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Exendin-4 protects retinal cells from early diabetes in Goto-Kakizaki rats by increasing the Bcl-2/Bax and Bcl-xL/Bax ratios and reducing reactive gliosis

Yichao Fan,[1] Kun Liu,[2] Qingping Wang,[1] Yuanyuan Ruan,[3,4] Yu Zhang,[1] Wen Ye[1]

[1]Department of Ophthalmology, Huashan Hospital Affiliated to Fudan University, Shanghai, China; [2]Department of Ophthalmology, First People's Hospital of Shanghai affiliated to Shanghai Jiaotong University, Shanghai, China; [3]Key Laboratory of Glycoconjugate Research, Ministry of Public Health, Shanghai Medical College of Fudan University, Shanghai, China; [4]Department of Biochemistry and Molecular Biology, Shanghai Medical College of Fudan University, Shanghai, China

Purpose: Exendin-4 (E4), a long-acting agonist of the hormone glucagon-like peptide 1 receptor (GLP-1R), is administered to treat type II diabetes in the clinical setting and also shows a neuroprotective effect. Our previous studies demonstrated its protective effect in early experimental diabetic retinopathy (DR), but the molecular and cellular mechanisms are largely unknown. This study aimed to investigate the protective mechanism of a GLP-1R agonist E4 against early DR in Goto-Kakizaki (GK) rats.

Methods: Diabetic GK rats and control animals were randomly assigned to receive E4 or vehicle by intravitreal injection. The retinal function and retinal cell counts were evaluated using an electroretinogram and light microscopy. The expressions of retinal GLP-1R, mitochondria-dependent apoptosis-associated genes, reactive gliosis markers, and endoplasmic reticulum stress-related pathway genes were studied by western blotting and immunohistochemistry in vivo and in vitro.

Results: E4 significantly prevented the reduction of the b-wave and oscillatory potential amplitudes and retinal cell loss and maintained the Bcl-2/Bax and Bcl-xL/Bax ratio balances in GK rats. It also downregulated the expression of glial fibrillary acidic protein and reduced retinal reactive gliosis. Similar results were found in primary rat Müller cells under high glucose culture in vitro.

Conclusions: E4 may protect retinal cells from diabetic attacks by activating GLP-1R, decreasing retinal cell apoptosis, and reducing retinal reactive gliosis. Thus, E4 treatment may be a novel approach for early DR.

Diabetic retinopathy (DR) remains the leading cause of blindness among working-age individuals in developed countries, and type II diabetes still accounts for most of these cases [1]. Retinal degeneration and related visual loss in proliferative diabetic retinopathy is known to be irreversible. Additionally, current treatments for DR are performed in advanced stages of the disease and are associated with significant adverse effects. Therefore, new pharmacological interventions for early stages are needed.

Researchers and ophthalmologists have accepted the concept that neurodegeneration of the retina occurs before vascular lesions in diabetes, and retinal neurodegeneration is an early event in the pathogenesis of DR that predates [2-4] and participates [5-7] in the microcirculatory abnormalities that occur in DR. Increasing evidence shows that retinal cell apoptosis and reactive gliosis are basic pathological features of early DR [8]. Before the observation of fundus changes, reactive gliosis and retinal cell apoptosis increase in early DR, and the corresponding functional and morphological changes have been demonstrated by electroretinogram (ERG) examination and retinal cell counting under a microscope [9-11].

The mitochondria-dependent apoptosis pathway has been demonstrated to be closely related to diabetic-induced retinal cell apoptosis [12]. As Bcl-2 family members, the anti-apoptotic factors Bcl-2 and Bcl-xL and pro-apoptotic factor Bax are innately balanced in the mitochondrial pathway, and this balance may determine the survival or death of retinal cells following a diabetic stimulus [13-17]. In addition, previous studies showed that the protein kinase B (AKT) may be activated at Ser473 in response to endoplasmic reticulum (ER) stress, which could also induce retinal cell apoptosis in response to diabetic attacks [18-20].

Müller cells are the principal glial cells in the retina. They envelop blood vessels and neuronal cell bodies and can modulate neuronal activity and blood flow [21]. Although microvascular cells have long been considered primary targets of hyperglycemia in diabetic retinopathy [22,23], early biochemical, physiologic, and morphological changes are also observed in nonvascular cells, including Müller cells

Correspondence to: Wen Ye, Department of Ophthalmology, Huashan Hospital Affiliated to Fudan University, 12 Middle Wulumuqi Road, Shanghai, China. Phone: 086-21-52888139; FAX: 086-21-62481019; email: yewen0412@hotmail.com

[24-29]. Retinal cell death and the development of diabetic retinopathy are strongly associated [28]. An increasing body of evidence indicates that hyperglycemia causes Müller cell death via apoptosis [30,31]. In diabetic rats, Müller cells markedly upregulate their expression of glial fibrillary acidic protein (GFAP) in the early stages of diabetes, and the ability of Müller cells to remove glutamate from the extracellular space appears to be compromised as well because the levels of this amino acid are elevated [29]. As intermediate filament proteins, GFAP and vimentin respond similarly to retinal attacks, and the former is the most sensitive reactive gliosis marker in DR [11,32]. Furthermore, Müller cell gliosis may impair neural-vascular relationships and contribute to neurodegeneration in the retina of DR patients [33]. Moreover, Müller cell gliosis also impedes regenerative processes in retinal tissue via the formation of a glial scar [34].

