Intravitreal Injection of Exendin-4 Analogue Protects Retinal Cells in Early Diabetic Rats

Yu Zhang, 1 Jingga Zhang, 2,3 Qingping Wang, 1 Xia Lei, 3 Qing Chu, 5 Guo-Tong Xu, 6,2,3,4 and Wen Ye 1,4

PURPOSE. To evaluate the protective effect of intravitreal injection of exendin-4 analogue (E4a) in early diabetic retinopathy (DR) and to explore its possible mechanism.

METHODS. Forty Sprague-Dawley rats were divided into three groups: normal (N), diabetic (D), and E4a-treated diabetic rats (E4a). Diabetes was induced by streptozotocin. Rats in the E4a group were treated with E4a (0.1 µg/2µl/eye), whereas the N and D groups were treated with the equivalent volume of normal saline. Electoretinography was performed at 1 month and 3 months after diabetes onset. Thicknesses and cell counts in each layer of the retina were evaluated. The concentration of glutamate was measured by high-performance liquid chromatography (HPLC). Expressions of glucagon-like peptide-1 receptor (GLP-1R) and GLAST (excitatory amino acid transporter) were detected at mRNA and protein levels and verified by immunohistochemistry in vitro and in vivo. The rMc-1 cells were cultured under high-glucose medium (25 mM), which mimicked diabetic conditions. Effects of E4a (10 µg/mL) were also tested in the rMc-1 culture system.

RESULTS. E4a prevented the reduction in b-wave amplitude and oscillatory potential amplitude caused by diabetes. It also prevented the loss of outer nuclear layer and inner nuclear layer; the thickness and cell count in the outer nuclear layer were decreased in 1-month diabetic rats. The concentration of glutamate in the retina was higher in diabetic rats and was significantly reduced in the E4a-treated group. Consistent with such changes, retinal GLP-1R and GLAST expression were reduced in the diabetic retina but upregulated in E4a-treated rats. No improvement was found in the retina in both functional and morphologic parameters 3 months after treatment.

CONCLUSIONS. Intravitreal administration of E4a can prevent the retina, functionally and morphologically, from the insults of diabetes in rats. GLP-1R and GLAST were proved to exist in the rat retina, and their lowered expressions in the diabetic retina might be related to retinal damage by increasing the retinal glutamate. E4a might protect the retina by reducing the glutamate level through upregulating GLP-1R and GLAST, as observed in retinal Müller cells in this study, but this protective effect was transient. Thus, this could be a potential approach for the treatment of DR. (Invest Ophthalmol Vis Sci. 2011;52:278–285) DOI:10.1167/iovs.09-4727

