Omega-3 Fatty Acid and its Metabolite 18-HEPE Ameliorate Retinal Neuronal Cell Dysfunction by Enhancing Müller BDNF in Diabetic Retinopathy

Short title

EPA and 18-HEPE induces Müller BDNF in DR

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Diabetic retinopathy, eicosapentaenoic acid, amacrine cell, Müller glia cell, brain-derived neurotrophic factor

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Abstract

Diabetic retinopathy (DR) is a widespread vision-threatening disease, and neuroretinal abnormality should be considered as an important problem. Brain-derived neurotrophic factor (BDNF) has recently been considered as a possible treatment to prevent DR-induced neuroretinal damage but how BDNF is upregulated in DR remains unclear. We found increase in hydrogen peroxide (H_2O_2) in the vitreous of patients with DR. We confirmed that human retinal endothelial cells secreted H_2O_2 by high glucose and H_2O_2 reduced cell viability of MIO-M1, Müller glia cell line, and PC12D, neuronal cell line, and lowered BDNF expression in MIO-M1, whereas BDNF administration recovered PC12D cell viability. Streptozocin (STZ)-induced diabetic rats showed reduced BDNF, which is mainly expressed in the Müller glia cell. Oral intake of Eicosapentaenoic acid ethyl ester (EPA-E) ameliorated BDNF reduction and oscillatory potentials (OPs) in electroretinography (ERG) in DR. Mass spectrometry revealed increase in several EPA metabolites in the eyes of EPA-E-fed rats. In particular, an EPA metabolite 18-hydroxyeicosapentaenoic acid (18-HEPE) induced BDNF upregulation in Müller glia cells and recovery of OPs in ERG. Our results indicated diabetes-induced oxidative stress attenuates neuroretinal function, but oral EPA-E intake prevents retinal neurodegeneration via BDNF in Müller glia cells by increasing 18-HEPE in the early-stages of DR.

Diabetic retinopathy (DR) is a potentially blinding complication of diabetes mellitus and is a significant cause of global vision impairment (1). DR is diagnosed by onset of clinical features, which are easily determined using fundus photography; however, DR-induced functional defects often precede these signs (2,3). Electroretinography (ERG) is widely used to assess retinal function not only in humans but also in animals and is sensitive enough to diagnose the early-stage DR. In particular, ERG can reveal neuroretinal abnormalities in diabetic model rodents and diabetic patients without gross changes by showing the reduced amplitudes of oscillatory potentials (OPs) and reduced b-wave/a-wave ratios (4-6).

The importance of detecting neuroretinal dysfunction is supported by the concept of "neurovascular unit impairment," which is characterized by the neuronal, glial, and vascular cells mutually influencing each other to cause subclinical changes during the early stages of DR (7,8). Previous studies have shown that high glucose levels induce the generation of reactive oxygen species (ROS) in vascular endothelial cells and ROS promote Müller glial and neuronal cell deaths (9,10). The corroboration of these studies introduced the concept of high crosstalk of high glucose–ROS–neuronal cell damage. This concept has gradually become more widespread due to the insufficiency of the present DR treatments. Although there are several therapies in this regard, including laser photocoagulation and intravitreal injections of anti-vascular endothelial growth factor drugs, and although the effectiveness of vitrectomy surgery is improving, the final visual outcomes for patients with DR remain disappointingly modest (11). Therefore, early treatment strategies focusing on neuroretinal protection can have substantial advantages in the prevention of public health disasters due to DR.

Brain-derived neurotrophic factor (BDNF) has a reportedly powerful neuroprotective effect by binding to its receptor TrkB, which amacrine cells and retinal ganglion cells express, and activates

ERK and PI3-kinase pathways (12,13). In the retina, glial cells provide structural and metabolic support for retinal neurons and blood vessels, and interestingly Müller glia cells are widely known as a source of BDNF secretion (14). Even in the early-stage of DR, Müller glia cell impairment and apoptosis reportedly occurs, followed by the dysregulation of neurotrophic factors, including BDNF, which leads to neurodegeneration in DR (15,16). However, little is known how, using the existing administration tools, can successfully increase BDNF expression in DR. Interestingly, oral administration of omega-3 fatty acids, mainly docosahexaenoic acid and EPA, was recently reported to reverse the downregulation of BDNF expression in the obese rat hypothalamus (17). In addition, the previous study which used fat-1 transgenic mice that endogenously synthesize omega-3 fatty acids showed that they had less Purkinje cell degeneration from BDNF-mediated autophagy under streptozocin (STZ)-induced diabetic conditions (18). In the brain, omega-3 fatty acid plays protective roles in neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease (19,20). The retina has fatty acid-rich photoreceptors and, similar to the brain, is a complex of neuronal, glial, and vascular cells. Therefore, it is possible that by applying a similar concept of "neurovascular unit impairment" for brain impairment and neuroretinal dysfunction during early DR, understanding the BDNF function in DR, and developing methods to increase BDNF in DR can provide strong tools for the treatment of DR neurodegeneration.

Based on these assessments, we hypothesized that neuroretinal dysfunction detected by the reduced OPs in the early-stage of DR is initiated by the downregulation of BDNF expression in Müller glia cells that was triggered by the exposure of ROS from retinal vascular endothelial cells, which could induce secondary impairment of neuroretinal cells, including amacrine cells. We also examined whether dietary omega-3 fatty acids, readily available for a therapeutic discipline, can

attenuate cellular damage in DR. In addition, we investigated whether omega-3 fatty acid or its metabolites play a pivotal role in DR using mass spectrometry.

Research Design and Methods

Data collection and measurement

We collected vitreous samples from patients with macular holes (MH) or epiretinal membranes (ERM), as controls, and DR who received vitrectomy using a vitrectomy cutter before starting infusion to keep them dry and immediately stored them at –80°C. The controls and patients with DR had no vitreous hemorrhages. This study was conducted following the guidelines of the Declaration of Helsinki; our protocol was registered with the Clinical Trial Registry of UMIN (registered number UMIN000024553) and was approved by the Nagoya University Hospital Ethics Review Board. We obtained written informed consent from all participating patients.

Animal diabetic model

Eight-week-old female Sprague−Dawley rats (CLEA) were used for these studies and divided into three categories: [a] control group without STZ injections and fed standard chow (CE-2, CLEA) without fishmeal but with sunflower oil (5%); [b] STZ group with intraperitoneal (i.p.) injections of STZ (65 mg/kg, Sigma-Aldrich) and fed the same chow as the control group; and [c] EPA group with i.p. injections of STZ (65 mg/kg, Sigma-Aldrich) and fed the standard chow without fishmeal but supplemented with 5% EPA-E (Mochida Pharmaceutical Co.) (Fig. 2A). The standard chow and the chow with EPA for one rat per day include 74 kcal and 77 kcal, respectively. All diets were stored at −80°C and were thawed at room temperature before use every day. To exclude the effect of omega-3 fatty acid in the food, we prepared CE-2-based chow without fishmeal and added sunflower oil 2 weeks before STZ induction (Fig. 2A, 4H). One week after STZ injection, STZ-treated rats with blood glucose levels under 350 mg/dL were excluded. The concentration of β-hydroxybutyrate (β-HB) in the plasma was measured according to the manufacturer's instructions (Cayman Chemical). All studies adhered to the ARVO Statement for the Use of Animals in

Ophthalmic and Vision Research and were conducted in accordance with the experimental protocol approved by the Nagoya University Animal Care Committee.

ERG measurement

The procedures we used here were similar to those described in detail in an earlier paper (21). Briefly, rats were dark-adapted for at least 2 hours and prepared under dim red illumination. Following anesthesia of the rats with an intramuscular injection of ketamine and xylazine, the pupils were dilated with topical 0.5% tropicamide/0.5% phenylephrine (Santen) and the animals were placed on a heating pad. Hydroxyethyl cellulose was used to hydrate the cornea and conjunctiva for better electrical conductivity of the electrodes. ERGs were recorded with a gold wire loop electrode on the cornea and gold wire reference electrode on the sclera. Responses were amplified and bandpass filtered between 0.3 and 1000 Hz. The ERGs were averaged using a computer-assisted signal averaging system (Power Lab). A ganzfeld bowl (LACE Electronica) with a xenon source was used for stimulation. The amplitude of the a- and b-waves and OPs were measured.