Unlike the streptozotocin (STZ)-induced type I diabetic model, the Goto-Kakizaki (GK) rat model was developed by selectively inbreeding Wistar rats and is a widely used as a model of spontaneous non-insulin-dependent diabetes mellitus [35]. In GK rats, glucose intolerance appears after two weeks of age [36], and significant hyperglycemia is found as early as four weeks of age [37]. Glucagon-like peptide 1 (GLP-1) is a gut hormone secreted from the L cells of the small intestine in response to food ingestion, facilitating glucose-dependent insulin secretion, while glucagon-like peptide 1 receptor (GLP-1R) is widely distributed in the organs of both humans and rats, such as the pancreas, brain, and lungs [38]. Growing evidence demonstrated that GLP-1R activation in the central neural system also exerted several protective effects on neural cells in neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, cerebral ischemia disease, and peripheral neuropathy induced by type II diabetes [39-41]. Studies have shown that GLP-1R exerts anti-apoptotic action by increasing the expression of anti-apoptotic proteins and inhibiting apoptotic proteins in insulin-secreting cells [42]. Exendin-4 (E4) is a "long-lasting" agonist of GLP-1R and has been administered to treat type II diabetes for several years [43]. Our previous studies [9,44] showed that GLP-1R activation significantly protected diabetic retinal cells, but the molecular and cellular mechanisms have remained largely unknown. The aim of this study was to verify the effect of E4 in type II diabetic GK rats and investigate the underlying mechanisms.

METHODS

Animal handling: All experiments in this study comply with the ARVO Statement for the "Use of Animals in Ophthalmic and Vision Research." All chemicals were of reagent grade quality and were purchased from Sigma Chemicals (St. Louis, MO) unless stated otherwise. Forty eight-week-old male GK rats and 20 age-matched male Wistar rats, each weighing approximately 250 g, were purchased from Slac Laboratory Animal (Shanghai, China). All rats were maintained on a standard diet and water ad libitum. The 20 Wistar rats were used as normal controls, and the 40 GK rats were randomly divided into two other groups: GK control and E4-treated GK group. The blood glucose (BG) levels and bodyweight of the rats were monitored weekly from eight to 16 weeks of age. The BG levels were measured using a Roche glucometer (Accu-Chek Active, Roche, Mannheim, Germany) at the same time of day. The rats' fasting BG levels were measured after overnight fasting for 18 h. E4 (Sigma-Aldrich, St. Louis, MO) was dissolved in normal saline (pH 5.2). Intravitreal injections of E4 were administered as described previously [9] at 12 weeks of age. Briefly, intravitreal injections of E4 were performed to both eyes with 30-gauge, half inch needle(Becton Dichinson, Franklin Lakes, NJ) on a microsyringe (Hamilton, Lausanne, Switzerland), using a temporal approach, 2 mm posterior to the limbus. The dosage of E4 was 0.1 µg/eye in a volume of 2 µl. Sham injections (2 µl of normal saline) were administered to both Wistar normal control and diabetic GK control rats. The vitreous pH in the GK control was 7.33±0.03 (n=3), while the pH of E4-treated GK rats was 7.36±0.02 (n=3). The rats were anesthetized by 2% pentobarbital(50mg/kg intraperitoneally) and killed at 16 weeks of age.

Electroretinography: The retinal neural functions were examined with ERG (EP-1000, Tomey, Erlangen, Germany), which was performed as described previously [45], at eight, 12, and 16 weeks of age. After overnight dark adaptation, the rats were anesthetized under dim red illumination and their pupils were fully dilated with 0.5% tropicamide (Wuxi Shanhe Group, Jiangsu, China). Topical anesthesia with 0.4% oxybuprocaine hydrochloride eye drops (Eisai Co., Ltd., Tokyo, Japan) was applied to reduce the animals' discomfort. The rats were placed in a stereotactic frame, lying on a heating blanket to maintain the body temperature at 37 °C, and facing the stimulus at a distance of 20 cm. The electrodes were manufactured by engineer Xiaoping Wu from the Ophthalmology Department of Shanghai First People's Hospital (Chinese invention patent ZL2006100305113). Stainless-steel wire (0.1 mm in diameter) loops were placed on the cornea to act as the corneal electrode after the topical application of ofloxacin ophthalmic gel (Sinqi Group, Shenyang, Liaoning, China). The reference electrode and ground electrode were also made of stainless steel. Corneal electrodes recorded the flash ERG responses from both eyes, with the reference electrode placed in the middle of the lower

eyelid and the ground electrode near the tail. The responses to a light flash (2.5 cd·s/m^2, self-calibrated) from a photic stimulator, Ganzfeld Q400 (Roland, Germany), were amplified, and the preamplifier bandwidth was set at 0.3–300 Hz. Full-field white-light stroboscopic flashes lasting 10 µs were presented at a rate of 1.0 per second. The oscillatory potentials (OPs) were automatically filtered online at 75–300 Hz. All recordings were taken in a darkened room under dim red illumination (5 lx) to ensure a completely dark-adapted state. Four recordings were acquired and averaged. The amplitude of the a-wave was measured from the baseline to the trough of the a-wave, while that of b-wave amplitudes were measured from the trough of the a-wave to the peak of the b-wave. The magnitude of the OPs was determined as the sum of the three major amplitudes [9,46].

