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RESEARCH ARTICLES

Docosahexaenoic Acid Protects against High Glucose-Induced Oxidative Stress in Human Retinal Pigment Epithelial Cells

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ABSTRACT | Diabetic retinopathy is a leading cause of vision loss and has been correlated with increased oxidative stress. The aim of the present study was to evaluate the protective properties of docosahexaenoic acid (DHA), an omega-3 fatty acid, in human retinal pigment epithelial (RPE) cells exposed to high glucose. Human RPE cell line (ARPE-19) was cultured for 4 days followed by 5 days of exposure to either a normal (5.5 mM) or a high (45 mM) D-glucose concentration in the absence and presence of DHA (100 µM). Reduced form of glutathione (GSH), total antioxidant capacity (TAC), total nitrites, and malodialdehyde (MDA) were assessed. 4-Hydroxynonenal (HNE), another lipid peroxidation product, was determined by immunocytochemistry. Cell viability was assessed by the MTT assay. The results showed that both TAC and GSH content were significantly decreased after the high glucose challenge. The presence of DHA prevented the reduction and maintained the TAC and GSH at the levels found in cells exposed to the normal glucose concentration (5.5 mM). Moreover, the levels of total nitrites and MDA were significantly increased after high glucose exposure compared to cell exposed to 5.5 mM glucose. Again, the presence of DHA prevented the increase and maintained the nitrites and MDA at the levels found in control cells. Notably, the high glucose condition led to a significantly increased number of HNE aggresomes as compared to control cells, and DHA completely prevented this increase. In line with the reduced oxidative stress, DHA treatment also completely prevented the high glucose-induced decrease in RPE cell viability. Taken together, this study demonstrated that DHA protected human retinal pigment epithelial ARPE-19 cells from high glucose-induced oxidative damage and cytotoxicity. The results of this study support a role for oxidative stress in high glucose-induced RPE injury and the potential use of omega-3 fatty acids to protect against diabetic retinopathy.

KEYWORDS | Diabetic retinopathy; Glutathione; High glucose; 4-Hydroxynonenal; Malodialdehyde; Oxidative stress; Nitrites; Retinal pigment epithelial cell

ABBREVIATIONS | CDNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DHA, docosahexaenoic acid; GSH, reduced glutathione; HNE, 4-hydroxynonenal; HO-1, heme oxygenase-1; iNOS, inducible NO synthase; MDA, malondialdehyde; MTT, 3-[4,5-dimethylthiazolyl-2-]-2,5-diphenyltetrazolium bromide; NO, nitric oxide; Nrf2, nuclear factor E2-related factor 2; ROS, reactive oxygen species; RPE, retinal pigment epithelium; TAC, total antioxidant capacity

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CONTENTS

1. INTRODUCTION

High circulating glucose levels are a typical feature of diabetes mellitus. This condition leads to metabolic abnormalities finally inducing, in many ways, the production of reactive oxygen species (ROS). Oxidative stress typically involves the formation of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), as well as peroxynitrite ($ONOO^-$) that occurs as a result of the reaction between nitric oxide (NO) and $O_2^{\bullet-}$. Increased lipid peroxidation and augmented decay of antioxidant enzymatic activities are commonly observed under hyperglycemic conditions. In this context, decreased content of reduced glutathione (GSH) and increased levels of malondialdehyde (MDA) or 4-hydroxynonenal (HNE) are frequently used to monitor oxidative stress-related cellular damage under various pathophysiological conditions [1–3].

Diabetic retinopathy is a common diabetic complication involving retinal injury and finally vision impairment. Previous studies demonstrated a close relationship between diabetic retinopathy and oxidative stress [4]. Vascular endothelial cells are most likely the cellular targets primarily affected by hyperglycemia. In fact, a positive correlation between peroxynitrite and retinal endothelial cell death with concomitant alteration of the retinal-blood barrier has been demonstrated in a number of studies [5–7].

