EPO and remained in the medium for 9 hours. (catalog no. 50649; Sigma) was added to the culture medium with or without EPO and remained in the medium for 9 hours.

**Rat Retinal Organ Culture**

Adult Sprague-Dawley rats (male, 160 g) were deeply anesthetized and then killed. The eyeballs were dissected, and the neurosensory retinas were washed twice and mounted onto the filter paper with the ganglion cell layer facing upward. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Cultured retinas were maintained in medium (Ames; catalog no. A1420; Sigma) at 37°C in a humidified 5% CO₂ atmosphere. Glyoxal (catalog no. 50649; Sigma) was added to the culture medium with or without EPO and remained in the medium for 9 hours.

**Primary Retinal Neuronal Cell Culture**

Retinal neuron cell cultures were prepared with slight modifications, as previously described.19 On postnatal day (P)1, Sprague-Dawley rats were anesthetized and killed; the retinas were isolated from ocular tissue in D-Hanks buffer and cut into small pieces. A single-cell suspension was prepared by digestion with 0.05% Trypsin-EDTA at 37°C for 10 minutes. The suspension was seeded onto coverslips or dishes precoated with poly-d-lysine (100 ng/mL; catalog no. A-003-E; Chemicon). Seeding was performed at 4 to 6 × 10⁶/60-mm dish for protein extraction and 1 × 10⁶/60-mm dish for immunostaining or TUNEL assay. The cells were maintained in medium (Ames; catalog no. A1420; Sigma) at 37°C in a humidified 5% CO₂ atmosphere. Glyoxal (catalog no. 50649; Sigma) was added to the culture medium with or without EPO and remained in the medium for 9 hours.

**In Situ Detection of Cell Death by TUNEL Assay**

Evaluation of apoptotic cells was performed using a TUNEL assay according to the manufacturer’s instruction. Briefly, paraffin sections of organ-cultured retinas were dewaxed, and the apoptotic cells were detected (ApopTag Kit; catalog no. S7100; Chemicon). Slides were counterstained by methyl green to show the nuclei. For each sample, the number of TUNEL-positive cells in each layer was determined in three meridian sections through the optic nerve head. Sections were examined, and color images were obtained with a digital camera (Nikon, Tokyo, Japan). Apoptotic cells were counted in three different microscopic fields of each section with an image analysis system (Image-Pro Plus 5.1; Media Cybernetics, Houston, TX). The number of TUNEL-positive cells was expressed as the ratio of TUNEL-labeled nuclei to the total (methyl green-stained) nuclei. The mean of the three sections was used as an independent data for each retinal organ culture. R28 cells on coverslips were fixed with 4% paraformaldehyde for 15 minutes at 4°C, the assay was performed with an in situ cell death detection kit (catalog no. 11684799510; Roche) according to the standard protocol. The nuclei were detected by 4',6-diamidino-2-phenylindole (DAPI) after TUNEL labeling. Stained cells were observed, and nonoverlapping fields (>4) in each slide were photographed under the microscope. More than 300 cells in each field were counted with image analysis software (Image-Pro Plus 5.1; Media Cybernetics). The results are expressed as relative percentage of TUNEL-positive cells based on the total (DAPI-stained) number of cells counted.

**Morphologic Analysis Using Ethidium Bromide and Acridine Orange Staining**

Cell death was detected and quantified using methods described previously.21–23 Briefly, a dye-mix solution of AO (100 μg/mL; catalog no. 0360; Genebase Gene-Tech, Co., Ltd., Shanghai, China) and EB (100 μg/mL; catalog no. 0492; Genebase Gene-Tech, Co., Ltd.) was prepared in phosphate-buffered saline (PBS; pH 7.2). After glyoxal or EPO treatments, a total volume of 5 μL mix was added to each coverslip on which the cells were cultured. This preparation was examined using a fluorescence microscope. More than 300 cells were counted per sample and were scored as follows: (1) viable cells with nonapoptotic nuclei (normal); (2) viable cells with apoptotic nuclei (early apoptosis); (3) nonviable cells with apoptotic nuclei (late apoptosis); (4) necrotic cells (necrosis). Because apoptosis and necrosis were very difficult to distinguish from each other by AO/EB staining, the results were expressed as the ratio of sum of cells in 2, 3, and 4 to the total cell number (labeled as apoptosis and necrosis).

**Cell Viability Assay**

Primary retinal neuronal cells or R28 cells were seeded in 96-well plates at a density of 2 × 10⁴ cells/well and were serum-starved for 12 hours before the experiments. Cells were treated with glyoxal or EPO, or both, for 9 hours. Then the medium was discarded, and the cells were washed with PBS. The fresh medium containing MTT (5 mg/mL) or assay (Alamar Blue; Invitrogen) was added for 4-hour incubation. Cell viability was determined by the relative decrease in OD value for treated samples to the value for control samples.