Retinal cell counts: The rats were killed after deep anesthesia. The eyes were enucleated and fixed in phosphate-buffered saline (PBS), buffered 4% paraformaldehyde for 24 h. A cutting marker was made at the 12 o'clock positions of the serial sections (5 µm) were assured by controlling that the cuttings passed through the optic nerve head and the cutting marker. The sections were then analyzed after they were stained with hematoxylin and eosin for morphologic studies by light microscopy for retinal cell counts in each layer. The retinal cell numbers in each layer were counted in the region within a fixed 50-µm column and centered with the reference lines, which were 1 mm away from the optic nerve on both the dorsal and ventral sides at 1000× magnification. The cell density was then expressed as the cell count/width (mm for cell counts in inner nuclear layer [INL] and outer nuclear layer [ONL] or 100 µm for ganglion cell layer [GCL]) of the retina in different layers.

Western blotting: Individual retinas from GK 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 containing 10mM Tris-HCl (pH 7.5),150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS),1% Nonidet P-40, and 1% sodium deoxycholate, for Western blot analysis. RIPA buffer enables efficient retinal tissue lysis and protein solubilization while avoiding protein degradation and interference with immunoreactivity. This buffer was supplemented with the protease inhibitor phenylmethysulfonyl fluoride (Shenergy Bicolor Bioscience Technology Company). After 15 min incubation on ice, the extracts were centrifuged at 12,000 ×g for 15 min at 4 °C and stored at -80 °C. Protein concentrations were determined by protein assay kit (Bio-Rad, CA), Equal amounts of protein were dissolved in SDS-polyacrylamide gels and transferred electrophoretically onto a polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). After blocking, for 1 h with 5% nonfat milk the PVDF membranes containing transferred protein were incubated overnight with the following primary antibodies: anti-GLP-1R (1:500, Abcam, Cambridge, UK), anti-Bcl-2 (1:500, Cell Signaling Technology (CST)), anti-Bcl-xL (1:500, CST), anti-Bax (1:500, CST), anti-Caspase-3 (1:1000, CST), anti-GFAP (1:1000, Epitomics, Burlingame, CA), anti-vimentin (1:1000, CST), anti-protein kinase B (AKT; 1:1000, CST), or anti-phospho-AKT (Ser473) (1:500, CST). β-actin (1:2000, Abmart, Shanghai, China) expression was used as an internal control. The optical density of each band was determined with analysis software (Quantity One, Bio-Rad).

Immunohistochemistry: The paraffin-embedded retinal sections were employed for immunohistochemical analysis using the avidin-biotin-peroxidase complex method (SABC kit, Zhongshan-Golden Bridge, China). The sections were incubated with a blocking solution (10% normal goat serum in PBS) for 30 min, followed by overnight incubation with polyclonal anti-GLP-1R antibody (1:200) or monoclonal anti-GFAP antibody (1:100) at 4 °C. Sections incubated with normal rabbit IgG (Boster, Wuhan, China) were used as negative controls. All sections were counterstained with hematoxylin. The results were evaluated under light microscopy (BX51, Olympus, Japan).

Cell culture: A culture of primary rat retinal Müller cells (RMCs) was prepared as described previously [47]. Briefly, eyes from postnatal day 5-7 Wistar rats were enucleated and incubated overnight in Dulbecco's Modified Eagle Medium (DMEM). Eyecups were transferred to dissociation solution (DMEM containing 0.1% Trypsin and 70 U/ml collagenase) and incubated at 37 °C in CO_2 incubator for 1 h. Eyecups were washed with DMEM and the retina was dissected out with care to avoid contamination from RPE and ciliary epithelium. The retina was mechanically dissociated into small aggregates and cultured in DMEM containing 10% FBS for 8-10 days. The floating retinal aggregates and debris were removed leaving purified flat cell population of Müller cells attached to the bottom of the dish. Cells were trypsinized and cultured in DMEM containing 10% FBS for another 5 days to get a further purified population. The purity of enriched Müller cells was determined via immunofluorescence microscopy using antibodies to glutamine synthetase (GS; 1:1000, Sigma) and GFAP (1:100, Epitomics) [48]. The RMCs were starved in medium without fetal bovine serum (FBS; High Clone Corporation, Logan, UT) for 8 h and then treated with different concentrations of glucose with or without E4 (normal group, 5.6 mM glucose; normal glucose+E4 treatment, 5.6 mM glucose+E4 (10 µg/ml); high glucose group, 20

mM glucose; high glucose+E4 treatment, 20 mM glucose+E4 (10 μg /ml)) in test medium (DMEM supplemented with 1% FBS) for 24, 48 and 72 h. The culture media plus E4 and glucose were changed every day to maintain constant E4 and glucose concentrations throughout the treatment period.

Statistical analysis: The data are expressed as the means ± SEM. The statistical analyses were performed using two-way ANOVA or one-way ANOVA as appropriate, followed by Bonferroni's test for multiple comparisons using SPSS 13.0 and GraphPad Prism 5.0. $p \leq 0.05$ was considered statistically significant.

RESULTS

Blood glucose level and bodyweight in GK rats before and after E4 intravitreal injection: Blood glucose level and bodyweight measurements were used to assess the systemic metabolism of the rats before and after E4 intravitreal injection. From eight to 16 weeks of age, the non-fasting and fasting BG levels in both the GK controls and the E4-treated GK group were approximately 1.9-fold to 2.4-fold and 1.3-fold to 1.6-fold higher than that in the Wistar control rats, respectively (Figure 1A,B; $p<0.001$ and $p<0.01$), while the bodyweight of the GK control and the E4-treated GK group was 3.6% to 11% lower than that of the Wistar control group, respectively (Figure 1C). The non-fasting and fasting BG levels and the bodyweight results did not differ significantly between the GK control and E4-treated group from 12 to 16 weeks of age (Figure 1, $p>0.05$).