Diabetic retinopathy (DR) is the leading cause of blindness in patients aged 20 to 70 years 1 in the United States. It was predicted that the numbers of Americans in the 40-year and older population with DR and vision-threatening DR (VTDR) would be triple in 2050 compared with the numbers in 2005, from 5.5 million to 16.0 million for DR and from 1.2 million to 3.4 million for VTDR. 2 The direct cause of DR is still largely unknown. The notion that DR is solely a microvascular complication in diabetes has been challenged in recent years.3–6 Evidence exists that all classes of cells within the retina are involved in a multiplicity of disease processes at early stage of diabetes.4–6 Glucagon-like peptide-1 (GLP-1), produced primarily by cells in the intestines and discovered for its effects on glucose metabolism,7 is an endogenous insulinotropic peptide that is secreted from the L-cells of the gastrointestinal tract in response to food. It has potent effects on glucose-dependent insulin secretion, insulin gene expression, and pancreatic islet cell formation,8 and it has been evaluated as a potential treatment for diabetes when given at pharmacologic concentrations.9,10 GLP-1 can also stimulate cellular differentiation11 and exert cytoprotective and antiapoptotic actions on pancreatic β-cells12 and myocardial cells.13 The GLP-1 receptor (GLP-1R) is expressed in many types of cells and tissues,14–18 such as pancreatic endocrine cells, intestinal epithelial cells, brain, lung, kidney, heart, muscle skin, and primary porcine proximal tubular cells as well as pancreatic islet-derived dedifferentiated cells.19 Recent studies showed that GLP-1R was also expressed in both rodent20 and human21 brain, such as in the hypothalamus, thalamus, brain stem, lateral septum, subfornical organ, and the area postrema, suggesting a central role for GLP-1 in the regulation of food intake and the response to other stress.22 It has been shown that GLP-1R activation induces neurite outgrowth in PC12 cells and SK-N-SH human neuroblastoma cells.23 Exendin-4 (E4), a 39-amino acid peptide, is a GLP-1R agonist that was found in the saliva of the Gila monster.24,25 E4 has been shown to bind to GLP-1R in pancreatic cells and to promote the proliferation of β-cells in the pancreas.24,26 It was reported that both GLP-1 and E4 had neuroprotective properties that could protect neurons against glutamate-induced apoptosis in cell culture and that could attenuate cholinergic neuron atrophy in the basal forebrain of the rat after excito-
toxic lesions.\textsuperscript{25} In our previous study, we demonstrated that GLP-1R was also expressed in the rat retina.\textsuperscript{27} Hence, we postulated that exendin-4 analogue (E4a), a polypeptide prepared from genetic engineering, might combine some features of GLP-1 and E4 through binding GLP-1R.

Glutamate is the main excitatory neurotransmitter in the retina, mediating neurotransmission among the major neuronal classes (photoreceptors, bipolar cells, and ganglion cells). Rapid removal or inactivation of glutamate is necessary to maintain the normal function of the retina. The retina has sodium-dependent, high-affinity uptake systems for glutamate.\textsuperscript{28–30} The removal of L-glutamate from the extracellular space by astrocytes through the astroglial glutamate transporter (GLT-1) and GLAST (excitatory amino acid transporter [EAAT1]) appears essential for maintaining a homeostatic milieu for neighboring neurons. These studies suggest a possible neurotrophic role of GLP in the central nervous system.

Based on the protective effect of systemic administration of E4a on DR, the present study was carried out to test the effects of E4a in retinal protection when delivered intravitreally, its effects on retinal glutamate level, and the expressions of GLP-1R and GLAST in rat retina and in retinal Müller cells.

**Materials and Methods**

**Reagents**

Streptozotocin (STZ; pH 4.5) was purchased from Sigma-Aldrich (Beijing, China). Rats were purchased from Shanghai SLAC Laboratory Animals Co., Ltd. (Shanghai, China). All other chemical and reagents were obtained from analytical grade. Dexamethasone sodium phosphate, insulin, and streptozotocin were purchased from Sigma-Aldrich (Shanghai, China). The amino acid sequence of the human GLP-1 receptor (GLP-1R) and EAAT1 was designed with the software (Primer Premier Version 5.0), were purchased from Shanghai DNA Biotechnology Corp., Ltd. (Shanghai, China). The primers for EAAT4 were 5′-GGACTCATCG-3′ (antisense) and 5′-GCAAACAGTTCAGGGTTG-3′ (sense), and the size of the amplified fragment was 296 bp. PCR products for EAAT4 were 5′-GTCTTGGGATCTCCTCCTTG-3′ (antisense) and 5′-GAAATTCGTTGATGGGTT-3′ (sense), and the size of the amplified fragment was 300 bp.

**Exendin-4 Analogue**

The E4a was a gift kindly provided by Yukun Sun (Shanghai Research Centre of Biotechnology, Chinese Academy of Sciences). The amino acid sequence of the peptide is HEGGTX1TDLSKQX2EEAXV-3LFIEWKLNGX4PX5 (patent no. CN 200510040823.8). E4a was dissolved in normal saline (pH 5) and was injected intravitreally into both eyes of the diabetic rats (0.1 μg/μL/eye). Diabetic and healthy controls were given normal saline (2 μL/eye).