Due to its anatomical location and function, retinal pigment epithelium (RPE) is in contact with choroid vessels, serving as part of the blood-retinal barrier. Indeed, hyperglycemia in type 1 and type 2 diabetes has been reported to be associated with RPE dysfunction [8, 9]. In view of the pivotal role of RPE in retinal homeostasis, it seems plausible that hyperglycemia-induced RPE oxidative stress may play a significant role in diabetic retinopathy and the development of vision impairment.

Docosahexaenoic acid (DHA) is an essential omega-3 fatty acid present in cellular membranes and can be found in the brain and retina [10]. DHA accumulation in the eye correlates with synaptogenesis, dendrite formation, and photoreceptor biogenesis during postnatal development, and is also involved in neuroprotection [10, 11]. DHA supplementation has also been reported to be beneficial in many systemic diseases as well as cancer [12–14]. However, little is known about the possible beneficial effects of DHA on the function and integrity of RPE under hyperglycemic conditions.

The present study was aimed to investigate the protective effects of DHA on oxidative stress injury in a human retinal pigment epithelial cell line (ARPE-19) under a high glucose condition and explore the potential value of using this omega-3 fatty acid in the intervention of diabetic retinopathy.

2. MATERIAL AND METHODS

2.1. Cell Culture

The human retinal pigment epithelial ARPE-19 cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in DMEM/F-12 containing 10% fetal bovine serum, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 2 mM L-glutamine. The medium was changed every 2 days and all studies were conducted by using confluent cells of passages 20–40 following 12-hr quiescence in serum-free medium. ARPE-19 cells were incubated with 5.5 or 45 mM D-glucose for 5 days. Two or three independent experiments were performed, giving an n = 8 experiments for cell viability and an n = 6 for immunocytochemistry, intracellular oxidative stress markers, total antioxidant capacity, and GSH determinations.

2.2. Assay for Cell Viability

ARPE-19 cells were grown to confluence in 96-well plates, and at the end of the experiment, the media were removed, and the cells were washed with ice-cold PBS three times. Cells were incubated with 3-[4,5-dimethylthiazolyl-2-]-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/ml in PBS) for 4 h at 37 ºC to determine cell viability using a Cell Proliferation Kit I from Roche Life Science (Indianapolis, IN, USA). Viable cells reduce MTT to purple formazan crystals which can be solubilized using the solubilisation solution provided in the kit. The absorbance of this formazan solution was measured spectrophotometrically at a dual wavelength of 550/650 nm using a micro-plate reader from Perkin Elmer (Waltham, MA, USA).

2.3. Assay for Total Antioxidant Capacity

The total antioxidant capacity (TAC) was measured with an Antioxidant Assay Kit from Cayman (Ann Arbor, MI, USA) that allows the measurement of the TAC of the samples. The assay relies on the ability of the antioxidants in the sample to inhibit the oxidation of ABTS (3-etilbenzothiazolin 6-sulfonic acid) to $ABTS^{\bullet+}$ by metmyoglobin. The amount of $ABTS^{\bullet+}$ can be monitored spectrophotometrically by reading the absorbance at 405 nm, which is proportional to its concentration.

2.4. Assay for Total Nitrites

Total nitrites were assessed by a commercial kit from R&D Systems (Minneapolis, IN, USA). The principle of this assay is the determination of nitric oxide concentrations based on the enzymatic conversion of nitrate to nitrite. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Griess reaction. The amount of total nitrites can be monitored by reading the absorbance at 540 nm, which is proportional to the nitric oxide concentration.

2.5. Assay for Lipid Peroxidation

The MDA level was measured using a Lipid Peroxidation Microplate Assay Kit from Oxford Biomedical Research (Rochester Hills, MI, USA) following the manufacturer's instructions. This assay is based on the reaction of two molecules of a chromogenic reagent, *N*-methyl-2-phenylindole, with one molecule of MDA, at 45^{o}C, to yield a stable chromophore with a maximal absorbance at 586 nm. The amount of MDA can be monitored spectrophotometrically by reading the absorbance at 586 nm, which is proportional to its concentration.