Protection of E4 on retinal neurons in GK rats: ERG was performed to objectively assess the functional status of the rat retina. From eight to 16 weeks of age, the b-wave and OP amplitudes of the GK control continuously decreased, and the b-wave, OP, and a-wave amplitudes of the GK control rats decreased to 48.1% (Figure 2A,B; $p<0.001$), 35.3% (Figure 2C,D; $p<0.001$), and 69.9% (Figure 2B,E; $p<0.05$) of those of the Wistar controls, respectively, at 16 weeks of age. The OP amplitudes significantly differed between the Wistar and GK control rats (or E4-treated group) at 12 weeks of age (before E4 injections; Figure 2C; $p<0.001$). The b-wave amplitudes did not statistically differ between the Wistar control and the GK group at 12 weeks of age (Figure 2A; $p>0.05$). The b-wave and OP amplitudes of the E4-treated group were significantly higher than those of the GK control (210.7±21.5 μV versus 131.2±12.9 μV, 92.7±9.0 μV versus 49.6±9.1 μV; $p<0.05$), indicating the prevention of DR progression by E4 in GK rats at 16 weeks of age (four weeks after E4 injection). The a-wave amplitudes did not significantly differ between the GK control and E4-treated GK group at 16 weeks of age (Figure 2E; 73.7±8.7 μV versus 88.1±7.8 μV; $p>0.05$). The a-wave and b-wave amplitudes did not significantly differ between the Wistar control and E4-treated GK group at 16 weeks of age, but the OP amplitude significantly differed between these groups (Figure 2C; $p<0.05$).

The morphological examination demonstrated a significant reduction in the number of retinal neurons in the GCL (61.3% of the Wistar control), INL (78.7% of the Wistar control), and ONL (72.5% of the Wistar control) at 16 weeks of age in GK rats (Figure 3A–C; $p<0.01$, $p<0.05$, and $p<0.001$, respectively). E4 treatment significantly prevented this loss in the GCL (147.8% of the GK control; Figure 3A,C) and ONL (131.6% of the GK control; Figure 3A,B) but not in the INL (119.6% of the GK control; Figure 3A,B), and the GCL, ONL, and INL did not differ significantly between the Wistar control and E4-treated GK rats (Figure 3, $p>0.05$).

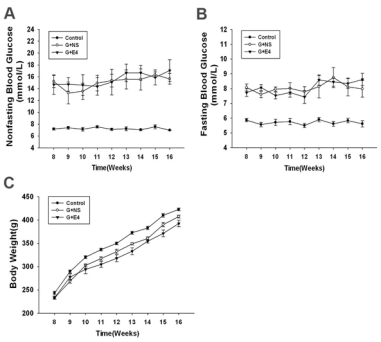

Figure 1. No influence on the blood glucose level or bodyweight of GK rats by E4 treatment. Non-fasting blood glucose (**A**), fasting blood glucose (**B**) and bodyweight (**C**) of rats with and without E4 intravitreal injection at 12 weeks of age (two-way repeated ANOVA for interaction: non-fasting blood glucose, $F(16,243)=1.112$, $p=0.344$; fasting blood glucose, $F(16,243)=1.157$, $p=0.304$; bodyweight, $F(16,243)=1.336$, $p=0.176$; n=10). Bodyweight and non-fasting and fasting blood glucose levels did not statistically differ between the GK control and E4-treated group from 12 to 16 weeks of age (two-way ANOVA followed by Bonferroni's test: non-fasting blood glucose, $p=0.43$; fasting blood glucose, $p=0.921$; bodyweight, $p=0.576$; n=10). Each datum point is expressed as the means ± SEM (n=10). GK rat: Goto-Kakizaki rat; E4: Exendin-4; Control: Wistar rats treated with sham injection (normal saline); G+NS: GK rats treated with normal saline; G+E4: GK rats treated with E4.

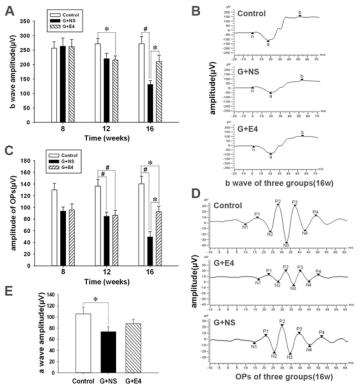

Figure 2. Prevention of the decrease in electroretinography (ERG) amplitudes of GK rats by E4 treatment. E4 intravitreal injection significantly prevents the decrease in the flash ERG b-wave (**A**, **B**) and Ops (**C**, **D**) amplitudes of GK rats at 16 weeks of age (one-way ANOVA followed by Bonferroni's test: b-wave, G+NS versus G+E4, t=3.023, p=0.016; OPs, G+NS versus G+E4, t=3.155, p=0.012; n=10). **E**: E4 prevents the decrease in the ERG a-wave amplitudes at 16 weeks of age, but the GK control and E4-treated GK group did not statistically differ (one-way ANOVA followed by Bonferroni's test: a-wave, G+NS versus G+E4, t=1.161, p=0.767, n=10). GK rat: Goto-Kakizaki rat; E4: Exendin-4; Control: Wistar rats; G+NS: GK rats treated with normal saline; G+E4: GK rats treated with E4. Data are presented as the means ± SEM (*: p<0.05, #: p<0.001, n=10). One-way ANOVA followed by Bonferroni's test.