**Experimental Animals and Intravitreal E4a Treatment**

Forty male Sprague-Dawley rats, each weighing approximately 200 g, were purchased from Slaccas (SIBS, 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/12-hour dark schedule with food and water ad libitum. Twelve rats were used as normal controls. Diabetes was induced by a single injection of STZ (administered intraperitoneally, 60 mg/kg body weight, freshly dissolved in citric buffer, pH 4.5) and was confirmed by the blood glucose concentration exceeding 250 mg/dL for 3 consecutive days. Four rats were excluded from the experiments because of their failure to develop diabetes. All rats were weighed weekly. The rats were killed 1 month and 3 months after diabetes onset. Normal control rats were maintained in the same conditions. The rats were anesthetized by 2% pentobarbital (50 mg/kg intraperitoneally). Intravitreal injections of E4a were performed to both eyes within 2 hours of STZ administration with a 30-gauge, half-inch needle (Becton Dickinson, Franklin Lakes, NJ) on a microsyringe (Hamilton, Lausanne, Switzerland), using a temporal approach, 2 mm posterior to the limbus. The dosage of E4a was 0.1 μg/eye, in a volume of 2 μL. Sham injections (2 μL normal saline) were performed to both normal control and diabetic control rats. The rats recovered spontaneously from the anesthesia and were sent back to room in which they were housed with food and water ad libitum.

**Electroretinography**

Retinal neuronal functions were examined with electroretinography (ERG) (EP-1000 System; Tomey, Nagoya, Japan), 1 month and 3 months after diabetes onset. The rats were anesthetized with 2% pentobarbital (50 mg/kg, intraperitoneally) after dark adaptation overnight. The 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. Flash ERG responses were recorded from both eyes by corneal electrodes, with the negative electrode placed in the subcutaneous space of the cheek and the ground electrode clipped to the back leg. Gel (Vidisc; Bausch & Lomb, Rochester, NY) was used as a conducting medium for the corneal electrode. The ERG signals were amplified (×20,000) and filtered (0.1–300 Hz) by differential amplifiers. Light stimuli were obtained from a Ganzfeld light source. Full-field white light stroboscopic flashes lasting 10 μs were presented at a distance of 30 cm and a rate of 1.0 per second. Pass bandwidth was set to 75–300 Hz. All recordings were taken in a darkened room, and the recordings were made under dim red illumination to ensure a completely dark-adapted state. ERG testing was begun when the electrical resistance was below 4 Ω with two repeats.

The b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave, as defined by the International Society for Clinical Electrophysiology of Vision.\textsuperscript{31} The implicit time of the b-wave was measured from the stimulus onset to the peak of the b-wave. Oscillatory potentials (OPs) are four to six waves in the electroretinogram that represent the rising phase of the b-wave.\textsuperscript{32} The magnitude of the OPs was determined as the sum of the three major amplitudes.\textsuperscript{33}

**RNA Isolation and Determination of Gene Expression**

Total RNA was extracted from neurosensory retinas. The reverse transcription product (1 μL) was then amplified by PCR. The specific primers, designed with the software (Primer Premier Version 5.0), were purchased from Shanghai DNA Biotechnology Corp., Ltd. (Shanghai, China). The primers for GLP-1R were 5′-GTCTCGAGGATCTCGAGCT-3′ (sense) and 5′-GAAACAGTTCAGGGTTG-3′ (antisense), and the size of the amplified fragment was 187 base pairs (bp). The primers for GLAST were 5′-CTCTGGGACATCCCTTCCTTG-3′ (sense) and 5′-GAAACAGTTCAGGGTTG-3′ (antisense), and the size of the amplified fragment was 300 bp. The primers for EAAT4 were 5′-GTCTTGGGATCTCCTCCTTG-3′ (sense) and 5′-GAAATTCGTTGATGGGTT-3′ (antisense), and the size of the amplified fragment was 296 bp. PCR products for β-actin were used as a positive control and the internal standard. The primers for β-actin were 5′-GAAACAGTTCAGGGTTG-3′ (sense) and 5′-GAAATTCGTTGATGGGTT-3′ (antisense). The size of the amplified fragment was 227 bp. Amplification conditions included an initial denaturation at 95°C for 5 minutes, followed by 39 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, an extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. PCR products were electrophoretically separated on 2% agarose gel in 1× TAE buffer. The optical densities of GLP-1R, GLAST, and EAAT4 were determined with analysis software (Quantity One; Bio-Rad, Hercules, CA).