**BrdU Incorporation Analysis**

R28 cells (1 × 10⁶ cells/60-mm dish) were serum-starved for 12 hours, and the glyoxal or EPO treatments were performed for the time indicated. BrdU (10 μM, final; catalog no. B9285; Sigma) was added and incubated for another 1.5 hours. Cells were fixed with 70% ethanol for 20 minutes at −20°C and then stained with anti-BrdU. Nuclei were labeled with DAPI (300 μM) for 10 minutes at room temperature. At least three slides in each group were examined, and five nonoverlapping fields per slide were photographed under a fluorescence microscope. The number of BrdU-positive cells and the total number of cells (>300) in each field were counted with image analysis software (Image-Pro Plus 5.1; Media Cybernetics). The index of BrdU incorporation rate was determined by the ratio of BrdU-positive cells to DAPI-stained cells.

**Cell Cycle Analysis Using PI Staining**

After treatments, the cells were collected and resuspended in PBS containing 70% cold absolute ethanol for fixation and permeabilization.
(4°C overnight). The cells were then washed twice with PBS and treated with RNase A (40 mg/mL) for 30 minutes at 37°C. Finally, the propidium iodide (PI; catalog no. 81841; Sigma) solution was added to the final concentration of 25 μg/mL. Cell cycle analysis was performed using a fluorescence-activated cell sorter (BD Biosciences), and PI fluorescence was measured at 488 nm.

**Caspase-like Activity Analysis**

Cells were lysed by the addition of 1 mL lysis buffer (1% NP40; 50 mM Tris-HCl, pH 7.6; 5 mM EDTA), followed by a 20-minute incubation on ice, and centrifugation for 15 minutes at 4°C, 12,000g. Protein concentrations were determined by protein assay kit (Bio-Rad, Hercules, CA). Samples containing 40 μg protein were mixed with 40 μL reaction mix (containing Zn2PnLD-aminoluciferin substrate) from a caspase-like assay kit (Proteasome-Glo; catalog no. G8641; Promega) and were incubated at room temperature for 30 minutes. Luminescence was measured by a 96-well plate reader, and results presented as the percentage normalized by group control.

**Quantitative Real-Time RT-PCR**

After treatments, total RNA of R28 cells was extracted, and the cDNAs were reverse transcribed. Real-time RT-PCR was performed in 384-well plates on a sequence-detection system (Prism 7900; Applied Biosystems, Inc. [ABI], Foster City, CA). Reverse transcription included an incubation period of 30-second hold at 95°C, followed by 40 cycles of 5 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C. Primers were as follows: for rat Bcl-xL (GenBank accession number NM_001053672), 5'-TCTAACATCGCCAGCTCAT-3' (sense), 5'-GGAA- TCCGACTCCAACTATA-3' (antisense); for rat Bax (GenBank accession number NM_017059), 5'-TCTAACATCGCCAGCTCAT-3' (sense), 5'-GGAA-TCCGACTCCAACTATA-3' (antisense); for rat Bcl (GenBank accession number NM_017059), 5'-GGAA-TCCGACTCCAACTATA-3' (sense), 5'-GGAA-TCCGACTCCAACTATA-3' (antisense); for β-actin (GenBank accession number NM_031144), 5'-GTAAGAACC T C TAT-GCCAACA-3' (sense), 5'-GGACTCA TGGTACTCCGT-3' (antisense; for β-actin (GenBank accession number NM_031144), 5'-GTAAGAACC T C TAT-GCCAACA-3' (sense), 5'-GGACTCA TGGTACTCCGT-3' (antisense; for β-actin). The comparative Ct (ΔΔCt) method was used to obtain quantitative data of relative gene expression, according to the manufacturer’s (ABI) instructions.

**Plasmids and Transfection**

The plasmids Ras dominant-negative form (ppp-S17N), Ras active form (ppp-F53S), and Akt active form (ppp-Akt) were gifts from Ying Jin’s laboratory (Institute of Health Sciences, Shanghai, China). All the plasmids were delivered into R28 cells by electroporation according to the manufacturer’s instructions (50 μg plasmid DNA/5 × 106 cells, 240 V, 500 μF; Bio-Rad). Cells transfected with the plasmids were selected by puromycin (1 μg/mL; catalog no. P9620; Sigma) for 3 days before expansion.

**Western Blot Analysis**

Cells were collected and lysed in ice-cold radioimmune precipitation assay buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% anhydrous liquid nonionic surface-active agent (Nonidet P-40; Roche), and 1% sodium deoxycholate, supplemented with protease inhibitor phenylmethylsulfonyl fluoride (Shenher Biocolor Bioscience Technology Company, Shanghai, China) and cocktail (catalog no. p2714; Sigma). After 25-minutes incubation on ice, the extracts were clarified by centrifugation at 12,000g for 20 minutes at 4°C and stored at −80°C. Protein concentrations were determined by protein assay kit (Bio-Rad). Equal amounts of protein were resolved in SDS-polyacrylamide gels and transferred electrophotically onto a nitrocellulose membrane (Bio-Rad). Immunoblot analyses were performed using specific antibodies as described in the figure legends. After detection with the phosphospecific antibodies, the blot was stripped and reprobed successively with antibodies against total proteins, as described, and the optical density of each band was determined (Quantity One Software; Bio-Rad). Densitometric values for the phospho-proteins of interest were normalized for protein loading with their total proteins. The relative expression (or phosphorylation) levels were determined by the fold changes of densitometric values in treated groups to the values in the control group.

**Statistical Analysis**

Values are given as mean ± SD; n represents the number of independent experiments. Statistical analyses were performed using the ANOVA Student’s t-test, with P < 0.05 considered statistically significant.