Upregulation of GLP-1R expression by E4 stimulation in vivo and in vitro: In 16-week-old GK rats, GLP-1R expression was downregulated to 72.4% of the level in the Wistar control rats (Figure 4A; p<0.01) but was maintained at normal levels under E4 treatment or increased to 132.2% of the GK control (Figure 4A; p<0.05). The changes in GLP-1R expression evidenced by retinal immunostaining were consistent with those shown by western blot analysis among the above three groups, and GLP-1R was expressed throughout the entire retina (GCL, inner plexiform layer [IPL], INL, outer plexiform layer [OPL], and inner segment [IS]; Figure 4B).

RMCs were identified by GS and GFAP immunostaining. GS was highly expressed in the cytoplasm in normal RMCs, while GFAP was less expressed (data not shown). In normal RMCs, GLP-1R was highly expressed in the cell membrane and the cytoplasm (Figure 4C). After 72 h of high glucose treatment (20 mM), its expression decreased to 72.9% of the normal control (5.6 mM; Figure 4D; p<0.05). In RMCs under E4 treatment (10 μg/mL), GLP-1R expression increased to 161.8% of the high glucose control level, and the expression of GLP-1R was maximized after 48 h of E4 treatment (Figure 4D; p<0.01). Moreover, the GLP-1R expression of the E4-treated group receiving normal glucose (5.6 mM) treatment only increased to 102.4% of the normal untreated control (5.6 mM) level, which showed that E4 did not affect the RMCs under normal glucose (5.6 mM) treatment after 48 h of treatment (Figure 4D; p>0.05).

Regulation of mitochondria-dependent apoptosis associated genes in GK rat retina: At 16 weeks of age, Bcl-2, Bcl-xL, and Bax expression were also evaluated by western blotting. In GK rats, the Bcl-xL/Bax and Bcl-2/Bax protein ratios decreased to 72.3% and 54.7% of the Wistar control level (Figure 5A; p<0.001 and p<0.01) but were maintained at normal levels under E4 treatment or increased to 131% and

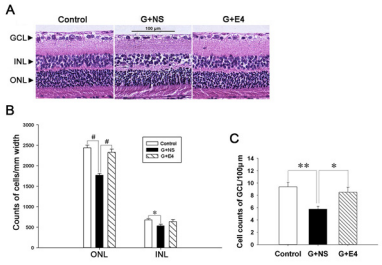

Figure 3. Prevention of GK rat retinal cell loss by E4 stimulation. **A**: Photomicrographs of retina cross-sections with hematoxylin-eosin (HE) staining of three groups: Control, G+NS, and G+E4. The scale bar represents 100 μm. **B**, **C**: E4 intravitreal injection prevented retinal cell loss in the GCL, INL, and ONL, but the INL did not statistically differ between the GK control and the E4-treated GK group (one-way ANOVA followed by Bonferroni's test: ONL, G+NS versus G+E4, t=11.6, p<0.001; INL, G+NS versus G+E4, t=2.369, p=0.083; GK rat: Goto-Kakizaki rat; E4: Exendin-4; GCL, G+NS versus G+E4, t=2.794, p=0.033; *: p<0.05, **: p<0.01, #: p<0.001, n=8). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. GK rat: Goto-Kakizaki rat; E4: Exendin-4; Control: Wistar rats treated with sham injection (normal saline); G+NS: GK rats treated with sham injection (normal saline); G+E4: GK rats treated with E4. GK rat: Goto-Kakizaki rat; E4: Exendin-4. Data are expressed as the means ± SEM. One-way ANOVA followed by Bonferroni's test.

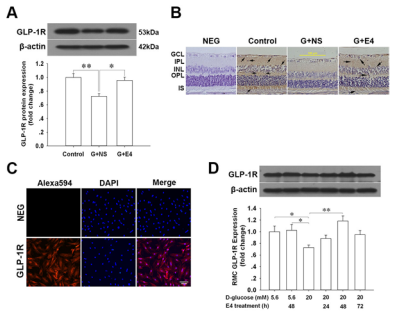

Figure 4. Upregulation of GLP-1R protein expression both in vivo and in vitro by E4 stimulation. (**A**, **B**) western blot analysis and immunohistochemistry result showed that E4 treatment prevented the downregulation of retinal GLP-1R expression in GK rats (one-way ANOVA followed by Bonferroni's test: western blot analysis, G+NS versus G+E4, t=3.792, p=0.013). GLP-1R was expressed in the inner retinal layers (GCL, IPL, and INL) and inner segment of photoreceptors (IS; n=6). The scale bar represents 100 µm. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer. NEG: negative control (Wistar control); Control: Wistar rats treated with sham injection (normal saline); G+NS: GK rats treated with sham injection (normal saline); G+E4: GK rats treated with E4. (*: p<0.05, **: p<0.01, #: p<0.001, n=4). **C**: Immunostaining of GLP-1R in primary retinal Müller cells (RMCs) from Wistar rats. The scale bar represents 100 µm. GLP-1R was highly expressed in RMCs membrane and cytoplasm (red). NEG: negative control. (**D**) Western blot analysis showed that E4 upregulated the GLP-1R protein expression of RMCs under high glucose culture (20 mM) at different time points (24 h, 48 h, and 72 h). One-way ANOVA followed by Bonferroni's test: Control (D-glucose, 5.6 mM) versus control (D-glucose, 20 mM, t=3.421, p=0.046; E4 control [5.6 mM D-glucose + 48 h E4 treatment] versus control [D-glucose, 20 mM], t=3.764, p=0.021; control [D-glucose, 20 mM] versus E4 treatment [20 mM D-glucose + 48 h E4 treatment], t=4.396, p=0.005; n=4). E4 had no effect on RMCs under normal glucose (5.6 mM) treatment after 48 h. Data are expressed as the means ± SEM. One-way ANOVA followed by Bonferroni's test.