Measuring lipid mediators in rat retina

Lipid mediators in each sample were quantified by high-performance liquid chromatography–tandem mass spectrometry (HPLC/MS/MS), as previously described (22,23). Eyes were collected, and retinal choroidal tissue was separated, frozen immediately in liquid nitrogen, and stored at −80°C. Samples of retinal choroidal tissue were homogenized with Automill (Tokken, Chiba, Japan) and the lipids were extracted with 1 mL of methanol at −20°C. After overnight incubation, the samples were centrifuged at 5,000 $\times g$ for 5 min at 4°C. The supernatants were mixed with

four volumes of water containing 0.1% formic acid with deuterium-labeled internal standards (Cayman Chemical). Diluted samples were loaded onto Oasis HLB cartridges (Waters, Milford, MA, USA) and washed with 0.1% formic acid, 15% methanol containing 0.1% formic acid, and petroleum ether containing 0.1% formic acid. The samples were eluted with 200 μl of methanol containing 0.1% formic acid. The lipids were injected into a Prominence HPLC system (Shimadzu, Kyoto, Japan) and a TSQ Quantum Ultra triple-stage quadrupole mass spectrometer equipped with a H-ESI interface (Thermo Fisher Scientific, Waltham, MA, USA). Mobile phases A and B comprised water and acetonitrile:formic acid (100:0.1 v/v), respectively. The gradient conditions were as follows: 0–7 min, 37% B; 7–19 min, 37–90% B (linear gradient); 19–21 min, 100% B; 21–22.5 min, 37% B; flow rate, 0.12 mL/min. The separation column was a Capcell Pak C18 MGS3 (1 × 100 mm, Shiseido, Tokyo, Japan) set at 45°C and the trapping column was an Opti-Guard Mini C18 (1 × 15 mm, Optimize Technologies, Oregon City, OR, USA). Lipids were analyzed in a negative-ion-polarity mode under the following conditions: spray voltage, 2500 V; capillary temperature, 225°C; vaporizer temperature, 250°C; sheath gas (N_2) pressure, 40 psi; ion sweep gas pressure, 0 psi; auxiliary gas pressure, 10 psi; collision gas (Ar) pressure, 1.5 mTorr; and the EZ Method (scheduled SRM method) with a cycle time of 1.0 s. For accurate quantification, calibration curves were generated for each target eicosanoid using known reference standards and the isotope-labeled internal standards. Data analysis and quantitative calculations were performed using Xcalibur 2.2 software (Thermo Fisher Scientific, Waltham, MA, USA).

Intravitreal 18-HEPE injection

All the schedule of this experiment is shown in Fig. 4H. Following an intramuscular injection of ketamine and xylazine, 18-HEPE [Cayman Chemical, containing equal amounts of 18(S)-HEPE

and 18(R)-HEPE; 10 ng/2 μL] in phosphate-buffered saline (PBS) was injected into the vitreous space of one eye and equal volume of PBS was injected into the other eye as a control. On the same day, a single intraperitoneal injection of STZ (65 mg/kg, Sigma) was also administered, and after 1 week, diabetes was confirmed using the same criterion for blood glucose level as mentioned above. Intravitreal injections of 18-HEPE or a control vehicle were administered on days 0 and 14. On day 28, ERG was measured, and the retinas were dissected.

Immunocytochemistry

After being fixed in 4% paraformaldehyde and cryoprotected in 30% sucrose, the eye cups were incubated with rabbit antibody against BDNF (EPR1292, 1:500, Abcam) and mouse antibody against glutamine synthetase (MAB302, 1:500, Millipore), followed by secondary antibodies with Alexa Fluor 488 and 594 conjugates (1:1000 dilution; Thermo Fisher Scientific) and Hoechst 33342 (1:5000 dilution; Invitrogen). Sections were analyzed using a fluorescence microscope (BZ-9000; Keyence).

Cell culture

Human retinal endothelial cells (HRECs) from Cell Systems (Kirkland) were cultured with EGM-2 medium (Lonza) as previously described (24). HRECs were incubated with or without vascular endothelial growth factor (VEGF) and with different doses of glucose (25 and 5.6 mM) after the adjustment of osmotic pressure as previously described (25). Cultured MIO-M1, from a human Müller glia cell line, and human retinal pigment epithelial (hRPE) cells were purchased from E-lucid (University College London) and the American Type Culture Collection (Rockville), respectively. MIO-M1 and hRPE cells were maintained in Dulbecco's Modified Eagle's Medium

(DMEM; Invitrogen) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, and 10% fetal bovine serum (Sigma-Aldrich). The neuronal cell line PC12D cells (26,27) were cultured in DMEM supplemented with 10% fetal bovine serum and 10% horse serum (Medical & Biological Laboratories) and then incubated for 2 days with 50 ng/mL of nerve growth factor (Millipore) for differentiation into neurons. The high and low glucose DMEM contained 25 and 5.6 mM glucose, respectively. We applied hydrogen peroxide (H_2O_2; Sigma-Aldrich) as oxidative stress. Recombinant BDNF (Abcam) resuspended in DMEM was incubated for 24 hours with PC12D, HRECs, and hRPE cells 1 hour after H_2O_2 administration, and we evaluated the cell viability. We exposed MIO-M1 to H_2O_2 for 24 hours when we evaluated cell viability and BDNF mRNA expression levels, respectively, and we exposed the cells to H_2O_2 for 72 hours when we prepared the sample for ELISA. We measured the relative changes in the BDNF mRNA expression levels in MIO-M1 under oxidative stress (200 µM H_2O_2) following the administration of 18-HEPE, 15-HEPE, 12-HEPE, and 5-HEPE (Cayman Chemical) for 48 hours to evaluate the effects of the lipid mediators. Additionally, we evaluated the expression levels of BDNF mRNA following the administration of 15-hydroxyeicosatetraenoic acid (15-HETE) and 12-HETE (Cayman Chemical) for 48 hours.

Protein and RNA isolation

For protein collection, rat retina and cultured cells were lysed in a RIPA buffer (Sigma-Aldrich) with a protease inhibitor cocktail (Roche). The lysate was centrifuged at 15,000 rpm for 15 min at 4°C and the supernatant was collected. Protein concentrations were determined using a Bradford Assay Kit (Bio-Rad). For quantitative reverse transcription-polymerase chain reaction (qRT-PCR), total RNA was purified using a Qiagen RNeasy Mini-kit (Qiagen). RNA concentration and quality

were assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

ELISA and real-time qPCR

MIO-M1 cells were cultured in 24-well culture plates at a concentration of 3.0×10^4/well in 500 μL of DMEM exposed to different concentration of H_2O_2 with or without 18-HEPE for 72 hours. In addition, MIO-M1 cells were cultured in six-well culture plates at a concentration of 6.0×10^4/well in 2 mL of DMEM exposed to different concentration of glucose for 7 days. The culture supernatant was used according to the manufacture's protocol. Protein lysates were prepared from the rat retinochoroidal tissue, and BDNF protein level was measured using an ELISA kit (Human Free BDNF Quantikine ELISA kit; R&B for human BDNF and Human BDNF ELISA kit: Abcam for rat BDNF). Total RNA was reverse transcribed using a Transcriptor Universal cDNA master (Roche) and RT-PCR was performed using the Thunderbird Probe qPCR mix (Toyobo Life Science, Osaka, Japan) and a Gene Expression Assay containing primers and FAM dye-labeled TaqMan probe to detect human BDNF (*Hs02718934_s1*; Applied Biosystems, Foster City, CA, USA), rat BDNF (*Rn02531967*_s1; Applied Biosystems), and eukaryotic 18S rRNA (Hs99999901_s1; Applied Biosystems). PCR cycles consisted of a pre-denaturation step at 95°C for 2 min, followed by 40 cycles of denaturing steps at 95°C for 15 s, and annealing and extending steps at 60°C for 60 s. The relative expressions of the target genes were determined by the $2^{-\Delta\Delta Ct}$ method.