**Western Immunoblot Analysis for GLP-1R and GLAST**

Individual retinas from experimental and control rats (four single retinas from four rats selected randomly per group) were isolated and homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer containing 10 mM 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 phenylmethylsulfonyl fluoride (Shenergy Bioscience Technology Company, Shanghai, China). After 15 minutes' incubation on ice, the extracts were centrifuged at 12,000g for 15 minutes at 4°C and stored at −80°C. Protein concentrations were determined by protein assay kit (Bio-Rad). Equal amounts of protein were dissolved in SDS-polyacrylamide gels and transferred electrophoretically onto a nitrocellulose membrane (Bio-Rad). The membranes were blocked for 30 minutes with 1% nonfat milk and then were incubated overnight with anti–GLP-1R antibody (1:250), anti–GLAST antibody (1:1000). After they were washed with (Tris-buffered saline Tween-20 (TBST), the membranes were incubated for 1 hour with horseradish peroxidase–conjugated anti–donkey antiseraum, anti–rabbit antiseraum in TBST and 1% nonfat milk. The membranes were washed three times with TBST, and proteins were visualized by enhanced chemiluminescence. The optical density of each band was determined with analysis software (Quantity One; Bio-Rad).

Immunohistochemistry for GLP-1R and GLAST in Retinal Layers

The rats were killed after deep anesthesia. The eyes were enucleated and fixed in phosphate-buffered saline (PBS), buffered 4% paraformaldehyde for 24 hours. A cutting marker was made at the 12 o’clock position of the limbus. The eyes were embedded in paraffin. Orientations 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 (retinal thickness measurement and cell counts in each layer). Specimens were used for immunohistochemical analysis using the avidin-biotin-peroxidase complex method (SABC kit; Boster). For experiments, retinal paraffin sections were dewaxed and rehydrated. After they were rinsed with PBS, the sections were incubated with blocking solution (10% normal goat serum in PBS) for 30 minutes at room temperature, followed by overnight incubation with polyclonal anti–GLP-1R antibody (1:100), and anti–GLAST antibody (1:100), separately, at 4°C. Sections without primary antibodies were used as negative controls. The following day, sections were washed in PBS three times, for 5 minutes each time, and were incubated with biotinylated donkey anti–goat IgG (1:200, GIA 7247; Santa Cruz) and biotinylated sheep anti–rabbit IgG (1:200, BA1003; Boster, China), separately, for 20 minutes at room temperature. After they were washed with PBS, the sections were incubated with avidin-horseradish peroxidase complex (SABC kit; Boster). The sections were washed again, and then the stainings were developed by 3,3-diaminobenzidine without nuclear staining and terminated by water. The sections were washed, dehydrated, passed through xylene, and coverslipped. Balsam neutral was used as mounting media. The results were evaluated under light microscopy.