150% of the GK control, respectively (Figure 5A; p<0.001 and p<0.05). Additionally, the expression of retinal caspase-3 protein increased to 148.5% of the Wistar control level in GK rats (Figure 5B; p<0.01), while it was suppressed to 114.3% after E4-treatment (Figure 5B; p<0.05).

Reduction of reactive gliosis by E4 treatment in vivo and in vitro: The expression of GFAP in the retinas of 16-week-old GK rats was dramatically upregulated to 72.9-fold the level of the Wistar control (Figure 6A). The GFAP expression of the E4-treated group was significantly reduced to 73.7% of the GK control level (Figure 6A, p<0.001). Similarly, the vimentin expression was upregulated to 116.6% of Wistar control level in the GK group and reduced to 87.3% of the GK control level in the E4-treated group, although the three groups did not significantly differ (Figure 6B; p>0.05). In addition, the retinal immunostaining changes in GFAP among the three groups were consistent with the western blotting analysis (Figure 6C). In vitro, the GFAP expression level in RMCs after 72 h of high glucose treatment was upregulated (5.2-fold of the normal control) and downregulated (2.8-fold of the normal control) after 72 h of E4 treatment (Figure 6D).

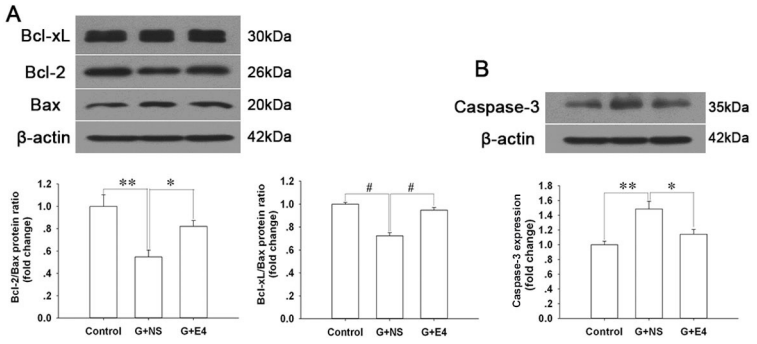

Figure 5. Maintenance of Bcl-xL/Bax ratio and Bcl-2/Bax ratio are E4 treatment in the retinas of GK rats. A: Western blot analysis showed that E4 maintained retinal the Bcl-xL/Bax ratio and Bcl-2/Bax ratio in GK rats (one-way ANOVA followed by Bonferroni's test: Bcl-xL/Bax ratio: G+NS versus G+E4, t=7.273, p<0.001; Bcl-2/Bax ratio: G+NS versus G+E4, t=3.076, p=0.040, n=4). **B**: Western blot analysis showed that the increase in the Caspase-3 expression was prevented by E4 treatment in GK rats (one-way ANOVA followed by Bonferroni's test: G+NS versus G+E4, t=3.876, p=0.011). *: p<0.05, **: p<0.01, #: p<0.001. GK rat: Goto-Kakizaki rat; E4: Exendin-4; Control: Wistar rats treated with sham injection (normal saline); G+NS: GK rats treated with sham injection (normal saline); G+E4: GK rats treated with E4 intravitreal injection. Data are expressed as the means ± SEM. One-way ANOVA followed by Bonferroni's test.