**RESULTS**

**Rescue of Retinal Neuronal Cells from Glyoxal Toxicity by EPO**

Intravitreal injection of EPO protected retinal neurons in a rat model of early diabetic retinopathy. Because accumulation of AGEs was observed in the diabetic retinas (Supplementary Fig. S1, http://www.iovs.org/cgi/content/full/51/1/35/DC1), it is possible that the neuroprotective function of EPO was due to reduced cellular injury from AGEs. To test this possibility, three in vitro models have been established to explore the protective role of EPO against the stress of glyoxal. First, TUNEL labeling was used to measure apoptosis in retina organ culture under glyoxal or EPO treatment, or both. The density of apoptotic cells was highest in the ganglion cell layer (GCL) and inner nuclear layer (INL) (Fig. 1A, left). There was a dose-dependent increase in apoptosis after 9-hour incubation in glyoxal compared with untreated control. Cotreatment with EPO and glyoxal (0.2 U/mL) significantly reduced the level of apoptosis (P < 0.05; Fig. 1A, right). Second, AO/EB staining was performed to further study the protective role of EPO in both primary retinal neurons and R28 cells. In primary culture, AO/EB staining demonstrated that glyoxal (0.15 and 2 mM) resulted in neuronal cell death up to 58.0% ± 4.1% (2 mM) at 9 hours, which was a significant increase compared with the background cell death in untreated control (0 mM, 10.7% ± 6.1%; P < 0.05). The addition of EPO (0.2 U/mL) reduced cell death significantly (to 36.7% ± 2.2%; P < 0.001; Fig. 1B). In R28 cells, incubation with glyoxal (2 mM) for 9 hours also produced significant increases in cell death (35.2% ± 2.2%; P < 0.001), with little background apoptosis detected in untreated control (0.3% ± 0.05%). Glyoxal-induced cell death could be dramatically abolished by the AGE trapper AG (to 1.5% ± 0.1%; P < 0.001). EPO (0.2 U/mL) was also able to reduce cell death but to a lesser extent than AG (to 10.8% ± 1.17%; P < 0.001; Fig. 1C). Third, in the viability assay, glyoxal (0.03–10 mM) reduced cell viability in a dose-dependent manner. Inclusion of EPO (0.2 U/mL) led to an obvious improvement in viability (P < 0.01). The rescue effect of EPO was also glyoxal dose dependent; from 0.03 to 2 mM glyoxal, EPO restored cell viability completely. However, EPO lost the protective function when higher doses of glyoxal (2 mM) were used. A minor increase of viability by EPO was also found in control cells (no glyoxal), but it was not statistically significant (Fig. 1D). The protective effect of EPO was found to be dose dependent: 0.2 U/mL EPO appeared to yield the greatest protection (from 79.3% ± 2.1% to 92.6% ± 1.3%; P < 0.05; Fig. 1E).

**Effects of EPO on R28 Cell Proliferation**

To determine whether EPO affects cell proliferation, we chose the retinal neuronal cell line R28. Although some biological features of R28 cells may be different from those of primary cells, they do proliferate well in cultures. To investigate whether the decreased cell death by EPO, as presented in
exposure to glyoxal significantly reduced the proportion of BrdU-positive cells (from 48.9% to 5.9%). However, EPO produced no effects on either glyoxal-treated cells (7.0% vs. 5.9%; P = 0.05) or control cells (48.8% vs. 48.9%; P = 0.05; Fig. 2A). PI cell cycle assay demonstrated that glyoxal decreased the number of G1-phase cells (from 42.1% to 24.9%) but significantly increased the number of S-phase cells (from 30.2% to 50.6%; P = 0.05). These effects could be largely reversed by AG (to 37.2%; P = 0.05) but not by EPO (51.0%; P = 0.05; Fig. 2B). These findings clearly indicated that, even for R28 cells in their proliferative phase, cell proliferation played little role in EPO neuroprotection against glyoxal. Therefore, we have focused on the study of antiapoptotic signaling pathways in the present study.

Figure 1C, was associated with increased cell proliferation, both DNA synthesis and cell cycle were measured by BrdU incorporation and PI flow cytometry assay, respectively. Exposure to glyoxal significantly reduced the proportion of BrdU-positive cells (from 48.9% to 5.9%). However, EPO produced no effects on either glyoxal-treated cells (7.0% vs. 5.9%; P = 0.05) or control cells (48.8% vs. 48.9%; P = 0.05; Fig. 2A). PI cell cycle assay demonstrated that glyoxal decreased the number of G1-phase cells (from 42.1% to 24.9%) but significantly increased the number of S-phase cells (from 30.2% to 50.6%; P = 0.05). These effects could be largely reversed by AG (to 37.2%; P = 0.05) but not by EPO (51.0%; P = 0.05; Fig. 2B). These findings clearly indicated that, even for R28 cells in their proliferative phase, cell proliferation played little role in EPO neuroprotection against glyoxal. Therefore, we have focused on the study of antiapoptotic signaling pathways in the present study.