Measurement of the Changes in Retinal Thickness and Cell Counts

The thickness of the different retinal layers was measured at 200× magnification, including the outer limiting membrane to the inner limiting membrane (OLMLM), the outer nuclear layer (ONL), the inner nuclear layer (INL), the ganglion cell layer (GCL), and the inner plexiform layer and inner nuclear layer (IPL+INL). Two measurements were taken on each section, at the two reference lines, which were 1 mm away from the optic nerve on both superior and inferior sides. Cell numbers in ONL and INL were counted in the same area at 1000× magnification. All the cell nuclei within a fixed 25-μm column, centered with the 1-mm reference lines, were counted. Cell density was then expressed as the cell count/width (mm) of the retina in different layers.

High-Performance Liquid Chromatography

Neuroretinal retinas were weighed and homogenized in perchloric acid (0.5 mL, 0.1 M) at 4°C. The extract was centrifuged at 13,600g for 20 minutes at 4°C. Supernatants (100 μL) were centrifuged again under the same condition, and a final 10-μL clarified liquid of each sample was analyzed by HPLC. Asparagusic acid, glutamic acid, glutamine, glycine, taurine, and g-aminobutyric acid were purchased from Sigma. The stock reagent contained 27 mg ortho-phthalaldehyde (OPA), 40 μL 2-mercaptoethanol (2-MCE), and 5 mL methanol. The working solution was prepared by diluting the stock solution with 5 mL of 0.1 M sodium tetraborate buffer (pH 9.6) (Fluka, Sigma-Aldrich, St. Louis, MO), Precolumn derivatization was performed by 10 μL retina sample volumes with 10 μL working-strength OPA. The levels of glutamate were analyzed fluorometrically after OPA/2-MCE derivatization and HPLC separation, as described by Sitges with some modifications. The amino acids were separated by a reverse-phase HPLC system (Rheodyne; Beckman Coulter, Brea, CA) composed of an ultraspHERE octadecyl silane (ODS) column (particle size, 5 mm; length, 250 mm; diameter, 4.6 mm) at 25°C and a fluorescence detector (model 157; Beckman Coulter) set at 280 nm (excitation wavelength) and 340 nm (emission wavelength). A linear gradient elution program carried out over 20 minutes was applied for amino acid elution with a flow rate of 1.0 mL/min. Eluent B (90% methanol) from 0% to 40% in 12 minutes was then applied for 5 minutes, followed by eluent A (0.1 M monopotassium phosphate buffer, pH 6.0, with 35% methanol and 2% tetrahydrofuran) for 5 minutes. The integration of the glutamate peak area and further calculations were carried out by System Gold software, and quantification was allowed by running standard amino acid solutions under the same conditions.

Culture of Rat Müller Cell Line rMC-1

The rMC-1 cells were kindly provided by Vijay Sarthry (Northwestern University, Evanston, IL). The cells were maintained in normal glucose (5 mM) Dulbecco’s modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS; High Clone Corporation, Logan, UT) and 1% penicillin/streptomycin (Sigma-Aldrich) at 37°C with 5% CO2 in a humidified incubator. For passaging the rMC-1 cells, cells were plated in six-well culture dishes at 8 × 10^5 cells/cm^2. When the cells reached 70% confluence, they were starved in medium without FBS for 8 hours and then were treated with different concentrations of glucose with or without E4a (normal group, 5 mM glucose; high glucose group, 25 mM glucose; high glucose+E4a treatment, 25 mM glucose + E4a [10 µg/mL] in test medium (DMEM supplemented with 1% FBS and 2% HS) for indicated time. The rMC-1 cells had a doubling time of approximately 70 to 72 hours and were passaged by trypsinization every 3 to 4 days. Total RNA was prepared using reagent (Trizol; Invitrogen-Life Technologies, Eggstein, Germany). Western blot analysis was performed on 20 µg protein from each cell lysate. Expression of glial fibrillary acidic protein (GFAP) was used as the marker for Müller cells. Immunostaining of GLP-1R and GLAST in different groups was studied.

Immunostaining

The rMC-1 cells were grown on cover slides and were stained with GLP-1R antibody (1:50), GLAST antibody (1:100), and GFAP antibody (1:100), separately, overnight at 4°C. After staining with Cy3-conjugated anti-rabbit IgG, the immunostaining was visualized and photographed under a fluorescence microscope.