Quantification of oxidative stress and oxidative stress-induced damage to DNA

H_2O_2 in the human vitreous samples, extracellular concentration of H_2O_2 from MIO-M1, and that from HREC cultured with high and low glucose for 72 hours were measured using the OxiSelect™ Hydrogen Peroxide/Peroxidase Assay Kit (Cell Biolabs), according to the manufacturer's

instructions. Oxidative stress in the retina and choroid of rats and the human vitreous sample were evaluated by quantifying the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) as oxidatively modified DNA. Total DNA was purified from the retina and choroid using the Qiagen DNeasy Blood & Tissue Kit (Qiagen), and purified DNA was digested with an 8-OHdG Assay Preparation Reagent Set (FUJIFILM Wako Pure Chemical Corporation Co.) according to the manufacturer's instructions. 8-OHdG levels were measured using an ELISA kit from Oxis Research.

Cell viability assay

The cultured cells were treated with different doses of H_2O_2, recombinant BDNF (Abcam), and 18-HEPE for 24 hours. MIO-M1 cells were treated with different doses of glucose for 3 and 7 days. Cell viability was determined by using the Cell Proliferation Kit 1 (Roche), according to the manufacturer's instruction. Additionally, we evaluated the effect of BDNF on PC12D cells to assess the neurite outgrowth of PC12D using the IncuCyte Zoom real-time imaging system (Essen Biosciences). We administered 100 ng/mL of recombinant BDNF prior to imaging (BDNF-treated group). Phase-contrast images were taken at 20× magnification 3 and 24 hours later. After imaging, IncuCyte Neurotrack Software (Essen Biosciences) was used to determine the length of the neurite outgrowth (28).

Statistics

Data are presented as means ± standard deviations. For comparisons between >2 groups, initial statistically significant results were further analyzed using Scheffe's test, and the Steel–Dwass test was used when significant differences were found. For comparisons between two groups, the Mann–Whitney U test or Wilcoxon signed-rank test were applied. A value of $p < 0.05$ was

considered statistically significant.

Data and resource availability statements

The datasets generated during and/or analyzed during the current study are available in the figshare: Kaneko H. “Omega-3 Fatty Acid and its Metabolite 18-HEPE Ameliorate Retinal Neuronal Cell Dysfunction by Enhancing Müller BDNF in Diabetic Retinopathy” , posted 12.10.2019, link (https://doi.org/10.6084/m9.figshare.9973616.v1).

No applicable resources were generated or analyzed during the current study.

Results

Reduced bioactivity of Müller glia and retinal neuronal cells by ROS and protection effect of BDNF from ROS induced damage

The levels of 8-OHdG and H_2O_2 significantly increased in human vitreous samples of the eyes of patients with DR compared with those from the eyes of patients with MH and ERM (Fig. 1A, B). After confirming the presence of ROS in DR, we verified the influence of high glucose on HREC and MIO-M1. The extracellular concentration of H_2O_2 levels from HRECs in 25 mM glucose without VEGF was not significantly higher than that in 5.6 mM glucose without VEGF (Supplementary Fig. S1). In contrast, the extracellular concentration of H_2O_2 levels from HRECs in 25 mM glucose containing VEGF significantly increased compared with that in 5.6 mM glucose with VEGF (Fig. 1C). In contrast, 25 mM glucose did not increase the extracellular concentration of H_2O_2 from MIO-M1 but significantly elevated the cell viability of MIO-M1 after exposure for 72 hours compared with 5.6 mM glucose (Fig. 1D, E). Based on these findings and previous studies which have shown that hyperglycemia itself directly induced neuroretinal damage (29,30), we assumed that ROS produced in HREC under high glucose conditions was another trigger for diabetic neuroretinal damage. Therefore, in this study, we focused on oxidative stress and provided medium containing 25 mM glucose within 72 hours for all the experiments and oxidative stress was induced to mimic the condition of early DR. In MIO-M1 cells, H_2O_2 significantly reduced cell viability and *BDNF* mRNA levels compared with that in the control as well as BDNF protein level in the culture medium compared with that in the control (Fig. 1F–H). Similarly, H_2O_2 significantly reduced the PC12D cell viability in a dose-dependent manner (Fig. 1I). Moreover, the administration of recombinant BDNF statistically ameliorated the reduction of cell viability in H_2O_2-treated PC12D cells dose-dependently and the decrease rate of the relative change in the

neurite length per cell body cluster from the beginning in PC12D cells time-dependently (Fig. 1J, K).

EPA protection effect on retinal function in DR rats

Based on previous results indicating that omega-3 fatty acid increased BDNF in other tissues (17,31), we investigated the effect of EPA-E on BDNF expression in the eye and on ERG in STZ-induced diabetic rats. STZ group showed significant differences in body weight, blood glucose and β-HB compared with the control group at 2 and 8 weeks after STZ injection, but the STZ and EPA groups did not show significant differences in all the three parameters including β-HB which is widely used as a diabetic ketoacidosis marker (32) (Table 1 and Supplementary Table S1). These findings indicated that treatment with EPA-E did not affect the metabolic variables or ketosis. To evaluate the neuroprotective effects of EPA on DR, full-field ERG was applied. We first examined when a significant change occurred in the OPs of ERG after STZ injection and confirmed that it occurred 4 weeks after compared with the control (Fig. 2B). Thereafter, we evaluated the effect of EPA-E oral administration for 8 weeks on ERG. The b/a-wave ratio of the ERG in the STZ group was significantly lower than that of the control group, and there was no significant difference between the b/a-wave ratio in the STZ and the EPA group (Fig. 2C). The OPs of ERG in the STZ group were significantly lower than those of the control group, but those in the EPA group were significantly higher than those in the STZ group (Fig. 2D). In summary, the decrease in b-wave/a-wave (b/a) ratio and OP amplitudes in the STZ group suggested a decreased neuroretinal function in DR (27), and significant improvement of the OPs in the EPA group indicated that oral EPA-E administration ameliorated the decreased neuroretinal function in DR. Immunohistochemistry showed that glutamine synthetase and BDNF were co-localized in the

inner retina (Fig. 2F), suggesting that BDNF is produced from Müller cells, ganglion cells, and astrocytes in the rat retina. Furthermore, 8-OHdG showed a significant increase in the STZ group compared with that in the control group and a significant decrease in the EPA group, indicating that oxidative stress was statistically suppressed by EPA-E (Fig. 2G). In addition, the *Bdnf* mRNA level and the relative BDNF protein level in the retina of the STZ group were significantly lower than those of the control and the EPA group (Fig. 2H, I). These results suggested that oral EPA-E administration reduced ROS and ameliorated BDNF reduction in the retina, resulting in the recovery of neuroretinal cell damage after ROS exposure in the early-stage of DR.

Improvement of BDNF expression and neuroretinal function by 18-HEPE

To further investigate the protective mechanisms of EPA in DR, we examined the lipid mediators in rat retinal tissue. Of the possible metabolites made from EPA, 18-HEPE, 15-HEPE, 12-HEPE, and 5-HEPE were significantly detected in the retinal tissue of EPA-E-fed rats (Fig. 3). In contrast, 12-HETE was significantly lower in the EPA group than in the control and STZ groups. The raw data for each subject is shown in Supplementary Table S2. Furthermore, we preformed single-cell culture of MIO-M1 to confirm that Müller cells is one of the sources of BDNF. Relative *BDNF* mRNA level and BDNF protein level in MIO-M1 cells treated with H_2O_2 were increased by 2 μM 18-HEPE but not by 0.5 μM 18-HEPE compared with the control (Fig. 4A, B). In contrast, relative *BDNF* mRNA was not significantly increased by 2 μM 15-HEPE, 2 μM 12-HEPE, or 2 μM 5-HEPE compared with the controls (Fig. 4C). Meanwhile, the relative *BDNF* mRNA level in MIO-M1 cells exposed to 2 μM 15-HETE and that exposed to 2 μM 12-HETE were significantly lower than those of the controls (Fig. 4D). Additionally, H_2O_2 significantly reduced the cell viabilities of all PC12D, HREC, and hRPE cells, but 18-HEPE did not inhibit these reductions (Fig. 4E–G).

These observations confirmed that 18-HEPE played the most prominent role in BDNF increase in Müller glia cells but did not affect the other ocular cell types. Moreover, OPs and the *Bdnf* mRNA level in the retina of STZ rat eyes with 18-HEPE intravitreal injection were significantly higher than those with PBS (Sham; Fig. 4J, K). These corroborating data suggested that 18-HEPE specifically induced BDNF expression in the STZ rat retina and that resulted in OP recovery in ERG as was seen after EPA-E administration in vivo.