**ERK and Akt Dependent Protection of EPO against Glyoxal in R28 Cells**

Multiple downstream signaling pathways (mainly ERK, Akt, and STAT5) of EPO receptor (EPOR) were reported to be involved in EPO protection. The expression of EPOR was found in primary retinal and R28 cells (Supplementary Fig. S2, http://www.iovs.org/cgi/content/full/51/1/35/DC1). However, the exact signaling pathways involved in EPO protection against glyoxal-AGEs remained largely unknown. To elucidate the signaling pathways in the current model, the major downstream pathways of EPOR signaling were selectively blocked by specific inhibitors. Unexpectedly, we did not observe the activation of STAT5 with EPO treatment (Supplementary Fig. S3, http://www.iovs.org/cgi/content/full/51/1/35/DC1), indicating that STAT5 signaling might not have been involved under our experimental conditions. Therefore, the functions of ERK and Akt signaling in the current model were investigated. In the absence of glyoxal, EPO itself significantly enhanced the
phosphorylation of ERK and Akt in a time-dependent manner in R28 cells (Fig. 3A, left) and in primary retinal cells (Fig. 3A, right). In R28 cells, glyoxal also stimulated ERK phosphorylation, but the elevated phosphorylation returned to normal level at 5 hours. Coincubation with EPO and glyoxal revealed no significant difference in ERK phosphorylation at 1 hour and 3 hours; however, the phosphorylation became much stronger at 5 hours in comparison with glyoxal treatment alone. For Akt, glyoxal gradually downregulated its phosphorylation, and this effect was rescued by EPO. It was noticed that 0.2 U/mL EPO was more powerful than 1 U/mL in enhancing ERK and Akt phosphorylation (Fig. 3B, upper). In primary retinal cells, glyoxal was able to stimulate ERK phosphorylation, whereas such phosphorylation was not further enhanced by EPO application. Of note, very weak phosphorylation of Akt was found in the control; however, EPO was able to upregulate the Akt phosphorylation compared with glyoxal alone. In contrast to the R28 cells, 0.2 U/mL EPO showed effects on ERK and Akt similar to those of 1 U/mL EPO in the primary cells (Fig. 3B, lower). Based on these observations, it was speculated that...
ERK and Akt pathways played important roles in glyoxal toxicity and EPO neuroprotection but with different temporal dependency. The ERK or Akt pathway was further studied with the use of specific inhibitors, such as U0126 (for ERK) or Wortmannin (WM; for Akt). Viability assay(s) demonstrated that glyoxal reduced cell viability to 67.2% ± 0.3% (R28 cells) and to 74.6% ± 1.9% (primary cells); this was significantly rescued by EPO (to 78.2% ± 1.7% in R28 cells and to 84.4% ± 1.8% in primary cells; *P < 0.05*). In R28 cells, the blockage of ERK signaling pathway by U0126 caused minor effects on glyoxal toxicity (glyoxal + DMSO, 67.2% ± 0.3% vs. glyoxal + U0126, 64.8% ± 1.2%; *P > 0.05*) while largely abolishing EPO protection (glyoxal + EPO + U0126, 64.1% ± 1.1% vs. glyoxal + EPO + DMSO, 80.0% ± 1.7%; *P < 0.01*). Of note, inhibition of the Akt pathway by WM led to more severe viability loss (glyoxal + WM, 55.6% ± 0.8%; *P < 0.01*) compared with glyoxal + DMSO. Furthermore, EPO-enhanced viability was also eliminated by WM (glyoxal + EPO + WM, 55.3% ± 0.8%; *P < 0.01*). Similar to the findings in R28 cells, the protection of EPO in primary cells was also inhibited by U0126 (EPO + DMSO, 84.4% ± 1.8% vs. EPO + U0126, 76.7% ± 1.8%; *P < 0.05*) or WM (EPO + WM, 73.8% ± 1.3%; *P < 0.05*). Of note, neither inhibitor affected primary cell viability when applied with glyoxal alone (Fig. 3C). These phenomena were confirmed by using different inhibitors of ERK (PD98059) and Akt (LY294002) signaling (data not shown) in R28 cells. None of these inhibitors caused significant toxicity to R28 cells (Supplementary Fig. S4, http://www.iovs.org/cgi/content/full/51/1/35/DC1). In addition, the viability results were supported by TUNEL assay; in R28 cells, glyoxal significantly increased apoptosis (7.9% ± 0.5%; *P < 0.01*). In contrast, only 0.23% ± 0.06% of apoptosis was found in untreated control. The apoptotic cell population caused by glyoxal was significantly reduced by EPO (1.7% ± 0.2%; *P < 0.01*). Coapplication of glyoxal with WM rather than U0126 further increased apoptosis to 10.3% ± 0.9% compared with DMSO. Both U0126 and WM were capable of eliminating the antiapoptotic effect of EPO: glyoxal + EPO + DMSO (1.7% ± 0.2%) compared with glyoxal + EPO + U0126 (7.8% ± 0.5%) compared with glyoxal + EPO + WM (8.2% ± 0.5%; *P < 0.01*). In primary cells, glyoxal also significantly increased apoptosis (41.8% ± 2.2%; *P < 0.01*), superimposing the background cell death (5.6% ± 1.2%) in control. The application of EPO, however, significantly alleviated the apoptosis induced by glyoxal (glyoxal + EPO + DMSO, 11.1% ± 1.2%; *P < 0.01*). EPO neuroprotection in primary cells was antagonized by either U0126 (glyoxal + EPO + U0126, 35.3% ± 1.9%; *P < 0.01*) or WM (glyoxal + EPO + WM, 42.4% ± 2.4%; *P < 0.01*). Neither U0126 nor WM affected the cell death induced by glyoxal alone (glyoxal + U0126, 41.9% ± 2.4% vs. glyoxal + WM, 44.4% ± 2.8%; *P > 0.05*) (Figs. 3D, 3E).