Statistical Analysis

Data were expressed as mean ± SE. Statistical significance was assessed applying one-way ANOVA or paired t-test where appropriate. *P ≤ 0.05* was considered statistically significant.

RESULTS

Establishment of Diabetic Rat Model

After diabetes onset, over a 1- to 4-week period, serum glucose levels in both the diabetic control and the E4a-treated group were approximately fourfold to sixfold higher than in healthy control rats (Fig. 1A). The body weight of the diabetic rats...
controls (Figs. 3A, 3C). For the diabetic rats, the cell number in the ONL between E4a-treated diabetic rats and healthy controls increased steadily and increased by 75.8% at 4 weeks (Fig. 1B). At 4 weeks, the body weight of the diabetic rats was approximately 63% that of the healthy control rats. In comparison, intravitreal E4a (0.1 μg/eye) had no effect on blood glucose level or body weight.

Protection of Retinal Function by E4a in Diabetic Rats as Measured with ERG

ERG was used as an objective method to evaluate the functional status of the retina. To evaluate the effects of E4a on DR, full-field flash ERG was applied. The amplitudes of b-waves and OPs decreased significantly in STZ-induced diabetic rats 1 month after diabetes onset compared with the controls (Fig. 2): the b-wave amplitudes and OP amplitudes of diabetic rats were approximately 51.4% (Fig. 2A) and 50% (Fig. 2B) those of the normal control, indicating the decreased function of the retina in diabetic rats. For the E4a-treated group, b-wave amplitudes and OPs were significantly higher than those of diabetic rats (n = 12; P < 0.05). There was no significant difference between the normal control and the E4a-treated group (n = 12; P > 0.05). Furthermore, after 3 months, there was no significant difference in the amplitudes of b-wave and OPs between diabetic rats and E4a-treated rats (data not shown).

Protection of E4a to Retinal Neurons in Diabetic Rats

Morphologic examination of hematoxylin and eosin-stained retinal paraffin sections demonstrated significant reduction of total retinal thickness in 1-month diabetic rats in comparison with the control rats (Fig. 3A). This reduction primarily occurred in the ONL (n = 12; P < 0.05, Fig. 3A). In addition, in the ONL of diabetic rats, the cell counts decreased significantly (76% of the control; n = 12; P < 0.05, Fig. 3A). However, no difference was found in total retinal thickness and cell counts in the ONL between E4a-treated diabetic rats and healthy controls (Figs. 3A, 3C). For the diabetic rats, the cell number in the GCL was much lower than that of the healthy control 1 month after diabetes onset, and E4a treatment significantly prevented such cell loss in the GCL (Fig. 3B). The thickness and cell count in the INL in 1-month diabetic rats were similar to those in normal rats.

At 3 months after diabetes onset, no significant differences were observed between E4a-treated rats and diabetic rats in all the parameters, including retinal thicknesses of OLM-ILM, ONL, and GCL, as well as the cell counts in ONL and GCL (data not shown). The few significant changes observed were the reduction in retinal thickness and the loss of cell count in the INL at this time point (n = 12; P < 0.05; data not shown). There was no significant difference in the thickness of the IPL or OPL between diabetic rats and healthy controls at both 1 month and 3 months (data not shown).

Reduction of Retinal Glutamate by Intravitreal E4a

The glutamate level in the neurosensory retina was determined by HPLC. At 1 month, the glutamate level in the diabetic retinas was 550.696 ± 135.928 nM, higher than that in normal control rats (336.672 ± 90.354 nM). In E4a-treated diabetic rats, the glutamate level was significantly decreased to 297.553 ± 86.777 nM (P < 0.05; Fig. 4). However, at 3 months after diabetes onset, the glutamate level in the E4a-treated group remained the same as that in the diabetic group (data not shown).