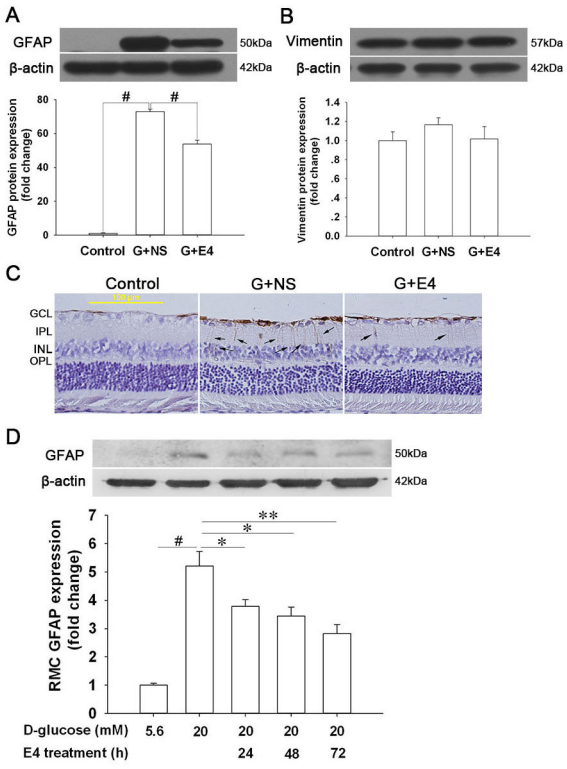

Figure 6. Reduction in the retinal reactive gliosis by E4 treatment in diabetic GK rats in vivo and in vitro. Western blot analysis of retinal GFAP (**A**) and vimentin (**B**) expression in three groups (one-way ANOVA followed by Bonferroni's test: GFAP, G+NS versus G +E4, t=6.501, p<0.001, n=4). **C**: Immunohistochemistry of retinal GFAP in three groups. **D**: Reduction of GFAP expression in primary Müller cells (RMCs) from Wistar rat at different time points (24 h, 48 h, and 72 h) by E4 treatment under high glucose culture (20 mM; one-way ANOVA followed by Bonferroni's test: control [D-glucose, 5.6 mM] versus control [D-glucose, 20 mM], t=11.5, p<0.001; control [D-glucose, 20 mM] versus E4 treatment [20 mM D-glucose + 24 h E4 treatment], t=3.396, p=0.040; control [D-glucose, 20 mM] versus E4 treatment [20 mM D-glucose + 48 h E4 treatment], t=3.957, p=0.013; control [D-glucose, 20 mM] versus E4 treatment [20 mM D-glucose + 72 h E4 treatment], t=5.157, p=0.001;n=4. (*: p<0.05, **: p<0.01, #: p<0.001). GK rat: Goto-Kakizaki rat; E4: Exendin-4; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer. The scale bar represents 100 µm. Control: Wistar rats treated with sham injection (normal saline); G+NS: GK rats treated with sham injection (normal saline); G+E4: GK rats treated with E4 intravitreal injection. Data are expressed as the means ± SEM. One-way ANOVA followed by Bonferroni's test.

Regulation of AKT (Ser473) activation and expression of Bcl-2 in Vitro: The phosphorylation level of AKT at Ser473 in RMCs after 72 h of high glucose treatment was upregulated to 196% of the normal control level and downregulated to 124.5% of the normal control level after 48 h of E4 treatment (Figure 7A). This finding was consistent with the GLP-1R expression over time that was observed in response to E4 treatment in vitro (Figure 4D). The Bcl-2 expression was downregulated to 51.7% of the normal control level after 72 h of high glucose treatment (Figure 7B, p<0.001) and was upregulated to 162.7% of the high glucose control in RMCs after 72 h of E4 treatment in vitro (Figure 7B, p<0.01).

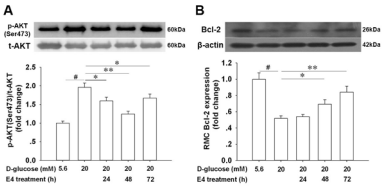

Figure 7. Modulation of p-AKT (Ser473) activation and Bcl-2 expression in primary retinal Müller cells (RMCs) by E4 under high glucose culture. E4 stimulation downregulated p-AKT (Ser473) activation (**A**) and upregulated Bcl-2 expression (**B**) in primary retinal Müller cells (RMCs) under high glucose culture at different time points (24 h, 48 h, and 72 h; one-way ANOVA followed by Bonferroni's test: p-AKT [Ser473], control [D-glucose, 5.6 mM] versus control [D-glucose, 20 mM], t=10.07, p<0.001; control [D-glucose, 20 mM] versus E4 treatment [20 mM D-glucose + 24 h E4 treatment], t=3.391, p=0.040; control [D-glucose, 20 mM] versus E4 treatment [20 mM D-glucose + 48 h E4 treatment], t=5.191, p=0.001; control [D-glucose, 20 mM] versus E4 treatment [20 mM D-glucose + 72 h E4 treatment], t=3.315, p=0.047; Bcl-2, control [D-glucose, 5.6 mM] versus control [D-glucose, 20 mM], t=10.76, p<0.001; control [D-glucose, 20 mM] versus E4 treatment [20 mM D-glucose + 48 h E4 treatment], t=3.876, p=0.015; control [D-glucose, 20 mM] versus E4 treatment [20 mM D-glucose + 72 h E4 treatment], t=5.213, p=0.001; n=4). *: p<0.05, **: p<0.01, #: p<0.001. E4: Exendin-4. Data are expressed as the means ± SEM. One-way ANOVA followed by Bonferroni's test.

DISCUSSION

DR is a neurodegenerative disease that includes microangiopathy and neurodegeneration. Retinal cell apoptosis (including vascular cells) and reactive gliosis are basic pathological features of early DR [8]. Our previous studies have shown that both the systemic administration [44] and intravitreal injection [9] of E4 analog (E4a) exerted protective effects on the diabetic retina. When injected intravitreally, E4a only locally exerted its protective effect on the retina and did not systemically reduce the blood glucose level. Therefore, we again chose to intravitreally inject E4 to focus on the underlying molecular and cellular mechanisms in the retina.

In the present study, we used type II diabetic GK rats as our diabetic animal model. This type of diabetic model consists of congenital spontaneous hyperglycemia and does not require the injection of pharmacologic substances to induce diabetes; it is also free from drug-induced neuronal damage [35,49] and provides a stable and moderate diabetic state, allowing relatively long-term follow-up [50]. Early DR has also been found in GK rat retinas with microcirculatory abnormalities [51,52]. We monitored the non-fasting blood glucose, fasting blood glucose, bodyweight, and ERG in GK rats from eight to 16 weeks of age, approximately one month before and after E4 treatment. Hyperglycemia was stable and moderate in the GK rats from eight to 16 weeks of age, which is similar to human type II diabetes. The results showed that the intravitreal injection of E4 did not significantly affect the systemic metabolism (bodyweight and blood glucose level) in the GK rats, which again demonstrated that the effect of E4 on the retina was local [9] and not systemic [44]. The immunohistological results confirmed that GLP-1R was diffusely distributed in the retina, including the IS and optic nerve (data not shown). We also observed retinal cell loss in the GCL, INL, and ONL of the diabetic GK rat retina, which was consistent with the decrease in the GLP-1R expression in the GCL, INL, and IS.