Discussion

It is already revealed that the oral administration of omega-3 fatty acids, including EPA, has anti-inflammation, anti-angiogenesis, and anti-oxidation effects in DR (33). However, only few studies focused on the neuroprotective effect of EPA (34). BDNF is one of the key factors in neuroprotection, and we showed the protective effect of BDNF on PC12D. Our study is the first to demonstrate that EPA has significant neuroprotective effects; specifically, it rescued amacrine cell function, which can be evaluated by OPs in ERG in DR by enhancing BDNF expression in Müller glia cells. We further found that some EPA metabolites including 18-HEPE were highly produced in the retina of rats administered EPA-E, and 18-HEPE specifically induced BDNF expression in Müller glia cells. The novel findings in our study were as follows: (i) Although previous studies showed a BDNF increase in other tissues following the administration of omega-3 fatty acid (17,31), the concurrent increase in BDNF in the retina has not been reported to date. (ii) Although EPA metabolites in the eyes have been analyzed already (35), we detected the increase in 18-HEPE in eyes with EPA treatment for the first time. (iii) Although the other HEPEs also increased in the retina after EPA administration, only 18-HEPE increased BDNF mRNA in Müller glia cells.

Oxidative stress plays an important role in DR (10). Indeed, we confirmed that H_2O_2, which production was elevated in DR and under high glucose condition *in vitro* study, reduced BDNF secretions in Müller glia cells and cell viability in both neuroretinal and Müller glia cells. However, EPA-E intake reduced oxidative stress in DR, which contributed to BDNF recovery, resulting in the amelioration of neuroretinal function. To the best of our knowledge, this finding has not been reported to date. STZ-induced diabetic models in rodents reportedly exhibited differences between the control and STZ groups with regard to their general condition, e.g., body weight (36). In our

study, the β-HB levels in the STZ and EPA groups were significantly higher than those in the control group, suggesting that STZ injection must be a trigger of ketoacidosis. In contrast, it is already known that the quantitative measurement of β-HB could be useful to diagnose diabetic ketoacidosis, and serum β-HB levels of ≥3.0 mmol/L was one of the criteria for the diagnosis in previous studies (37-39). In addition, a study defined serum β-HB levels of ≥1.0 mmol/L as a ketosis model (40). Therefore, in our study, we considered that the animals developed ketosis instead of ketoacidosis. Nevertheless, we did not find significant systemic improvement in STZ-induced rats by EPA-E, suggesting that EPA-induced retinal recovery was not only due to an improvement in the general condition. Nevertheless, it was unclear whether the EPA-induced retinal recovery was due to other specific factors in addition to the reduction in oxidative stress in DR. To answer this question, we further investigated which EPA metabolites were increased in DR and specifically induced BDNF expression in the retina. For the first time in our study, we showed that an increase in 18-HEPE particularly contributed to an increase in the Müller glia cell-derived BDNF, both *in vivo* and *in vitro*. Recent studies have demonstrated that EPA is metabolized by vascular endothelial cells and Macrophages introduced from bone marrow may locally produce 18-HEPE (41,42). Consistent with the previous study which demonstrated that vascular endothelial cell injury in diabetic mice was not improved by oral administration of omega-3 fatty acids (43), we confirmed that 18-HEPE did not improve decrease in HREC cell viability by oxidative stress as well as PC12D and hRPE cells. However, 18-HEPE induced a BDNF increase in Müller glia cells *in vitro*. On the other hand, the potential roles of 12/15-lipoxygenase (12/15-LOX) and LOX-derived eicosanoids in DR have been documented in several articles and it has been reported that the treatment of HREC with 12- and 15-HETE increased ROS generation (44,45). In contrast to the present study, other studies showed a significant increase in 12/15-LOX

and its metabolites in patients and mice with DR. Indeed, 8-OHdG was improved by EPA-E administration in the present study. It is possible that the oral administration of EPA-E did not only increase 18-HEPE and Müller glia BDNF but also promoted a change in the activity of 12/15-LOX from metabolizing arachidonic acid to EPA (46,47) and further reduction in 12-HETE, resulting in the protection of Müller glia BDNF.

The other questions and the limitations of the study are as follows: (a) The precise mechanisms of Omega-3 fatty acids, including their metabolites, remain unclear and specific receptors for 18-HEPE have not been identified. Resolvin E1, an 18-HEPE metabolite, reportedly binds to the receptor ChemR23 and plays a pivotal role in suppressing neovascularization and inflammation (41). However, we did not detect resolvin E1 in our retinal samples. Nevertheless, it is unclear whether the metabolite was present in undetectable quantities due to the limited sample volume of the rat retina. Further analysis is necessary to determine the precise mechanism involving lipid mediators in DR. (b) Although OP reduction was reportedly observed 6 weeks after STZ administration (48), we noted OP reduction 4 weeks after STZ administration. This discrepancy may be due to the following reasons: (i) The previous study was performed with 60 mg/kg STZ, whereas we used 65 mg/kg STZ to induce the diabetic condition, and the higher STZ dose resulted in faster ERG change. (ii) We created a special chow by removing fishmeal and adding sunflower oil and started using chow 2 weeks before STZ induction. The effects of EPA reportedly began 2 weeks after administration (49). Sunflower oil contains large amounts of omega-6 fatty acid, whereas CE-2, the basal chow that we modified for this study, contains fishmeal, which is rich in omega-3 fatty acid. Therefore, our STZ rats had a large amount of omega-6 fatty acid but no omega-3 fatty acid for 6 weeks before ERG measurement. The difference in the amount of omega-6 and omega-3 fatty acid in the food could affect the time of OP change. (iii) In our study, the food

was not available *al libitum* and the total calories were controlled. The relationship between total calories and diabetes prevalence has been reported (50,51), and the difference in calorie control might affect the difference in the time of OP reduction. (c) Although BDNF was reportedly detected in human vitreous fluid (52), we did not detect BDNF in the vitreous of patients with DR or that of the control subjects (data not shown) as previous studies using ELISA presented (53,54). It is not ethically possible to collect human retina samples during surgery. Therefore, to confirm BDNF expression in the diabetic eye, we measured the increase in BDNF in the eyes of rats in our study. (d) Various studies have reported that high glucose concentrations do not cause oxidative stress in HRECs when they are pure and properly cultured (55). However, we detected a slight but significant increase in ROS production from the HRECs in a high glucose concentration compared to those in a lower concentration. This was consistent with the findings of several previous studies (56-58). Two reasons might explain this divergence in findings between studies. First, the previous studies used primary HRECs obtained from donors, whereas we used HRECs from a manufacturer. Supplementation with VEGF may also have affected the ROS production; there was no increase in the extracellular concentration of H_2O_2 from HRECs in the medium without VEGF, even with 25 mM glucose. VEGF can promote the production of ROS by HRECs (59). Nevertheless, we considered it reasonable to use supplementation with VEGF to mimic the intraocular condition of retinal damage from diabetes. A second potential reason for the divergence between studies may have been the difference in the method used to detect ROS. We used OxiSelect™ Hydrogen Peroxide/Peroxidase Assay Kits (Cell Biolabs) to detect extracellular ROS, whereas most of the previous studies used 2′7′-dichloro-dihydro-fluorescin diacetate to detect intracellular ROS. (e) Many studies have reported high glucose-induced apoptosis in Müller cells, but a further distinction between types of cell death has been proposed. Many studies have reported that Müller

cells die in a hyperglycemic environment via pyroptotic cell death (60). In the present study, MTT assay showed that high glucose exposure for 3 days increased MIO-M1 cell viability, whereas exposure for 7 days significantly reduced cell viability, consistent with the reduced secretion of BDNF in MIO-M1 (Supplementary Fig. S2). Thus, the decrease in BDNF production by Müller cells in diabetic retinopathy may be due to oxidative stress caused by hyperglycemia and also due to hyperglycemia. Further studies are needed to clarify the mechanism underlying the damage to Müller cells in a diabetic environment.