2.6. Assay for Glutathione Content

An NWLSS Glutathione Assay kit from Northwest Life Science (Vancouver, Washington, USA) was used to measure the content of total cellular GSH following the manufacturer´s instructions. This assay kit is a modification of the method first described by Tietze [15]. The general thiol reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, also known as Ellman's reagent) reacts with GSH to form 5-thionitrobenzoic acid (TNB) and GS-TNB. GS-TNB is a chromophore that absorbs light maximally at 412 nm. The amount of cellular GSH was estimated by measuring the formation of GS-TNB using a microplate reader at 405 nm.

2.7. Assay for HNE Immunocytochemistry

ARPE-19 cells were prepared for immunocytochemical staining by fixation in 4% fresh formaldehyde. Primary HNE antibody (rabbit HNE11-S, Alpha Diagnostic International, San Antonio, TX, USA), 1:200 in PBS containing 0.3% Triton X-100, was

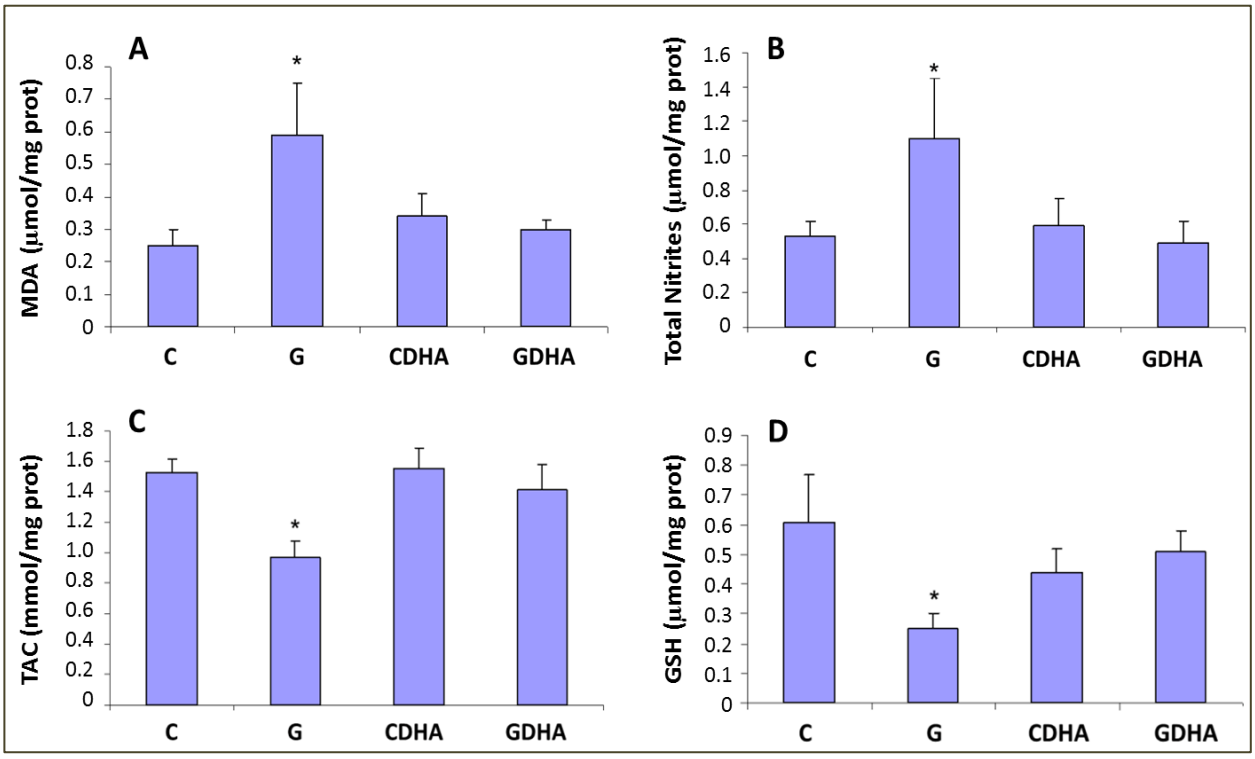

FIGURE 1. High glucose-induced oxidative stress and the protective effects of DHA in human retinal pigment epithelial ARPE-19 cells. Cells were cultured with 5.5 mM or 45 mM glucose in the absence and presence of 100 µM DHA for 5 days followed by measurements of the cellular levels of MDA (panel A), total nitrites (panel B), total antioxidant capacity (TAC) (panel C), and GSH (panel D) according to the procedures described in the Materials and Methods section. Data represent