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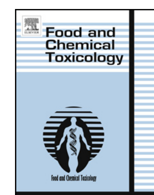

Photooxidative damage in retinal pigment epithelial cells via GRP78 and the protective role of grape skin polyphenols

Zhao Zhao [a,1], Tao Sun [a,1], Yun Jiang [a], Lijiang Wu [a], Xiangzhong Cai [a], Xiaodong Sun [b,c,*], Xiangjun Sun [a,*]

[a] *College of Agriculture and Biology, Shanghai Jiaotong University, Shanghai, China*
[b] *Department of Ophthalmology, Shanghai First People's Hospital, School of Medicine, Shanghai JiaoTong University, Shanghai, China*
[c] *Eye Research Institute, Shanghai JiaoTong University, Shanghai, China*

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Blue light induced oxidative damage and ER stress are related to the pathogenesis of age-related macular degeneration (AMD). However, the mechanism of blue light-induced damage remained obscure. The objective of this work is to assess the photooxidative damage to retinal pigment epithelial cells (RPE) and oxidation-induced changes in expression of ER stress associated apoptotic proteins, and investigate the mechanism underlying the protective effects of grape skin extracts. To mimic lipofuscin-mediated photooxidation in vivo, ARPE-19 cells that accumulated A2E, one of lipofuscin fluorophores, were used as a model system to investigate the mechanism of photooxidative damage and the protective effects of grape skin polyphenols. Exposure of A2E containing ARPE-19 cells to blue light resulted in significant apoptosis and increases in levels of GRP78, CHOP, p-JNK, Bax, cleaved caspase-9, and cleaved caspase-3, indicating that photooxidative damage to RPE cells is mediated by the ER-stress-induced intrinsic apoptotic pathway. Cells in which GRP78 had been knocked down with shRNA were more vulnerable to photooxidative damage. Pre-treatment of blue-light-exposed A2E containing ARPE-19 cells, with grape skin extracts, inhibited apoptosis, in a dose dependent manner. Knockdown GRP78 blocked the protective effect of grape skin extracts.

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1. Introduction

The progressive accumulation of autofluorescent lipofuscin in the retinal pigment epithelium (RPE) is one of the hallmarks of aging (Sparrow et al., 1999). It is related to increased risk of age-related macular degeneration (AMD). AMD is the main cause of the irreversible loss of vision that affects millions of elderly worldwide. Oxidative stress, particularly lipofuscin-mediated photooxidative damage, contributes to the onset and progress of AMD (Schütt et al., 2000; Sparrow and Boulton, 2005). It is well known that visible and UV radiation can induce photochemical lesions in RPE. Indeed, RPE are particularly susceptible to wavelengths within the blue region of the spectrum. Lipofuscin has been regarded as the mediator of blue light damage (Sparrow et al., 2000). RPE lipofuscin is a byproduct of the phagocytosis of outer segments of lipid-rich photoreceptors. It comprises a complex mixture of pigments. Studies have demonstrated that lipofuscin is a potent photoinducible generator of reactive oxygen species and that it is phototoxic to RPE cells (Rozanowska et al., 1998; Wassell et al., 1999). Lipofuscin represents a mixture of fluorophores. One of the fluorophore of RPE lipofuscin is N-retinyledin- N-retinylethanolamin (A2E), a result of all-*trans*-retinal reacting with ethanolamine (Sparrow et al., 1999). Although certain levels of A2E are tolerated by RPE cells, excessive accumulation has been shown to be detrimental. A2E plays a proapoptotic role via a mitochondria-related mechanism and confers a sensitivity to blue light damage in RPE cells (Schütt et al., 2000; Suter et al., 2000). Evidence indicates that the generation of reactive oxygen species upon photoexcitation of A2E causes serious toxicity to RPE cells (Kurz et al., 2008). Although some reports demonstrated that there was no correlation between the spatial distribution of A2E and lipofuscin fluorescence in the human retinal pigment epithelium (Ablonczy et al., 2013), A2E has been confirmed as a potent photoinducible generator of reactive oxygen species and phototoxic to RPE cells (Sparrow et al., 2000). These reports indicate that chronic or acute photooxidative damage related to A2E may contribute to the pathogenesis of AMD.

Oxidative stress induces endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in retinal cells (Cano et al., 2014).

Abbreviations: RPE, retinal pigmented epithelium; AMD, age-related macular degeneration; A2E, N-retinyledin- N-retinylethanolamin; GSEs, grape skin extracts.

* Corresponding authors. Eye Research Institute of Shanghai JiaoTong University, Shanghai, China. Tel.: +86 21 63240090 Ext 6822; fax: +86 21 63240090 Ext 6822 (X.D. Sun). College of Agriculture and Biology, Shanghai Jiaotong University, Shanghai, China. Tel.: +86 2134205711; fax: +86 2134205711 (X.J. Sun).
E-mail addresses: xdsun@sjtu.edu.cn (X.D. Sun) xjsun@sjtu.edu.cn (X.J. Sun).
[1] These authors contributed equally to the study.