BDNF is reported to be upregulated by anti-oxidants (61). According to the recent study which indicated that BDNF is expressed not only in Müller glia cells but also in retinal ganglion cells, consistent with our immunohistochemistry result, BDNF expression from both the retinal ganglion and the retinal neuronal cells may be reduced under diabetic conditions (14). Therefore, further studies are required to elucidate EPA involvement with these other cells in DR. Although previous studies have shown BDNF neuroprotective role in the eyes, they used subretinal transplantation of the transfected cells that can express BDNF or BDNF intravitreal injection and, thus, many problems need to be overcome for clinical applications (14, 62). However, our study successfully suggests a very safe and effective drug delivery method: straightforward oral administration of EPA-E is effective for neuroprotection at early-stage DR.

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H.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflict of interest

All authors declare no conflict of interest related to this project, and no external funding was received for the conduct of this study.

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<References>

1. Antonetti DA, Klein R, Gardner TW: Diabetic retinopathy. N Engl J Med 2012;366:1227-1239
2. Yonemura D, Aoki T, Tsuzuki K: Electroretinogram in diabetic retinopathy. Arch Ophthalmol-Chic 1962;68:19-24
3. Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW: Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. The Journal of clinical investigation 1998;102:783-791
4. Li Q, Zemel E, Miller B, Perlman I: Early retinal damage in experimental diabetes: electroretinographical and morphological observations. Experimental eye research 2002;74:615-625
5. Di Leo M, Falsini B, Caputo S, Ghirlanda G, Porciatti V, Greco A: Spatial frequency-selective losses with pattern electroretinogram in type 1 (insulin-dependent) diabetic patients without retinopathy. Diabetologia 1990;33:726-730
6. Holopigian K, Seiple W, Lorenzo M, Carr R: A comparison of photopic and scotopic electroretinographic changes in early diabetic retinopathy. Investigative ophthalmology & visual science 1992;33:2773-2780
7. Hammes H-P: Diabetic retinopathy: hyperglycaemia, oxidative stress and beyond. Diabetologia 2018;61:29-38
8. Okabe K, Kobayashi S, Yamada T, Kurihara T, Tai-Nagara I, Miyamoto T, Mukouyama Y-s, Sato TN, Suda T, Ema M: Neurons limit angiogenesis by titrating VEGF in retina. Cell 2014;159:584-596
9. Giardino I, Fard AK, Hatchell DL, Brownlee M: Aminoguanidine inhibits reactive oxygen species formation, lipid peroxidation, and oxidant-induced apoptosis. Diabetes 1998;47:1114-1120
10. Roy S, Kern TS, Song B, Stuebe C: Mechanistic insights into pathological changes in the diabetic retina: implications for targeting diabetic retinopathy. The American journal of pathology 2017;187:9-19
11. Willis JR, Doan QV, Gleeson M, Haskova Z, Ramulu P, Morse L, Cantrell RA: Vision-related functional burden of diabetic retinopathy across severity levels in the United States. JAMA ophthalmology 2017;135:926-932
12. Cusato K, Bosco A, Linden R, Reese BE: Cell death in the inner nuclear layer of the retina is modulated by BDNF. Developmental brain research 2002;139:325-330
13. Almeida R, Manadas B, Melo C, Gomes J, Mendes C, Graos M, Carvalho R, Carvalho A, Duarte C: Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. Cell death and differentiation 2005;12:1329
14. Seki M, Tanaka T, Nawa H, Usui T, Fukuchi T, Ikeda K, Abe H, Takei N: Involvement of brain-derived neurotrophic factor in early retinal neuropathy of streptozotocin-induced diabetes in rats: therapeutic potential of brain-derived neurotrophic factor for dopaminergic amacrine cells. Diabetes 2004;53:2412-2419
15. Lieth E, Barber AJ, Xu B, Dice C, Ratz MJ, Tanase D, Strother JM: Glial reactivity and impaired glutamate metabolism in short-term experimental diabetic retinopathy. Penn State Retina Research Group. Diabetes 1998;47:815-820
16. Ola MS, Nawaz MI, El-Asrar AA, Abouammoh M, Alhomida AS: Reduced levels of brain derived neurotrophic factor (BDNF) in the serum of diabetic retinopathy patients and in the retina of diabetic rats. Cellular and molecular neurobiology 2013;33:359-367
17. Abdel-Maksoud SM, Hassanein SI, Gohar NA, Attia SM, Gad MZ: Investigation of brain-

derived neurotrophic factor (BDNF) gene expression in hypothalamus of obese rats: Modulation by omega-3 fatty acids. Nutritional neuroscience 2017;20:443-448
18. Bak DH, Zhang E, Yi M-H, Kim D-K, Lim K, Kim J-J, Kim DW: High ω3-polyunsaturated fatty acids in fat-1 mice prevent streptozotocin-induced Purkinje cell degeneration through BDNF-mediated autophagy. Scientific reports 2015;5:15465
19. Bousquet M, Saint-Pierre M, Julien C, Salem Jr N, Cicchetti F, Calon F: Beneficial effects of dietary omega-3 polyunsaturated fatty acid on toxin-induced neuronal degeneration in an animal model of Parkinson's disease. The FASEB Journal 2008;22:1213-1225
20. Calon F, Lim GP, Yang F, Morihara T, Teter B, Ubeda O, Rostaing P, Triller A, Salem Jr N, Ashe KH, Frautschy SA, Cole GM: Docosahexaenoic acid protects from dendritic pathology in an Alzheimer's disease mouse model. Neuron 2004;43:633-645
21. Nagaya M, Ueno S, Kominami T, Nakanishi A, Koyasu T, Kondo M, Furukawa T, Terasaki H: Pikachurin protein required for increase of cone electroretinogram b-wave during light adaptation. PLoS One 2015;10:e0128921
22. Ohba M, Saeki K, Koga T, Okuno T, Kobayashi Y, Yokomizo T: Profiling of bioactive lipids in different dendritic cell subsets using an improved multiplex quantitative LC-MS/MS method. Biochemical and biophysical research communications 2018;504:562-568
23. Hirakata T, Lee H-C, Ohba M, Saeki K, Okuno T, Murakami A, Matsuda A, Yokomizo T: Dietary ω-3 fatty acids alter the lipid mediator profile and alleviate allergic conjunctivitis without modulating Th2 immune responses. The FASEB Journal 2018;33:3392-3403
24. Kaneko H, Ye F, Ijima R, Kachi S, Kato S, Nagaya M, Higuchi A, Terasaki H: Histamine receptor h4 as a new therapeutic target for choroidal neovascularization in age-related macular degeneration. Br J Pharmacol 2014;171:3754-3763
25. Ihnat M, Thorpe J, Kamat C, Szabo C, Green D, Warnke L, Lacza Z, Cselenyak A, Ross K, Shakir S: Reactive oxygen species mediate a cellular 'memory'of high glucose stress signalling. Diabetologia 2007;50:1523-1531
26. Miyake S, Kobayashi S, Tsubota K, Ozawa Y: Phase II enzyme induction by a carotenoid, lutein, in a PC12D neuronal cell line. Biochemical and biophysical research communications 2014;446:535-540
27. Kurihara T, Ozawa Y, Nagai N, Shinoda K, Noda K, Imamura Y, Tsubota K, Okano H, Oike Y, Ishida S: Angiotensin II type 1 receptor signaling contributes to synaptophysin degradation and neuronal dysfunction in the diabetic retina. Diabetes 2008;57:2191-2198
28. Ano Y, Ohya R, Kita M, Taniguchi Y, Kondo K: Theaflavins Improve Memory Impairment and Depression-Like Behavior by Regulating Microglial Activation. Molecules 2019;24:467
29. Liu MH, Yuan C, He J, Tan TP, Wu SJ, Fu HY, Liu J, Yu S, Chen YD, Le QF, Tian W, Hu HJ, Zhang Y, Lin XL: Resveratrol protects PC12 cells from high glucose-induced neurotoxicity via PI3K/Akt/FoxO3a pathway. Cell Mol Neurobiol. 2015;35(4):513-22
30. Hao M, Li Y, Lin W, Xu Q, Shao N, Zhang Y, Kuang H: Estrogen prevents high-glucose-induced damage of retinal ganglion cells via mitochondrial pathway. Graefes Arch Clin Exp Ophthalmol. 2015;253(1):83-90
31. Ansari S, Djalali M, Mohammadzadeh N, Mohammadzadeh Honarvar N, Mazaherioun M, Zarei M, Gholampour Z, Javanbakht M: Assessing the effect of omega-3 fatty acids supplementation on serum BDNF (brain derived neurotrophic factor) in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled study. Int Res J Appl Basic Sci 2016;10:380-383
32. Yuen N, Anderson SE, Glaser N, Tancredi DJ, O'Donnell ME: Cerebral blood flow and cerebral