mean ± SD from 6 samples per group and the experiments were repeated twice (*$p < 0.05$ versus the rest of the groups). C, G, CDHA, and GDHA denote control (5.5 mM glucose), high glucose (45 mM), control plus DHA (100 µM), and high glucose plus DHA (100 µM), respectively.

incubated for 1 hr. After this incubation, the samples were rinsed and incubated in darkness with secondary anti-rabbit Alexa Fluor 488 for 1 hr (1:2000 in PBS, Molecular Probes, Invitrogen, Carlsbad, CA, USA). Confocal images were obtained with Ar-Kr 488-nm laser and voxel resolution of 1.5 × 1.5 × 3 µm. For all confocal scans, 1024 × 1024 and 8 bit intensity resolution were used.

2.8. Statistical Analysis

Data are expressed as means ± SD. Comparisons between groups were done using 1- and 2-way ANOVA, and Student's two tailed unpaired *t* test. Statistical differences were set at $p < 0.05$.

3. RESULTS

3.1. Lipid Peroxidation

ROS-mediated peroxidation of polyunsaturated fatty acids in biomembranes is a classical manifestation of oxidative stress injury. MDA is a major reactive aldehyde produced during lipid peroxidation. Exposure to the high glucose concentration (45 mM) resulted in a significant increase in the MDA level (more than two fold) in ARPE-19 cells, compared to the normal glucose concentration (5.5 mM) (**Figure 1A**). Notably, the presence of DHA (100 µM) completely prevented the high glucose-induced elevation of MDA level (**Figure 1A**).