Uprogulation of GLAST, EAAT4, and GS after E4a Treatment

The expression of GLAST was examined by RT-PCR, Western immunoblot analysis, and immunohistochemistry. The expression of GLAST was significantly up-regulated in E4a-treated diabetic retinas at both mRNA and protein levels 4 weeks after diabetes onset (Fig. 5). The levels for mRNA and protein were increased by 11.4% and 16.5% those in diabetic rats (Figs. 5B, 5C). The retinal expression of GLAST, confirmed by immunohistochemistry (Fig. 5A), was stronger in E4a-treated diabetic rats.
GLAST was expressed in the inner layers of the retina, primarily in the INL and retinal ganglion cells (Fig 5A). Three months after diabetes onset, there was no difference in GLAST expression in diabetic rats treated with or without E4a. The same findings were confirmed by experiments with the Müller cell line (rMc-1). GLAST immunostaining level in Müller cells was weaker than in the control but was enhanced under E4a treatment (data not shown). Such changes of GLAST were also validated by mRNA and protein examinations. When compared with the control (5 mM glucose), the mRNA level of GLAST in the high-glucose group (25 mM) decreased by 7.81% but increased by 13.0% after E4a treatment (Fig. 6A). The GLAST protein level decreased by 56.2% in the high-glucose group and increased by 21.8% with E4a treatment (Fig. 6B). No significant changes in the expressions of glutamine synthetase (GS) and EAAT4 were observed among the three groups (Figs. 5B, 6A).

**In Vitro and In Vivo GLP-1R Expression**

A previous study demonstrated GLP-1R expression in rat retina. In the present study, we examined the changes in GLP-1R expression in both rMc-1 cells and retinas of healthy and diabetic rats treated with or without E4a (Figs. 7A, 7C). GLP-1R was expressed in the inner layers of the retina, primarily in the INL and ganglion cells (Fig. 7C). In the 1-month diabetic retina, GLP-1R expression was downregulated by 21.2% compared with the normal control group (Fig. 7D) but was maintained at normal levels under E4a treatment or increased by 28.7% compared with the diabetic rats (Fig. 7D). In vitro study also showed that the expression level of GLP-1R under high-glucose conditions decreased by 24.5% compared with the normal control group and increased by 32.9% after E4a treatment compared with the high-glucose group (Fig. 7B).

**DISCUSSION**

It has been reported that E4a is a potential therapy for diabetes. In the present study, intravitreal injection of E4a was tested for its possible protection to the retina in diabetic rats. The data showed that intravitreal injection of E4a could protect the retinas from diabetic insults, but the effect was transient. The amplitudes of both b-waves and OPs were reduced in diabetic rats, which were greatly improved at 1 month after E4a treatment. However, such protective effects disappeared at 3 months after the treatment. Even though the detailed mechanisms were largely unknown, we believe that such action might have occurred through a local effect of E4a by its receptor GLP-1R because intravitreal injection of E4a had no effect on body weight or blood glucose level (Fig. 1). In comparison with systemic administration, the amount of E4a for intravitreal injection was much smaller, and its leakage into the blood could be ignored. E4a might function by an ON-OFF mechanism in the rat retina. Once E4a binds to the GLP-1R, it might trigger the ON-OFF switch to activate intracellular signal pathway(s) and prevent the retina from diabetic insults. However, its effect does not last long. It would be interesting to further study E4a pharmacokinetics and toxicity in the eye to optimize its regimen in potential diabetic retinopathy therapy.

Morphologic examination also confirmed the protective effects of E4a. Our previous study showed that retinal neurons were damaged significantly 1 month after diabetes onset. In this study, the retinal thickness reduction and cell loss
might be well explained (Fig. 3). Again, the protective effect was observed at 1 month, but not at 3 months, after E4a treatment. The maintenance of retinal neuronal cells by E4a might be achieved by its antiapoptosis function, but the pathways involved merit further exploration.