edema in rats with diabetic ketoacidosis. Diabetes 2008;57:2588-2594
33. Behl T, Kotwani A: Omega-3 fatty acids in prevention of diabetic retinopathy. Journal of Pharmacy and Pharmacology 2017;69:946-954
34. Dong Y, Pu K, Duan W, Chen H, Chen L, Wang Y: Involvement of Akt/CREB signaling pathways in the protective effect of EPA against interleukin-1β-induced cytotoxicity and BDNF down-regulation in cultured rat hippocampal neurons. BMC neuroscience 2018;19:52
35. Connor KM, SanGiovanni JP, Lofqvist C, Aderman CM, Chen J, Higuchi A, Hong S, Pravda EA, Majchrzak S, Carper D: Increased dietary intake of ω-3-polyunsaturated fatty acids reduces pathological retinal angiogenesis. Nature medicine 2007;13:868
36. Biessels G-J, Cristino NA, Rutten G-J, Hamers FP, Erkelens DW, Gispen WH: Neurophysiological changes in the central and peripheral nervous system of streptozotocin-diabetic rats: course of development and effects of insulin treatment. Brain 1999;122:757-768
37. Sheikh-Ali M1, Karon BS, Basu A, Kudva YC, Muller LA, Xu J, Schwenk WF, Miles JM: Can serum beta-hydroxybutyrate be used to diagnose diabetic ketoacidosis? Diabetes Care 2008;31(4):643-7
38. Lo W, O'Donnell M, Tancredi D, Orgain M, Glaser N: Diabetic ketoacidosis in juvenile rats is associated with reactive gliosis and activation of microglia in the hippocampus. Pediatr Diabetes 2016;17(2):127-39
39. Glaser N, Anderson S, Leong W, Tancredi D, O'Donnell M: Cognitive dysfunction associated with diabetic ketoacidosis in rats. Neurosci Lett 2012;510(2):110-4
40. Glaser N, Ngo C, Anderson S, Yuen N, Trifu A, O'Donnell M: Effects of hyperglycemia and effects of ketosis on cerebral perfusion, cerebral water distribution, and cerebral metabolism. Diabetes 2012;61(7):1831-7
41. Serhan C. Pro-resolving lipid mediators are leads for resolution physiology. Nature. nature; 2014;510(7503):92–101
42. Endo J, Sano M, Isobe Y, Fukuda K, Kang JX, Arai H, Arita M: 18-HEPE, an n-3 fatty acid metabolite released by macrophages, prevents pressure overload–induced maladaptive cardiac remodeling. Journal of Experimental Medicine 2014;211:1673-1687
43. Sapieha P, Chen J, Stahl A, Seaward M, Favazza T, Juan A, Hatton C, Joyal J, Krah N, Dennison RJ: Omega-3 polyunsaturated fatty acids preserve retinal function in type 2 diabetic mice. Nutrition & diabetes 2012;2:e36
44. Ibrahim AS, Elshafey S, Sellak H, Hussein KA, El-Sherbiny M, Abdelsaid M, Rizk N, Beasley S, Tawfik AM, Smith SB: A lipidomic screen of hyperglycemia-treated HRECs links 12/15-Lipoxygenase to microvascular dysfunction during diabetic retinopathy via NADPH oxidase. Journal of lipid research 2015;56:599-611
45. Othman A, Ahmad S, Megyerdi S, Mussell R, Choksi K, Maddipati KR, Elmarakby A, Rizk N, Al-Shabrawey M: 12/15-Lipoxygenase-derived lipid metabolites induce retinal endothelial cell barrier dysfunction: contribution of NADPH oxidase. PloS one 2013;8:e57254
46. Simopoulos AP: The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomedicine & pharmacotherapy 2002;56:365-379
47. Simopoulos AP: The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Experimental biology and medicine 2008;233:674-688
48. Sakai H, Tani Y, Shirasawa E, Shirao Y, Kawasaki K: Development of Electroretinographic Alterations in Streptozotocin-lnduced Diabetes in Rats. Ophthalmic research 1995;27:57-63
49. Neschen S, Morino K, Dong J, Wang-Fischer Y, Cline GW, Romanelli AJ, Rossbacher JC, Moore IK, Regittnig W, Munoz DS: n-3 Fatty acids preserve insulin sensitivity in vivo in a

peroxisome proliferator–activated receptor-α–dependent manner. Diabetes 2007;56:1034-1041
50. Roy MS, Janal MN: High caloric and sodium intakes as risk factors for progression of retinopathy in type 1 diabetes mellitus. Arch Ophthalmol-Chic 2010;128:33-39
51. Baumeier C, Kaiser D, Heeren J, Scheja L, John C, Weise C, Eravci M, Lagerpusch M, Schulze G, Joost H-G: Caloric restriction and intermittent fasting alter hepatic lipid droplet proteome and diacylglycerol species and prevent diabetes in NZO mice. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 2015;1851:566-576
52. Kaviarasan K, Jithu M, Mulla MA, Sharma T, Sivasankar S, Das UN, Angayarkanni N: Low blood and vitreal BDNF, LXA4 and altered Th1/Th2 cytokine balance are potential risk factors for diabetic retinopathy. Metabolism 2015;64:958-966
53. El-Asrar A, Ahmed M, Nawaz MI, Siddiquei MM, Al-Kharashi AS, Kangave D, Mohammad G: High-mobility group box-1 induces decreased brain-derived neurotrophic factor-mediated neuroprotection in the diabetic retina. Mediators of inflammation 2013;2013
54. El-Asrar AMA, Mohammad G, De Hertogh G, Nawaz MI, Van Den Eynde K, Siddiquei MM, Struyf S, Opdenakker G, Geboes K: Neurotrophins and neurotrophin receptors in proliferative diabetic retinopathy. PLoS One 2013;8:e65472
55. Busik JV, Mohr S, Grant MB: Hyperglycemia-induced reactive oxygen species toxicity to endothelial cells is dependent on paracrine mediators. Diabetes 2008;57(7):1952-65.
56. Aranda J, Motiejunaite R, Im E, Kazlauskas A: Diabetes disrupts the response of retinal endothelial cells to the angiomodulator lysophosphatidic acid. Diabetes 2012;61(5):1225-33
57. Xie L, Zhu X, Hu Y, Li T, Gao Y, Shi Y, Tang S: Mitochondrial DNA oxidative damage triggering mitochondrial dysfunction and apoptosis in high glucose-induced HRECs. Invest Ophthalmol Vis Sci 2008;49(9):4203-9
58. Safi SZ, Qvist R, Yan GO, Ismail IS: Differential expression and role of hyperglycemia induced oxidative stress in epigenetic regulation of β1, β2 and β3-adrenergic receptors in retinal endothelial cells. BMC Med Genomics 2014;7:29
59. Lee YJ, Kim M, Lee JY, Jung SH, Jeon HY, Lee SA, Kang S, Han ET, Park WS, Hong SH, Kim YM, Ha KS: The benzodiazepine anesthetic midazolam prevents hyperglycemia-induced microvascular leakage in the retinas of diabetic mice. FASEB J 2018:fj201800014RR
60. Coughlin BA, Feenstra DJ, Mohr S: Müller cells and diabetic retinopathy. Vision Res 2017;139:93-100
61. Sasaki M, Ozawa Y, Kurihara T, Kubota S, Yuki K, Noda K, Kobayashi S, Ishida S, Tsubota K: Neurodegenerative influence of oxidative stress in the retina of a murine model of diabetes. Diabetologia 2010;53:971-979
62. Abe T, Wakusawa R, Seto H, Asai N, Saito T, Nishida K: Topical doxycycline can induce expression of BDNF in transduced retinal pigment epithelial cells transplanted into the subretinal space. Invest Ophthalmol Vis Sci 2008;49:3631-3639