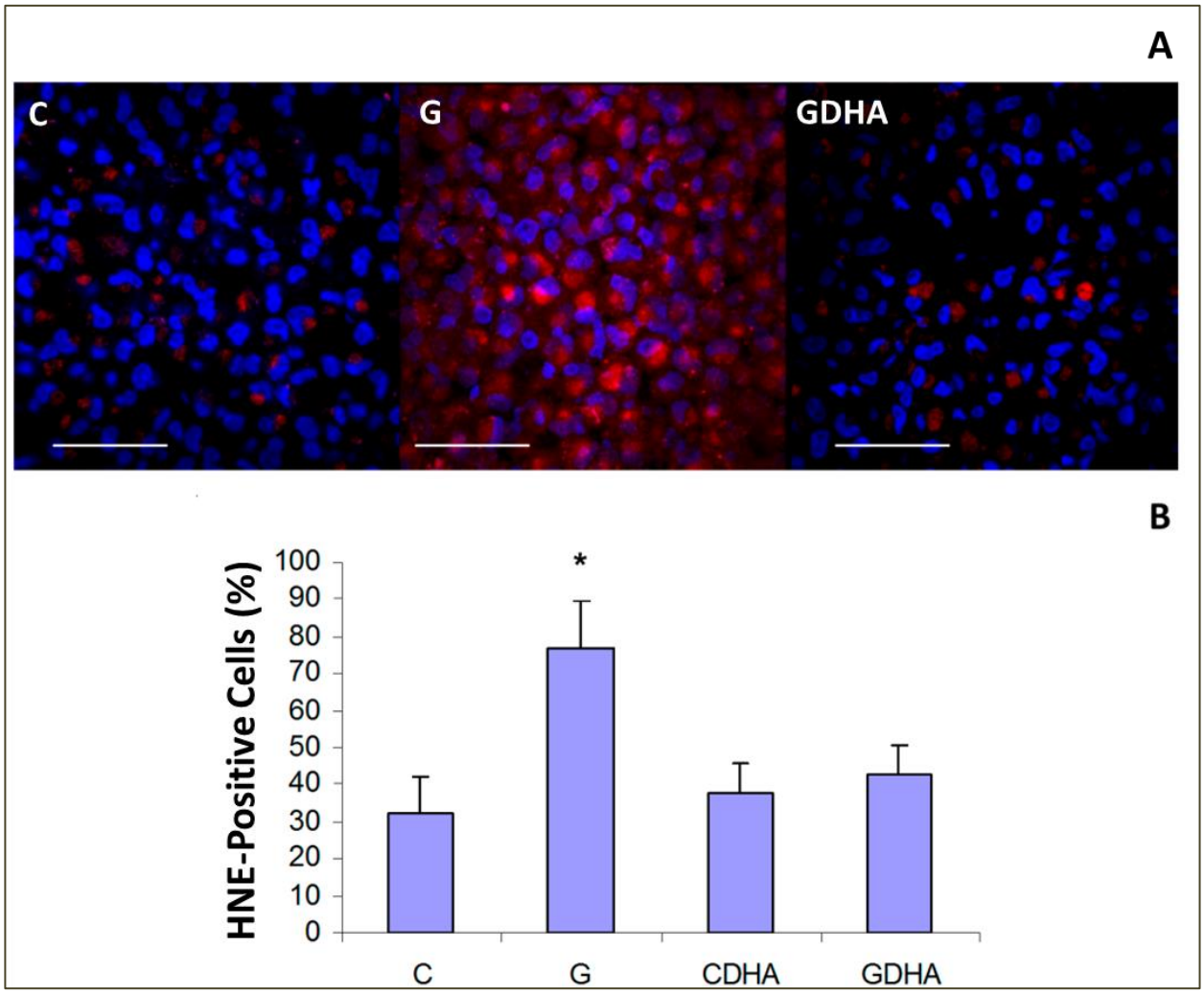

FIGURE 2. High glucose-induced HNE aggregates and the protective effects of DHA in human retinal pigment epithelial ARPE-19 cells. Cells were cultured with 5.5 mM or 45 mM glucose in the absence and presence of 100 µM DHA for 5 days followed by detection of the HNE aggregates immunocytochemically according to the procedures described in the Materials and Methods section. The representative confocal images and the quantitative data are shown in panels A and B, respectively. In panel B, data represent mean ± SD from 3 samples per group and the experiments were repeated twice (*$p < 0.05$ versus the rest of the groups). C, G, CDHA, and GDHA denote control (5.5 mM glucose), high glucose (45 mM), control plus DHA (100 µM), and high glucose plus DHA (100 µM), respectively. In panel A, bar = 100 µm.

3.2. Total Nitrites

The level of total nitrites is considered as a biomarker of NO activity. The level of total nitrites was significantly increased in the high glucose exposed cells (1.10 ± 0.35 µmol/mg protein) as compared to control cells (0.53 ± 0.08 µmol/mg protein). In line with the effect on MDA level, the presence of DHA also completely prevented the high glucose-induced increase in the level of total nitrites (0.49 ± 0.13 µmol/mg protein; $p < 0.05$ compared with high glucose alone) (**Figure 1B**).