**The Impact of Alteration in ERK and Akt Signaling on Glyoxal Toxicity and EPO Neuroprotection**

To further investigate the functions of ERK and Akt signaling in glyoxal toxicity and EPO neuroprotection, an overexpression method was used. The dominant/negative and active forms of Ras and the active form of Akt were overexpressed in R28 cells. Gene overexpression was determined by detecting the flag-tag (Fig. 4A) and the phosphorylation of ERK1/2 and Akt. Glyoxal caused a marked increase of ERK1/2 phosphorylation in control cells (transfected with ppy vector) but weaker phosphorylation in S17N (dominant/negative form of ras) cells. Similarly, EPO-enhanced ERK1/2 phosphorylation was significantly reduced by S17N expression compared with vector. Nevertheless, no obvious effects on Akt phosphorylation were found. The active form of Akt induced dramatic Akt phosphorylation but showed no influence on ERK1/2 phosphorylation (Fig. 4B). The viability assay revealed that both active ERK (T35S) and active Akt caused strong resistance to glyoxal toxicity (84.7% ± 1.9%; *P < 0.01*). Neither U0126 nor WM affected the cell death induced by glyoxal alone (glyoxal + U0126, 41.9% ± 2.4% vs. glyoxal + WM, 44.4% ± 2.8%; *P > 0.05*) (Figs. 3D, 3E).
The Rescue Effects of EPO on Glyoxal-Regulated Bcl-xl and Bax Expression

The role of Bcl-2 family proteins in glyoxal toxicity and EPO neuroprotection was investigated. Quantitative real-time RT-PCR was performed to evaluate mRNA expression of the gene of interest. Results were presented as fold change relative to group control, and relative expression was normalized to β-actin mRNA expression in each group. Summarized relative mRNA expression for Bcl-xl, Bax, and Bcl-2 is shown in R28 cells (A–C, respectively) and in primary cells (D–F, respectively) (*n > 3). Representative Western blots of Bcl-xl, Bax, and Bcl-2 protein expression under glyoxal or EPO treatment in R28 cells (G) or in primary cells (H). β-Actin served as a protein loading control. Summarized quantification of (I) Bcl-xl, (J) Bax, and (K) Bcl-2 protein expression in R28 cells from four experiments. (L) Ratio of Bcl-xl to Bax in R28 cells under treatment. Data were presented as the ratio of protein expression in treated groups to that in control groups after normalization with β-actin (n ≥ 3; *P < 0.05; **P < 0.01; ***P < 0.001).