As an agonist of GLP-1R, the similar function of intravitreal injection of E4a might be extrapolated by GLP-1. Increasing evidence suggests that GLP-1 plays an important role in the central nervous system in response to stress or aversive stimuli. Perry reported that both GLP-1 and E4 mediated reduction in secreted derivatives and mature cellular forms of \( \beta \)-amyloid precursor protein in PC12 cells. More interesting, when GLP-1 or E4 was infused chronically into the lateral ventricles of healthy mice, endogenous antibody peptide levels in whole brain homogenates were significantly reduced. In a well-established rodent model of cholinergic neurodegeneration, the infusion of GLP-1 showed complete amelioration of an ibotenic acid-induced cholinergic marker deficit. Although the molecular mechanism of GLP-1 in neurons is also unclear, GLP-1R activation might play a key role in modulating neuronal vulnerability in neurodegenerative disorders such as Alzheimer’s disease. It was also reported that GLP-1R was involved in learning and neuroprotection. We reported GLP-1R expression in rat retinas in our previous study, and here we showed that it was primarily Müller cells that expressed GLP-1R (Fig. 7A). The protein examination of GLP-1R showed that it was reduced in diabetic retinas and in Müller cells cultured with high-glucose medium. When treated with E4a, GLP-1R expression was greatly improved at both transcriptional and translational levels (Fig. 7), indicating that E4a might exert its protective function by upregulating its cognate receptor (GLP-1R), especially through Müller cells.

It should be emphasized that the major difference between the present study and our previous report is the drug delivery route. In the previous study, E4a was injected subcutaneously, whereas in this study it was injected intravitreally. After systemic administration, E4a exerted its protective effects by both lowering the blood glucose level and directly protecting the retinal neurons. By intravitreal injection, E4a only exerts its protective effect locally, such as by upregulating the expression of GLP-1R.

In this study, we also monitored glutamate and its receptors to evaluate the effect of E4a both in vivo and in vitro. Glutamate is the principal excitatory neurotransmitter in the central nervous system. Excessive levels of extracellular glutamate have been implicated in the pathogenesis of many neurologic and ophthalmic diseases. Increased extracellular glutamate is assumed to result from the failure or the reversed operation of glutamate transporters and from the death of neurons, with subsequent release of intracellular contents (containing approximately 10 M glutamate). Five excitatory amino acid transporters have been identified. In the retina, GLAST is expressed by Müller cells and astrocytes. EAAT2 (GLT-1) is localized in cones and two types of bipolar cells. EAAT3 (EAAC1) is found on horizontal cells, amacrine cells, and retinal ganglion cells, and, rarely, on bipolar cells. EAAT5 is localized to photoreceptors and bipolar cells; and EAAT4 expression in the retina has not yet been reported. In this study, Müller cells and rat retina were found to express...
EAAT4 but with no change under diabetic conditions treated with or without E4a (Figs. 5B, 6A). The most prominent change found was GLAST expression, which was downregulated 4 weeks after diabetes onset and was evidently reversed by intravitreal E4a. We also used high-glucose levels (25 mM) to mimic diabetic stress and tested the effects of E4a on the cell responses. The same finding indicates its role in maintaining normal glutamate levels and in ensuring RGC viability (Figs. 3B, 6).

In summary, the present study showed that intravitreal injection of E4a protected the retina from diabetic insults in early diabetic rat models. E4a might exert its protective effect locally through binding to GLP-1R and up-regulating GLAST expression in the retina, primarily the Müller cells. The retinal glutamate level was lowered as a result. Thus, this could be a new approach for treating diabetic retinopathy. The short-term effect of intravitreal E4a on DR should be a concern because it suggested that for the effect to be maintained, repeated injections into the eye would be required. Ocular pharmacokinetic and toxicity studies should be conducted to optimize the future uses of E4a.

References