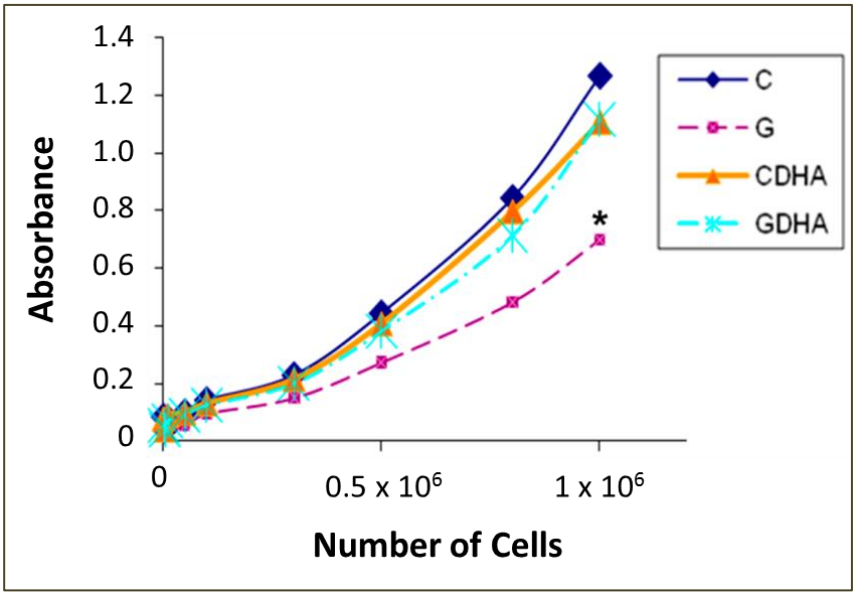

FIGURE 3. High glucose-induced reduction of cell viability and the protective effects of DHA in human retinal pigment epithelial ARPE-19 cells. Cells were cultured with 5.5 mM or 45 mM glucose in the absence and presence of 100 µM DHA for 5 days followed by detection of cell viability with the MTT assay according to the procedures described in the Materials and methods section. Viable cells that reduce MTT to purple formazan crystals as monitored spectrophotometrically at 550/650 nm were represented by the absorbance in the presence of different total numbers of cells (*$p < 0.05$ versus the rest of the groups). C, G, CDHA, and GDHA denote control (5.5 mM glucose), high glucose (45 mM), control plus DHA (100 µM), and high glucose plus DHA (100 µM), respectively.

3.3. TAC

The TAC of ARPE-19 cells exposed to the high glucose concentration was significantly decreased (0.97 ± 0.10 mmol/mg protein) as compared to that in control cells (1.52 ± 0.09 mmol/mg protein; $p < 0.05$ versus control) (**Figure 1C**). The presence of DHA prevented the high glucose exposure-induced decrease in TAC (1.41 ± 0.16 mmol/mg protein; $p < 0.05$ compared with high glucose alone).

3.4. Glutathione

Exposure to the high glucose concentration (45 mM) also significantly decreased GSH content in ARPE-19 cells (0.25 ± 0.05 µmol/mg protein) as compared to the control group (0.61 ± 0.16 µmol/mg protein; $p < 0.05$) (**Figure 1D**). Again, the presence of DHA prevented the decrease in GSH in the high glucose cultured cells. The GSH content was unaffected by DHA in the control group (0.51 ± 0.07 µmol/mg protein).

3.5. HNE Immunocytochemistry

HNE is a typical end-product of lipid peroxidation, which has been widely used as a marker for cellular oxidative stress. HNE immunocytochemistry revealed dense HNE-positive aggregates apparently close to the nucleus in control cells (**Figure 2A**), suggesting a basal level of oxidative stress and lipid peroxidation in ARPE-19 cells cultured in the presence of a physiological concentration of glucose. In line with the increased level of MDA, a classical end product of lipid peroxidation, the high glucose concentration caused a significant increase in the number of HNE-positive aggregates in ARPE-19 cells ($p < 0.05$). The presence of DHA significantly de-

creased the number of HNE-positive cell aggregates in high glucose-treated cells ($p < 0.05$) (**Figure 2**).