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The ER is responsible for protein translocation, protein folding, and protein post-translational modifications. It is susceptible to oxidative injury. When injury occurs, cells activate a self-protective mechanism called the ER stress response, which prevents cells from death pathway (Sano and Reed, 2013). During ER stress, the ER chaperone protein, GR78/BiP, binds to unfolded proteins which cause GRP78/BiP to dissociate from partners such as PERK, ATF6, and IRE1 and initiate a survival signaling pathway. Up-regulation of the molecular chaperone GRP78/BiP, attenuates ER stress and restores homeostasis by enhancing the machinery for protein folding and degradation by the UPR (Chang et al., 2013). However, if the ER stress is severe or prolonged, signaling switches to a proapoptotic pathway.

So far, there is no efficient therapy or preventive measures for early AMD other than blue-light protection. In contradistinction, higher dosed dietary antioxidants and mineral cofactors (i.e. Vitamins C, E, lutein, zeaxanthin, zinc) play an important role in patients with high risk AMD retinopathy, in preventing catastrophic loss of vision, as demonstrated in two large scale US studies known as AREDS (Age-Related Eye Disease Study) 1 (National Eye Institute, 2001) and AREDS 2 (National Eye Institute, 2013). The protective effects of dietary antioxidants on eye health have also been observed in other reports (Jomova and Valko, 2013; Ma and Lin, 2010; Olmedilla-Alonso et al., 2014). There has been increasing interest in the use of dietary countermeasures, in particular polyphenols, for preventing the progression of AMD (Kalt et al., 2010; Laabich et al., 2007; Xu et al., 2010). The complex biochemical matrix of grape skin contains large amounts of polyphenols of different classes, including resveratrol, catechin, epicatechin, anthocyanins, quercetin, oligomeric procyanidins, and phenolic acid, which have strong in vivo and in vitro antioxidant properties (Katalinić et al., 2010). Resveratrol and quercetin activate the Sirtuin 1 gene and chelate metal from retina. This may inhibit lipofuscin before loss of vision and achieve visual regeneration among patients already experiencing pathologic visual decline (Harris et al., 2013; Richer et al., 2009, 2013). Resveratrol and quercetin were found to significantly improve RPE cell survival from oxidative damage induced by either H_2O_2, t-BOOH, or depletion of GSH depletion (Kalt et al., 2010; King et al., 2005). In addition, resveratrol exerts protection against acrolein-induced cytotoxicity in human ARPE-19 cells via increases in the mitochondrial bioenergetics, and attenuates laser-induced choroidal neovascularization in animals exposed to cigarette smoke (Sheu et al., 2013). Anthocyanins exhibit protective effects on visual signal transduction in vitro. Hydrocaffeic and p-coumaric acid significantly reduced UV-B-induced damage to DNA in cornea and sclera tissue (Kalt et al., 2010). Dietary enrichment with grape extract was sufficient to prevent lipofuscin accumulation and age-related cone and rod photoreceptor dysfunction in β5–/– mice. This indicated that the oxidative burden of RPE cells in vivo that is associated with age related blindness may be prevented by consumption of a grape-polyphenol-rich diet (Yu et al., 2012). However, the mechanism by which grape skin polyphenols protect RPE cells from lipofuscin-mediated photooxidative damage and thus prevent AMD need to be further explored.

Oxidative stress and ER stress are closely related. Whereas oxidative stress triggers ER stress and UPR, excessive or prolonged ER stress can decrease mitochondrial membrane potential, limit bioenergetic changes, and foster the generation of ROS generation, and ultimately induce apoptosis (Apostolova et al., 2013). Because RPE is a primary target of photooxidative insult, oxidative stress induced ER stress and UPR may contribute to apoptosis of RPE and AMD-related lesions. To explore the mechanisms by which photooxidative damage induces apoptosis and grape skin polyphenols protect RPE cells, the effects of grape skin extracts on blue-light-induced changes of expression of the ER chaperone protein, GR78/BiP, and the consequent apoptosis pathway in cultured RPE were assessed.

2. Materials and methods

2.1. Materials

The grape skin extracts (GSEs) extracted from grape (*Vitis vinifera*) with ethanol and water were obtained from JF-Natural Corporation (Tianjin, China). Within the GSEs, total phenolic concentration (expressed as gallic acid equivalents) was 352.5 g/kg, with 8.5 g/kg resveratrol, 44.5 g/kg catechin, 23.2 g/kg epicatechin, 140.5 g/kg anthocyanins (expressed as malvidin-3-glucoside), 18.8 g/kg quercetin derivatives, 28.5 g/kg proanthocyanidin dimer, 15.1 g/kg gallic acid, 10.8 g/kg ferulic acid, and 6.2 g/kg caffeic acid. A stock solution of GSEs was prepared in dimethyl sulfoxide (DMSO) and diluted with Dulbecco's modified Eagle medium (DMEM). The highest DMSO concentration in the final solution was 0.1%. This concentration has been shown to not affect cell viability.