The Rescue Effects of EPO on Glyoxal-Regulated Bcl-xl and Bax Expression

The role of Bcl-2 family proteins in glyoxal toxicity and EPO neuroprotection was investigated. Quantitative real-time PCR revealed that glyoxal induced a time-dependent decrease in Bcl-xl mRNA. The strongest inhibition was observed at 5 hours (0.43 ± 0.08 in R28 cells and 0.18 ± 0.06 in primary cells compared with control; P < 0.01; Figs. 5A, 5D, black bar). EPO significantly enhanced Bcl-xl mRNA expression, peaking at 1 hour in R28 cells (glyoxal + EPO, 1.2 ± 0.2, vs. glyoxal, 0.8 ± 0.05; P < 0.05) and at 0.5 hour in primary cells (glyoxal + EPO, 0.90 ± 0.03, vs. glyoxal, 0.57 ± 0.09; P < 0.05), respectively (Figs. 5A, 5D, gray bar). In addition, glyoxal reduced Bcl-2 mRNA expression in a time-dependent manner, as seen at 3 and 5 hours in R28 cells and at 1, 3, and 5 hours in primary cells (P < 0.01), respectively. To our surprise, EPO failed to influence Bcl-2 mRNA expression by either R28 or primary cells under glyoxal treatment (Figs. 5C, 5F). Not surprisingly, the mRNA expression of Bax, a proapoptotic factor, was upregulated by glyoxal (1.5-fold increase at 1 hour; P < 0.01). The increased Bax expression returned to normal at 3 and 5 hours. At 1 hour, the addition of EPO significantly alleviated the upregulated Bax mRNA in R28 cells (glyoxal + EPO, 0.70 ± 0.21, vs. glyoxal, 1.5 ± 0.12; P < 0.05; Fig. 5B). In primary cells, the upregulation of Bax mRNA by glyoxal appeared to be delayed, with a significant increase found only at 5 hours (1.70 ± 0.01 vs. control; P < 0.01). The application of EPO significantly reduced Bax mRNA expression (0.86 ± 0.2; P < 0.05; Fig. 5E). In terms of the protein expression of Bcl-xl, Bcl-2, and Bax in R28 cells (Fig. 5G), glyoxal significantly downregulated Bcl-xl protein expression and upregulated Bax protein expression compared with control (P < 0.01 at 1
hour). These effects could be changed by EPO. For instance, relative Bcl-xL expression was increased from 0.7 ± 0.06 to 1.10 ± 0.14 (P < 0.05), and Bax expression was reduced from 1.52 ± 0.16 to 0.86 ± 0.08 (P < 0.01; Figs. 5I, 5J) in the presence of EPO. Bcl-2 protein expression did not change under either gly oxal or EPO treatment (Fig. 5K). The ratio of Bcl-xL/Bax was a sensitive determinant for the regulatory effect of gly oxal or EPO, or both (gly oxal + EPO, 1.32 ± 0.17, vs. gly oxal, 0.61 ± 0.08; Fig. 5L). In primary cells, the change in Bcl-xL protein expression was consistent with that observed in R28 cells (Fig. 5H). A time-dependent downregulation of Bcl-xL protein by gly oxal was observed, peaking at 3 hours (0.55 ± 0.16; P < 0.05). EPO showed significant upregulation of Bcl-xL at 1 hour (gly oxal + 0.2 U/mL EPO, 1.13 ± 0.06, and gly oxal + 1 U/mL EPO, 1.04 ± 0.07, vs. gly oxal, 0.76 ± 0.05; P < 0.05) (Fig. 5M). Bax, however, remained largely unchanged under all these treatments (Fig. 5N). Gly oxal and EPO also regulated Bcl-2 protein expression in a way that resembled the regulation of Bcl-xL. The reduced Bcl-2 expression by gly oxal could be alleviated by EPO application at 1 hour (gly oxal + 0.2 U/mL EPO, 1.18 ± 0.13, and gly oxal + 1 U/mL EPO, 1.05 ± 0.09, vs. gly oxal, 0.75 ± 0.07; P < 0.05; Fig. 5O). For the ratio of Bcl-xL/Bax, significant downregulation by gly oxal at 1 hour (0.58 ± 0.13; P < 0.05) and 3 hours (0.49 ± 0.16; P < 0.05) was observed. However, no regulatory effect by EPO on the ratio of Bcl-xL/Bax was revealed (Fig. 5P).

Enhanced BAD Phosphorylation and Reduced Gly oxal-Induced Caspase-like Activity under EPO Stimulation

BAD phosphorylation is critical in the modulation of Bcl-xL/Bcl-2 and Bax. In the present study, EPO caused a remarkable increase in BAD phosphorylation at both Ser112 and Ser136, and these effects remained throughout the course of the treatments (Fig. 6A). Moreover, the time-dependent induction of phospho-p90RSK, a key molecule in the ERK/BAD pathway, regulating BAD phosphorylation at Ser112, was observed (Fig. 6A). Interestingly, the baseline expression of both phospho-BAD (Ser112) and (Ser136) was diminished by gly oxal, though a recovery effect appeared at 5 hours. EPO (0.2 U/mL) enhanced the phosphorylation of BAD (Ser112 and Ser136) throughout gly oxal treatment. The regulatory effect of gly oxal and EPO on the phosphorylation of BAD showed a different temporal dependency. This regulation by EPO was also dose dependent because the effects of 1 U/mL EPO were negligible compared with 0.2 U/mL EPO (Fig. 6B). Both Bcl-xL/Bax expression and BAD phosphorylation are involved in controlling the release of apoptotic factors (such as AIF and cytochrome c) from mitochondria to cytoplasm, which in turn may activate caspas es and initiate apoptotic events. Therefore, the activation of caspas es was measured. Exposure to gly oxal resulted in a significant increase of caspase-like activity (149.1% ± 3.4% at 1 hour and 120.4% ± 5.8% at 3 hours vs. untreated as 100%; P < 0.05). EPO completely reversed the gly oxal-induced increase in caspase-like activity (87.4% ± 2.0% at 1 hour and 94.9% ± 4.2% at 3 hours; P < 0.01; Fig. 6C).

Essential Role of ERK and Akt Signaling in the Regulation of Bcl-xL, Bax, and BAD

The observed importance of ERK and Akt signaling in EPO neuroprotection against gly oxal prompted us to try to determine whether the two pathways were important to the regulation of Bcl-xL, Bax, and BAD observed in the present experiments. First, the expression of Bcl-xL and Bax and the phosphorylation of BAD under ERK and Akt overexpression were studied. Slight upregulation of Bcl-xL but downregulation of Bax were detected when the active form of Ras (T35S) was overexpressed. In the parallel experiment, the active form of Akt caused moderate reduction of Bax expression but no effect on Bcl-xL expression. ERK or Akt overexpression had little effect on Bcl-2. T35S induced significant p90RSK phosphorylation and, subsequently, BAD phosphorylation at Ser112. Of note, the active Akt resulted in a dramatic increase in BAD phosphorylation at both Ser112 and Ser136 without activation of p90RSK, indicating a p90RSK-independent pathway for BAD phosphorylation at Ser112 by Akt activation. The dominant/negative form of ERK (S17N) had no obvious effect on any of these proteins (Fig. 7A). The regulatory roles of ERK and Akt signaling were corroborated with the use of specific inhibitors.