The electrophysiological changes in diabetes reportedly occur before the appearance of visible lesions [53]. Moreover, OP waves are more vulnerable to disturbances of the retinal circulation than b-waves [53]. In our present study, the ERG b-wave amplitudes in the GK rats were nearly identical to those of the Wistar controls at eight weeks of age, while the OP amplitudes were smaller than those of the controls. This finding indicated that the total OP wave change occurred before that of the b-wave and that the retinal microcirculation may be already influenced by diabetic attacks [26] as early as eight weeks of age in GK rats. However, E4 treatment prevented the decreases in the b-wave and OPs wave amplitudes at 16 weeks of age, which indicated the protection of bipolar cells [54] and inner retina (including microcirculation) [26]. Similarly, the a-wave amplitudes of the GK rats decreased significantly at 16 weeks of age compared to those of the Wistar control, but this decrease was not observed at eight and 12 weeks of age (data not shown). This finding indicated that photoreceptors are also affected in diabetic GK rats at 16 weeks of age. The protective effect on the a-waves at 16 weeks of age was not significant in the E4-treated GK group. The inconsistent changes between the a-wave amplitude and the number of photoreceptors in the E4-treated GK group compared to the GK control may arise because changes in the ERG amplitude did not completely correspond to losses in the photoreceptors [55]. Alternatively, the ERG a-wave may not be exclusively attributed to photoreceptors [56].

GLP-1R was detected in the retina in vivo and in vitro in previous studies [9,44,57]. In our previous studies [9,44], the GLP-1R agonist E4a significantly prevented retinal cell loss in the GCL and ONL in the STZ-induced diabetes model. This protective effect persisted for subcutaneous or intravitreal administration and may be mediated by a decreased extracellular glutamate level. Hao's study [57] indicated that E4 protected RGCs from apoptosis due to high glucose culture by upregulating the expression of the anti-apoptotic protein Bcl-2 and downregulating the pro-apoptotic proteins Bax and caspase-3 in vitro as part of a mitochondria-dependent pathway. The mitochondria-dependent apoptosis pathway plays an important role in diabetic-induced retinal cell apoptosis. Chronic hyperglycemia stress induces cellular oxidative stress and nitric oxide synthesis via the polyol pathway, hexosamine pathway, advanced glycation end products pathway, and protein kinase C pathway, which ultimately activate the mitochondria-mediated apoptosis pathway [58]. In retinal ischemia-induced apoptosis, an imbalance in the Bax/Bcl-xL ratio induced retinal cell apoptosis [59]. In addition, AKT activated at Ser473 under ER stress could induce cell apoptosis in response to diabetic attacks [18-20]. In our present study, the balance between the Bcl-2/Bax and Bcl-xL/Bax ratios, which was significantly disturbed in the GK rats, was maintained in response to E4 treatment. These changes contributed to a reduction in caspase-3, which could preserve neurons [60] or retinal cells in GK rats. Similar effects of E4 on AKT(Ser473) activation and Bcl-2 expression were also detected in primary Müller cells in vitro. Therefore, we postulated that E4 may decrease AKT (Ser473) activation by alleviating retinal endoplasmic reticulum stress, which could ultimately reduce retinal neurons apoptosis. These results illustrated that E4 likely protected retinal cells from apoptosis via mitochondria-mediated and endoplasmic reticulum stress pathways.

Excess reactive gliosis exerts detrimental effects on retinal neurons and vascular cells [11,34]. GFAP was dramatically upregulated to levels 72.9-fold of the Wistar control level in the GK rats, primarily in the inner retinal layer, and E4 prevented this increase. Furthermore, the ERG data were consistent with the GFAP immunostaining area in the retina. Vimentin was also evaluated, but the change was not as significant as that of GFAP. The absence of a significant change in vimentin may be due to its higher abundance in the normal retina which relatively reduced its sensitivity to damage stimuli in the early stages of retinal injury compared to GFAP [32,34]. Alternatively, DR is not as severe in GK rats as in the STZ-induced model [61]. Müller cells are the principal glial cells of the neural retina and play a wealth of crucial roles in supporting neuronal function. Müller cells become reactivated in response to almost every pathological alteration of the retina, including photic damage, retinal trauma, ischemia, retinal detachment, glaucoma, DR, and age-related macular degeneration [62,63]. Reactive Müller cells may directly (e.g., via the release of toxic molecules) and indirectly (by impairing neuron-supportive functions) contribute to retinal damage [34]. In addition, our previous study demonstrated the elevation of the glutamate level in diabetic retina [9], which could ultimately induce ER stress and retinal reactive gliosis [64]. Because Müller cell gliosis is an important feature of early DR, the control of excessive gliosis may be a useful therapeutic approach to protecting retinal neurons. Our present study showed that Müller cells were highly reactivated in high glucose culture in vitro, and the result illustrated that E4 may directly affect reactive gliosis in Müller cells as well as affect the entire retina in vivo. Moreover, the downregulation of AKT (Ser473) activation in response to E4 treatment was consistent with the GLP-1R expression in vitro over time, which reinforced this conclusion.

In the present study, we evaluated the effect of E4 on retinal neurons in GK rats. It caused significant morphological and functional reduction of the apoptosis of retinal neurons. However, its effect on retinal vascular cells, which are important components of the blood-retinal barrier, remains unclear and should be further investigated. In summary, we identified the possible molecular and cellular mechanisms underlying the protective effects of E4 on retinal cell apoptosis and reactive gliosis induced by type II diabetes. E4 may exert its protective effects locally by activating GLP-1R and maintaining the balance of the Bcl-2/Bax and Bcl-xL/Bax ratios and by reducing retinal Müller cell reactive gliosis. These protective effects appeared to be linked to preventing the activation of the mitochondria-mediated apoptosis and endoplasmic reticulum stress pathways. These data reinforce the effect of E4 in early DR. Thus, E4 treatment could be a novel approach for DR.

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