Antibodies against GRP78, CHOP, JNK, p-JNK, Bcl-2, Bax, caspase-9, and caspase-3 were from Cell Signaling Technology (Beverly, MA, U.S.). The respective secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.). APC Annexin V was purchased from BD Biosciences (Franklin Lakes, NJ, U.S.) and DAPI was from Sigma-Aldrich (St. Louis, MO, U.S.).

2.2. Exposure to A2E and blue light

A2E was prepared as described by Parish et al. (1998) with some minor modifications. Briefly, a mixture of all-*trans*-retinal (Sigma-Aldrich) and ethanolamine (a molar ratio of 2:1) in ethanol was stirred in the presence of acetic acid at room temperature for 7 days in the dark. A2E was purified using a silica gel column (ANPEL Corp, Shanghai, China), and gradient elution was achieved with MeOH:CH_2Cl_2 (5:95–8:92) in the presence of 0.1% trifluoroacetic acid (TFA). A2E was stored as a stock solution in DMSO and kept at –80 °C in the dark.

ARPE-19 cells (American Type Culture Collection, Manassas, VA, U.S.) were grown in DMEM/F12 (volumetric ratio of 1:1; Hyclone, Logan, UT, U.S.) supplemented with 10% fetal bovine serum (FBS). For uptake into cultured RPEs, A2E was delivered at concentrations of 5, 10, or 25 μM in culture media. Confluent cultures were incubated with A2E for 2 h, washed with PBS to remove any A2E that had not been internalized, and cultured in DMEM/F12 for 24 h. The medium was then changed to DMEM/F12 with 10% FBS. Cells were incubated for additional 6–24 h after exposure to 2000 Lux blue light for 30 min from Kyoritsu Model 5202 in the presence or absence of grape skin extracts. Cells that had neither accumulated A2E nor been exposed to blue light were used as controls. Apoptosis of ARPE-19 was analyzed by flow cytometry. Cell viability was detected using MTT (3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide) assay. Levels of GRP78, CHOP, Bcl-2, Bax, caspase-9, and caspase-3 protein in ARPE-19 were determined by Western blotting. Levels of HSPA5 (GRP78) mRNA were analyzed using real time PCR. ROS levels were detected with dihydroethidium (DHE) using flow cytometry.

2.3. Detection of A2E accumulation in cultured ARPE-19 with confocal microscopy

A2E accumulation in cultured ARPE-19 cells was determined using a TCS SP5-II confocal laser-scanning microscope (Leica, Olms, Germany). RPE samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) containing 1 mM $MgCl_2$ and 0.2 mM $CaCl_2$. A2E fluorescence was detected at 510–560 nm after excitation at 460–550 nm. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI), and visualized at 488 nm after excitation at 340 nm.

2.4. Quantitation of A2E in ARPE-19 by LC-MS

Internalized A2E in ARPE-19 was quantified by liquid chromatography-mass spectrometry (LC-MS). Pelleted RPE cells were homogenized with a chloroform:methanol (2:1) solution to extract A2E. Internalized A2E was quantified with LC-MS. Extracted samples were injected onto a reversed-phase column (Xtimate C18, 30 × 2.1 mm, 3 μm) and eluted with a gradient of acetonitrile in water (30–90% acetonitrile with 0.5% TFA; Shimadzu LC-MS 2010 EV system). The flow rate was 1.2 ml/min, and detection time was 2 min. A photodiode array detector was used to obtain a UV spectrum for each eluted fraction. The quantities of A2E in cultured RPE cells were determined from integrated peak intensities and expressed as ng/10^5 cells.

2.5. Cell apoptosis assays

APC Annexin V (BD Biosciences) and DAPI (Sigma-Aldrich) were used to determine apoptosis of ARPE-19 cells. Briefly, RPE cells were seeded in 6-cm dishes and treated with A2E for 2 h, washed with PBS to remove any A2E that had not been internalized, and cultured in DMEM/F12 for 24 h. The medium was then changed to DMEM/F12 with 10% FBS. Cells were incubated for additional 12 h after exposure to 2000 Lux blue light for 30 min from Kyoritsu Model 5202 in the presence or absence of grape skin extracts. Cells that had neither accumulated A2E nor been exposed to blue light were used as controls. Cells were detached from the dish and incubated with APC Annexin V (5%) and DAPI (0.05 μg/ml) for 5 min. APC was excited at 633 nm and emitted a signal at 660 nm, and DAPI was excited at 340 nm and emitted a signal at 488 nm. Both APC and DAPI were unaffected by A2E fluores-

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