**Figure 6.** EPO enhances BAD phosphorylation and decreases the gly oxal-induced caspase-like activity in R28 cells. EPO (0.2 U/mL) was applied to R28 cells and remained in the culture medium for the time indicated. Phosphorylation of p90RSK and BAD was detected by antibodies specifically recognizing phospho-p90RSK, phospho-BAD (Ser112), and (Ser136). β-Actin served as an internal loading control. (A) Western blot determination of p90RSK and BAD phosphorylation by EPO. (B) Western blot analysis of BAD phosphorylation after treatment with gly oxal, EPO, or both. Relative phosphorylation levels were determined by the ratio of densitometric values of phospho-proteins to those of total proteins and were normalized by the values in control (shown at the end of the image) (n ≥ 3). (C) Caspase-like activity measured in R28 cells after the treatment of gly oxal (2 mM) or gly oxal + EPO (0.2 U/mL) for 9 hours. All values were normalized and expressed as the percentage relative to untreated control (n = 3; *P < 0.05, **P < 0.01).
The application of ERK inhibitor U0126 (10 μM, 1 hour earlier) significantly abolished the EPO-enhanced BAD phosphorylation at Ser112, but the phosphorylation at Ser136 was largely unaffected (Fig. 7B, left). On the other hand, WM (1 μM, 1 hour earlier) effectively eliminated the EPO-enhanced BAD phosphorylation at Ser136 but caused only a minor effect on phosphorylation at Ser112 (Fig. 7B, right). Quantitative analysis showed that inhibition by either U0126 or WM on EPO-regulated Bcl-xL expression (U0126, 0.70 ± 0.15, and WM, 0.79 ± 0.08, vs. DMSO, 1.12 ± 0.15; Fig. 7C). For Bax expression, U0126 failed to reverse the effects of EPO; however, WM led to a dramatic increase of Bax expression, even more than glyoxal itself (1.0 ± 0.14 vs. 1.2 ± 0.07; P < 0.05; Fig. 7D). Based on the Bcl-xL/Bax ratio, the inhibition of either ERK or Akt signaling led to deleterious effects on EPO-modulated Bcl-xL and Bax expression (Fig. 7E).

**DISCUSSION**

Neuronal apoptosis is a “final common pathway” of a variety of neurologic and retinal diseases, including diabetic retinopathy (DR). EPO, with a defined antiapoptotic property, has been shown to be a potential therapeutic approach for neurodegenerative diseases. In our laboratory, experimental data showed that EPO protects retinal neurons in an animal model of early DR. However, the underlying molecular mechanism of this effect has not been completely understood.

Growing evidence indicates that AGES may play a causative role in DR. Our data showing the elevation of AGES in the retinas of diabetic rats have prompted us to consider whether the neuroprotective effect of EPO may result from an ability to reduce the toxicity of AGES. Here we have provided evidence to show the protective function of EPO against glyoxal-AGES in cell culture models. Although minor differences exist among the different models, the consistent pattern of EPO neuroprotection observed with both R28 and primary cells has greatly strengthened the biological relevance of neuroprotection by EPO. The antiapoptotic properties of EPO have been extensively studied with regard to its neuroprotection. To explore the possible mechanisms of EPO protection in our models, we targeted cell proliferation, the clearance of intracellular Ne-(carboxymethyl)lysine (CML) formation, and the regulation of proapoptotic or antiapoptotic proteins. In contrast to previous reports showing EPO that supports erythropoiesis and neuron progenitor cell proliferation, stimulation of cell proliferation by EPO was not observed, suggesting that enhanced proliferation is not the mechanism of cellular protection by EPO in our model. Moreover, no regulation of EPO on intracellular CML accumulation was found (data not shown). These findings indicate that in our model EPO directly targeted neither cell proliferation nor AGE formation. In contrast, aminoguanidine (AG), a direct inhibitor of AGES, was able to restore the cell cycle distribution induced by glyoxal and to remove CML accumulation.

Therefore, we focused on the effects of EPO on proapoptotic or antiapoptotic proteins. Glyoxal-induced oxidative stress may cause functional changes to mitochondria, thus activating the intrinsic death pathway. Bcl-2 family proteins, composed of both proapoptotic and antiapoptotic proteins, play important roles in governing the intrinsic mitochondria death pathway: the proapoptotic BH3-only proteins (Bax and Bak) bind and sequester BH3-only molecules, thus preventing activation, whereas the antiapoptotic members (Bcl-2 and Bcl-xL) bind and sequester BH3-only molecules, thus preventing activation. Bcl-2 family proteins, composed of both proapoptotic and antiapoptotic proteins, bind and sequester BH3-only molecules, thus preventing activation. Bax, whose proapoptotic activity is regulated through phosphorylation at Ser136 and Ser112, is able to bind to Bcl-2/Bcl-xL, thereby antagonizing their antiapoptotic function. Of note, our data demonstrate that EPO was sufficient to enhance the EPO neuroprotective effect of EPO and to remove CML accumulation.
phosphorylation of BAD at these two sites, where phosphorylation was suppressed by glyoxal. As would be expected, the activation of caspases was also suppressed by EPO. These results show that EPO facilitates antiapoptotic proteins but suppresses proapoptotic proteins in the intrinsic mitochondrial pathway, thereby protecting the cells.