Tables

Table 1. Changes in body weight, blood sugar, and β-Hydroxybutyrate levels

	2 weeks			8 weeks		
Group name	Control	STZ	EPA	Control	STZ	EPA
STZ administration	(-)	(+)	(+)	(-)	(+)	(+)
Diet	Sunflower oil		EPA ethyl ester	Sunflower oil		EPA ethyl ester
Number of samples	6	6	5	4	6	6
Body weight (g) before study	187 ± 5.5	197 ± 3.7	182 ± 11.7	192 ± 17.0	201 ± 15.6	185 ± 15.1
Body weight (g) after study	216 ± 8.1	181 ± 6.1*	186 ± 17.7	323 ± 11.9	217 ± 15.9*	216 ± 26.4*
Blood glucose (mg/dL)	125 ± 19.5	404 ± 29.8*	422 ± 30.4*	158 ± 51.7	401 ± 39.2*	420 ± 37.8*
β-Hydroxybutyrate (mM)	0.23 ± 0.044	0.51 ± 0.12*	0.36 ± 0.19	0.24 ± 0.029	2.06 ± 0.17*	1.78 ± 0.29*

STZ = streptozocin, EPA = Eicosapentaenoic acid. Values are mean ± SD. *P-value was < 0.05 compared to those with control.

Figures legends

Figure 1. ROS reduced bioactivity in Müller glia and retinal neuronal cells, and BDNF protected neuronal cells from oxidative stress

A, B: The levels of 8-OHdG and H_2O_2 were significantly elevated in the human vitreous samples from the eyes with DR compared to control. ***C***: The exposure to 25 mM glucose with VEGF for 72 hours significantly increased the extracellular concentration of H_2O_2 from HREC compared with 5.6 mM glucose. ***D***: The exposure to 25 mM glucose for 72 hours significantly suppressed the extracellular concentration of H_2O_2 from MIO-M1 compared with 5.6 mM glucose. ***E***: The exposure to 25 mM glucose for 72 hours significantly elevated the cell viability of MIO-M1 compared with 5.6 mM glucose. ***F***: H_2O_2 exposure for 24 hours significantly reduced the cell viability of MIO-M1. ***G***: *BDNF* mRNA level in MIO-M1 was significantly decreased by H_2O_2 exposure for 24 hours. ***H***: BDNF protein level in the culture media of MIO-M1 was significantly decreased by H_2O_2 exposure for 72 hours. ***I***: H_2O_2 decreased PC12D cell viability in a dose-dependent manner. ***J***: The administration of recombinant BDNF dose-dependently inhibited the reduction of PC12D cell viability by H_2O_2. ***K***: The administration of recombinant BDNF statistically ameliorated the decrease rate of the relative change in the neurite length per cell body cluster from the beginning in PC12D cells time-dependently (3 hours: n = 18; 24 hours: n = 16, respectively). ***L***: Representative images of PC12D cells with or without the BDNF treatment. Values are mean ± SD. Scale bar in (***L***) = 50 μm.

Figure 2. EPA-E oral administration protected retinal function of diabetic rats

A: Schedule of the *in vivo* study in STZ-induced diabetic rats with EPA-E oral administration. ***B***: The OPs of ERG in the STZ group were significantly lower than those in the control group 4 weeks after STZ induction. ***C***: The b/a-wave ratio of ERG in the STZ group was significantly lower than that in the control group 8 weeks after STZ induction, but there was no significant difference between the STZ and the EPA group. ***D***: The OPs of ERG in the STZ group were significantly lower than those in the control and the EPA groups 8 weeks after STZ induction, and EPA-E treatment for the same period statistically improved the OPs of diabetic rats. ***E***: Representative images of ERG in the three groups. ***F*:** Colocalization of BDNF and GS, a marker for Müller cells, immunoreactivity in the control rat retina. GS was used as a marker for Müller cell. GS-positive cells were vertically located from GCL to IPL, and co-localized with BDNF-positive cells. ***G***: 8-OHdG showed a significant increase in the STZ group compared with that in the control group, but it significantly decreased in the EPA group. ***H, I***: The *Bdnf* mRNA level and the relative BDNF protein level in the retina of the STZ group was significantly lower than that of the control group, but that of the EPA group was significantly higher than that of the STZ group. Values are mean ± SD. Control group = rats with no STZ, no EPA-E treatment; STZ group = rats with STZ, but no EPA-E treatment; EPA group = rats with STZ and EPA-E treatment; GS = glutamine synthetase; RGC = retinal ganglion cell; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer. Scale bar in (***F***) = 50 μm.

Figure 3. Quantification of lipid mediators in rat retina

The levels of 18-HEPE, 15-HEPE, 12-HEPE and 5-HEPE in the rat retina of the EPA group was significantly higher than that of the control and the STZ groups. In contrast, 12-HETE and 11-HETE values in the rat retina of the EPA group were significantly lower than those of the control and the STZ groups. The raw data in each subject is shown in Supplementary Table S2. Control = rats with no STZ and EPA-E treatments, *n*=7; STZ = rats with STZ but no EPA-E treatment, *n*=7; EPA = rats with STZ and EPA-E treatments, *n*=8. 18HEPE = 18-hydroxyeicosapentaenoic acid; HETE = hydroxyeicosatetraenoic acid. Values are mean ± SD.

Figure 4. 18-HEPE induced BDNF in Müller glia cells and rescued neuronal retinal cell bioactivity

A, B: The relative *BDNF* mRNA and BDNF protein level in the culture media of MIO-M1 treated with H_2O_2 was increased by 2 μM 18-HEPE, but not by 0.5 μM 18-HEPE. ***C***: 2 μM 15-HEPE, 12-HEPE, and 5-HEPE did not improve the decreased *BDNF* mRNA level in MIO-M1 treated with H_2O_2. ***D***: 2μM 15-HETE and 12-HETE reduced the relative *BDNF* mRNA level in MIO-M1 cells even without oxidative stress. ***E, F, G***: The H_2O_2 exposure reduced the cell viability of PC12D, HREC, and hRPE cells, but 18-HEPE did not inhibit these reductions. ***H***: Schedule of the *in vivo* study in streptozocin (STZ)-induced diabetic rats with 18-HEPE intravitreal injection. ***I***: Representative ERG wave of STZ rat eyes with 18-HEPE intravitreal injection and those with PBS (Sham). ***J***: OPs of STZ rat eyes with 18-HEPE intravitreal injection were statistically higher than those with PBS. ***K***: The *Bdnf* mRNA level in the retina of the eyes with 18-HEPE was statistically higher than those with PBS. Values are mean ± SD.

Figure 1. ROS reduced bioactivity in Müller glia and retinal neuronal cells, and BDNF protected neuronal cells from oxidative stress