3.6. Cell Viability

The MTT assay was performed to determine the changes in cell viability following exposure to the high concentration of glucose. As expected, exposure of ARPE-19 cells to 45 mM glucose resulted in a significant reduction in cell viability. Consistent with its protective effects on high glucose-induced cellular oxidative stress, the presence of DHA significantly prevented the high glucose-elicited decrease in cell viability (**Figure 3**).

4. DISCUSSION

Oxidative stress plays a critical role in retinal degeneration under hyperglycemic conditions [4]. In line with this notion, antioxidant compounds have been studies with regard to their ability to inhibit retinal degeneration and diabetic retinopathy [16]. However, effective antioxidant therapies remain to be developed, which can be used clinically to protect against vision impairment in diabetic patients. Accordingly, in this study, we have investigated the protective effects of DHA, an omega-3 fatty acid with antioxidant properties, on high glucose-induced oxidative stress in retinal pigment epithelial cells, an important target of hyperglycemia-induced retinal degeneration.

Our results demonstrated that the levels of lipid peroxidation products and total nitrites were significantly increased, and on the other hand, cellular TAC and GSH content were markedly decreased in high glucose-treated human retinal pigment epithelial ARPE-19 cells. In line with the increased oxidative stress markers, cell viability was decreased following high glucose exposure. Importantly, we showed that DHA treatment normalized the above oxidative stress makers and prevented the decrease in cell viability caused by the high glucose exposure.

Total nitrites are considered as a fingerprint of NO activity, and in this regard, the significantly elevated level of total nitrites could be due to the increased activity of inducible NO synthase (iNOS) and the consequently augmented formation of NO in ARPE-19 cells following high glucose exposure. Increased formation of NO seems to be related to cell damage under hyperglycemic conditions [17]. In line with the increased MDA level, HNE-positive aggregates were also elevated upon high glucose exposure. The HNE-positive aggregates observed in the present study were similar to those found in our previous studies with ARPE-19 cells after ethanol exposure, and in these studies, HNE positive aggregates were identified as aggresomes and this process was proposed to involve both mitochondrial alterations and autophagy responses [18–20]. In this regard, we proposed that diabetes and ethanol exposure might share similar pathophysiological mechanisms related to oxidative stress [21]. It is known that reactive aldehydes can bind to proteins and other molecules by covalent modifications. In fact, reactive aldehydes, especially HNE, have been suggested to play a critical role in the pathophysiology of diabetes [22, 23].

DHA shows cytoprotective properties and is widely used as an antioxidant under various conditions. Previous studies from our laboratory demonstrated a protective role for DHA in diabetic conditions in rat brain and retina [24, 25]. DHA is typically present in the eye, protecting retina from oxidative damage and also preventing diabetes-related rod photoreceptor dysfunction [26, 27]. Notably, the content of DHA in both retina and RPE was decreased in diabetes [26]. DHA seemed to exert its protective effects by different ways, including serving passively as part of cell membranes and impacting transcription of redox-sensitive genes [10]. Indeed, DHA inhibited NO-iNOS production and the transcription of other pro-inflammatory genes [28–30]. The anti-inflammatory and antioxidative action of DHA seemed to occur through a mechanism that involves the activation of nuclear factor E2-related factor 2 (Nrf-2) and the up-regulation of heme oxygenase-1 (HO-1) [31]. Interestingly, 4-hydroxy hexenal derived from omega-3 fatty acids was found to be responsible for the activation of Nrf-2 and HO-1 [32, 33]. Hence, in the present study, the DHA-mediated protection against high glucose-induced oxidative stress injury in human retinal pigment epithelial cells may involve multifactorial complex mechanisms.

In summary, our study demonstrated that DHA effectively protected human retinal pigment epithelial ARPE-19 cells from high glucose-induced oxidative stress, including formation of HNE aggregates. The cytoprotective effects of HNE appeared to result from its potential anti-inflammatory and antioxidative properties, which might make DHA a promising agent for the intervention in diabetic retinopathy.

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