EPO exerts its function by binding to the receptor (EPOR), which is universally expressed in the brain and retina. Several intracellular signal transduction cascades are activated when EPO binds to EPOR, including ERK/MAPK, PI3K/Akt, and STAT5. A previous study reported that STAT5 signaling is important in EPO neuroprotection. However, the present study demonstrated no detectable activation of STAT5, suggesting a STAT5-independent pathway of EPO neuroprotection. It should be noted that a recent study reported that the activation of STAT5 was essential to the neurotrophic, but not to the antiapoptotic, role of EPO. Based on our data, it is unlikely that STAT5 is activated in the rescue mission of EPO for glyoxal-injured cells.

Evidence indicates that in different cell types and experimental models, the ERK and Akt signaling cascades may play critical roles in the mechanism of EPO neuroprotection. Therefore, their roles in EPO protection against glyoxal have been probed. First, we illustrated that both ERK and Akt pathways were essential to EPO neuroprotection. In the present study, the inhibition of ERK signaling did not block glyoxal-induced apoptosis. Thus it can be speculated that the activation of ERK by glyoxal may be a parallel response rather than a key process of glyoxal-induced apoptosis. On the other hand, the inhibition of Akt signaling in R28 cells resulted in more apoptosis with glyoxal treatment, suggesting the existence of an internal self-protective role of Akt. This type of self-protective function by Akt may vary in individual cell types. For instance, the complete inhibition of Akt signaling in primary cells exposed to glyoxal alone did not result in further loss of cell viability. This discrepancy is probably attributed to the different neuron composition in primary retinal neuron culture compared with that reported in R28 cell line. However, the essential role of Akt signaling in EPO neuroprotection was unchanged both in primary culture and R28 cell line. Interestingly, the protective role of EPO in both R28 and primary cells can be significantly suppressed by either ERK or Akt inhibition. The roles of ERK and Akt signaling in EPO neuroprotection have been confirmed by the overexpression experiments reported here. Our initial experiments showed that the ERK upstream molecule MEK was also regulated by glyoxal and EPO in a way similar to that of ERK (data not shown). Therefore, we proposed that the ERK signaling rather than ERK per se was the key element. To test this idea, overexpression of both dominant-negative and active Ras, which were reported to be potent in altering the ERK pathway, was performed. Overexpression studies corroborated the roles of ERK and Akt signaling that had been demonstrated by the inhibitor studies as well. In addition, the overexpression of active Ras or Akt provided us the opportunity to study the critical role of ERK and Akt signaling without EPO treatment. The present data revealed that in the absence of EPO, the activation of either ERK or Akt signaling was sufficient to antagonize the toxicity of glyoxal, which strengthened the importance of ERK and Akt signaling downstream of EPOR. We also found that the ERK and Akt pathways play a role in the BAD phosphorylation increase produced by EPO. To our knowledge, it is the first time that glyoxal and EPO have been shown to differentially regulate BAD phosphorylation. We have also provided evidence that ERK signaling is essential to the activation of BAD at Ser112, but not at Ser136, with EPO treatment. This phenomenon suggests that the functions of EPO against glyoxal have a spatial preference, such as the specific phosphorylation site of BAD. Given that, the inhibition of Akt signaling completely abolished the enhanced BAD phosphorylation at Ser136 by EPO but only moderately suppressed Ser112 phosphorylation. The overexpression of an active form of Akt also suggests that Akt signaling plays a dual role in BAD phosphorylation. Interestingly, phosphorylation of BAD at Ser112 by Akt signaling without the activation of the traditional ERK/p90RSK signaling indicated that other mechanisms may be involved. Therefore, our findings may revive the previous belief that Akt signaling and phosphorylation of Ser136 are primarily involved in EPO-induced BAD phosphorylation. Finally, we have illustrated the importance of ERK and Akt in EPO-modulated Bcl-xL and Bax expression. Inhibition of either ERK or Akt suppressed the regulatory function of EPO on Bcl-xL and Bax expression in the presence of glyoxal. Moreover, the EPO-regulated Bcl-xL/Bax ratio, a more precise parameter in determining cell death or survival, was also altered by ERK or Akt inhibition. The effects of Akt signaling on Bax expression were shown to be most significant because the inhibition of Akt caused a dramatic increase in Bax expression, and the overexpression of Akt led to greater reduction of Bax than did ERK overexpression. This work revealed a naturally inhibitory role of Akt on Bax, which is in accordance with previous reports. Neuroprotection also has been widely found in experimental glaucoma models. However, no consensus has been reached yet with regard to the specific signaling pathways (ERK and Akt) or Bcl-2 family protein expression (Bcl-2, Bcl-xL, and Bax) responsible for EPO protection in various models.

Taken together, our work has provided better understanding of EPO neuroprotection based on its ability to antagonize glyoxal-AGEs toxicity, as illustrated in Figure 8. For the underlying mechanism, the regulation of Bcl-2 family proteins by EPO in a glyoxal-AGEs model was elucidated. The critical role of ERK and Akt was also identified. These findings may assist future designs of therapeutic targets for diabetic retinopathy by EPO/EPOR-related mechanisms.
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References