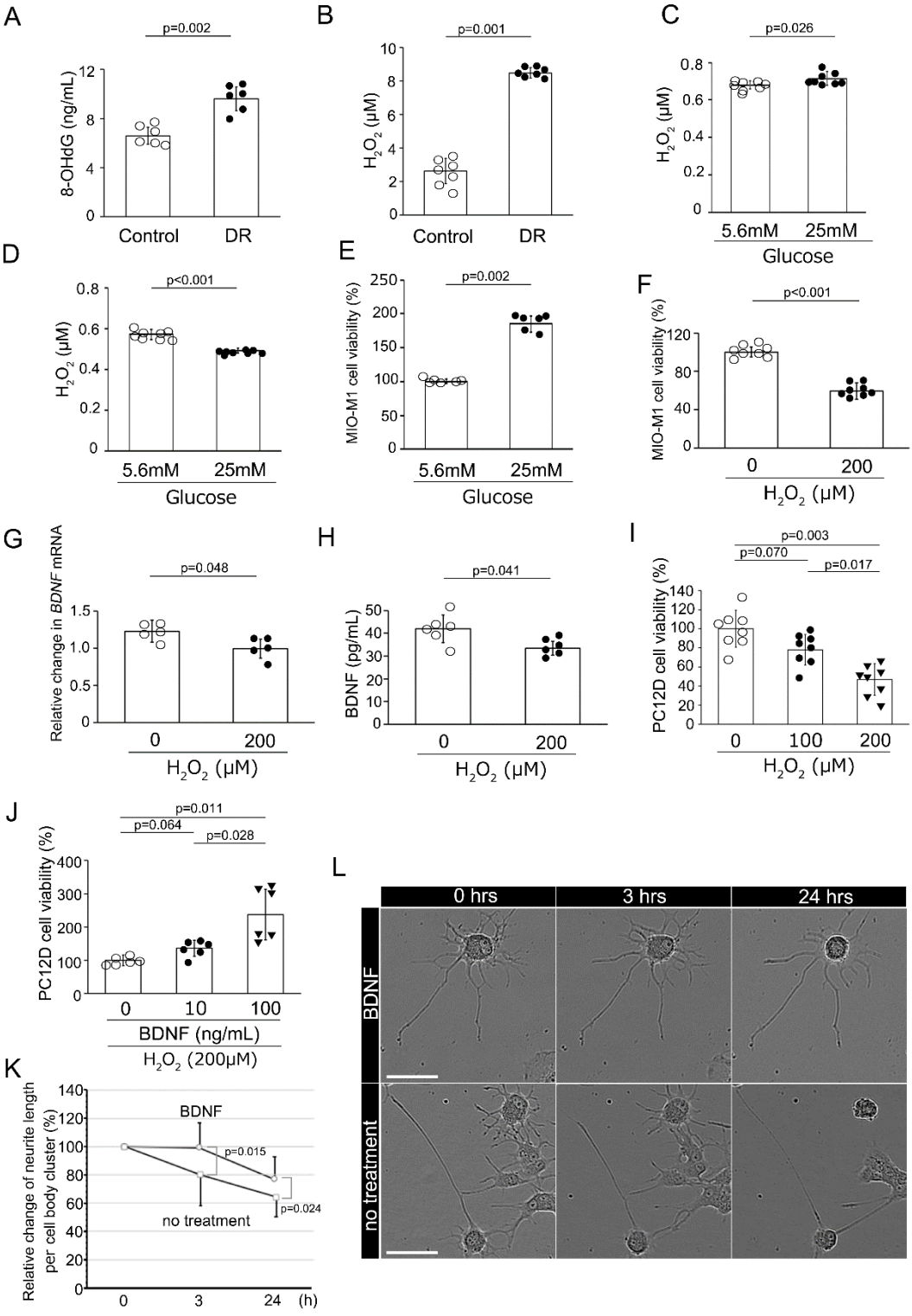

Figure 2. EPA-E oral administration protected retinal function of diabetic rats

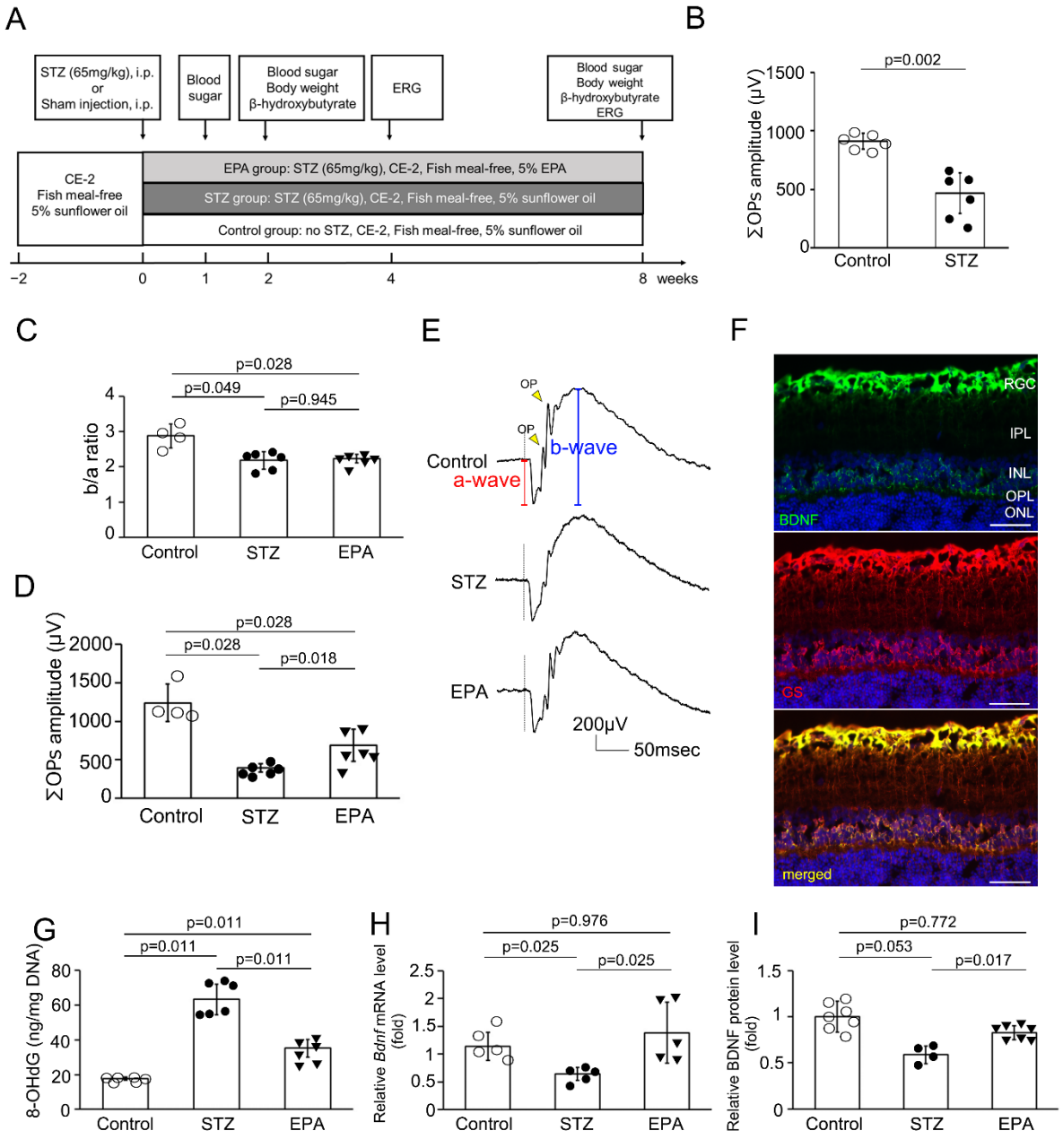

Figure 3. Quantification of lipid mediators in rat retina

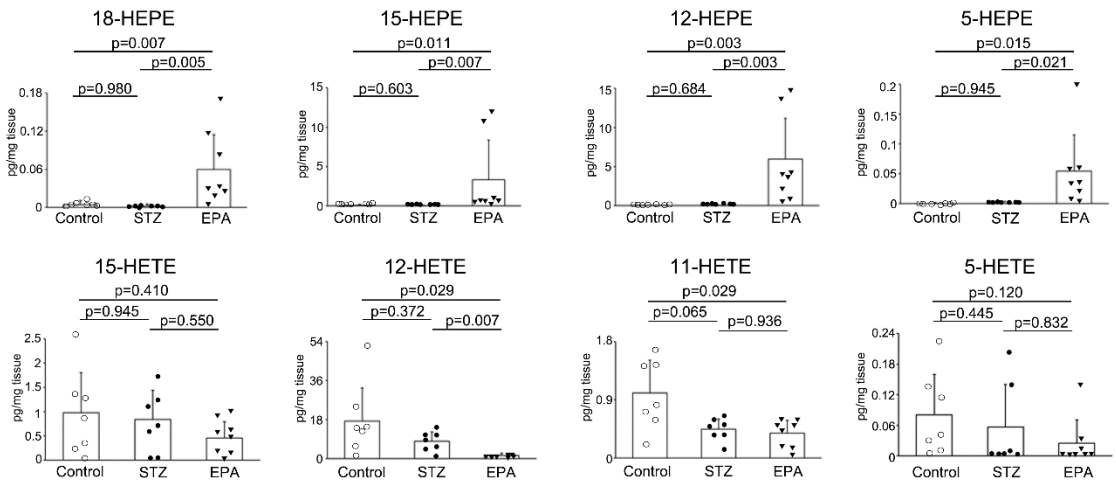

Figure 4. 18-HEPE induced BDNF in Müller glia cells and rescued neuronal retinal cell bioactivity