Indirect Antioxidant Protection Against Photooxidative Processes Initiated in Retinal Pigment Epithelial Cells by a Lipofuscin Pigment

JILIN ZHOU,1 XIANGQUN GAO,2 BOLIN CAI,1 and JANET R. SPARROW1,3

ABSTRACT

Oxidative mechanisms are considered to contribute to the aging changes in retinal pigment epithelial (RPE) cells that underlie the pathogenesis of age-related macular degeneration. An important source of oxidative damage is likely to be the photoreactive pigments that progressively accumulate and constitute the lipofuscin of retinal pigment epithelial cells. Evidence for a link between RPE lipofuscin and cellular dysfunction is also provided by the understanding of disease progression in Stargardt disease. Using a culture model previously used to demonstrate photooxidative damage to retinal pigment epithelial cells that have accumulated the lipofuscin fluorophore A2E, it was shown that the propensity for cell death is increased under conditions that deplete cellular levels of glutathione. Additionally, sulforaphane, a phytochemical and inducer of phase 2 enzymes, protected RPE cells that accumulated A2E and were irradiated at 430 nm. The protection afforded by sulforaphane was paralleled by elevated levels of glutathione and increases in the activities of the phase 2 enzymes NAD(P)H:quinone reductase and glutathione-S-transferases. Moreover, transcriptional induction of NAD(P)H:quinone reductase was indicated by the increases in mRNA determined by real time RT-PCR. There has been considerable interest in the intake of carotenoids and antioxidant vitamins and the related incidence of age-related macular degeneration. The present results indicate that the indirect antioxidant activity of plant-derived phase 2 inducers also may be potentially important.

INTRODUCTION

REACTIVE FORMS OF OXYGEN are generated in all mammalian cells largely as a byproduct of aerobic metabolism and as a result cells are equipped with multiple forms of defense.1 For instance, enzymes such as superoxide dismutase, catalase, and various types of peroxidases have the capacity to directly inactivate oxi-

1Department of Ophthalmology, Columbia University, New York, New York.
2Department of Pharmacology and Molecular Science, The Johns Hopkins University School of Medicine, Baltimore, Maryland.
3Department of Pathology and Cell Biology, Columbia University, New York, New York.

256
(GSSG) by NADPH-dependent GSSG reductase. The conjugation of glutathione with a wide variety of electrophiles is catalyzed by glutathione transferases. Other compounds that can serve as direct antioxidants in nonenzymatic reactions are available from the diet and include tocopherols, ascorbic acid, and carotenoids. All of these exogenous antioxidants are consumed stoichiometrically during quenching or scavenging reactions. Important additional protection comes from the transcriptional induction of enzymes of the phase 2 group (phase 2 enzymes) by a variety of natural and synthetic chemical agents. The enzymes in this class include glutathione-S-transferases (GSTs) NAD(P)H:quinone reductase (NQO1), epoxide hydrolase, \( \gamma \)-glutamylcysteine synthetase, UDP-glucuronosyl transferases. One particularly potent inducer of phase 2 enzymes is sulforaphane, an isothiocyanate that is abundant in cruciferous plants such as broccoli. Sulforaphane does not take part in oxidation/reduction reactions, directly, but instead induces phase 2 enzymes. Sulforaphane is taken up into cultured cells in abundance with intracellular levels reaching concentrations that are 40 to 180 times higher than the extracellular concentrations.

Besides the metabolic sources of reactive forms of oxygen, retinal pigment epithelial (RPE) cells are susceptible to oxidative damage because of an exposure to high oxygen partial pressure and light. Indeed, age-related reduction in the antioxidant capacity of the RPE is suggested as one reason for the demise of these cells during the early stages of age-related macular degeneration. That RPE cells are exposed to light is of particular importance because these cells also accumulate photoreactive compounds that constitute the lipofuscin of the cells. As of now, the lipofuscin fluorophores that have been characterized are A2E and its isomers and all-trans-retinal dimer—phosphatidylethanolamine conjugate. A2E and ATR-dimer-PE have absorbance maxima in the visible range at \( \approx 440 \) nm and 500 nm, respectively. The proposed biosynthetic pathways for both of these bis-retinoid pigments involve all-trans-retinals as precursors. At least in the case of A2E, photosensitization of the pigment leads to the generation of singlet oxygen and perhaps other reactive forms of oxygen. Additionally, the photooxidation processes initiated by A2E and ATR dimer-PE involve the addition of oxygen at double bonds along the retinoid-derived side-arms, with the result that reactive photolytic products are generated.

A number of antioxidants including the singlet oxygen quenchers histidine, DABCO (diazabicyclooctane), and azide and naturally occurring compounds such as vitamins E and C have been shown to confer resistance to blue light-induced death of A2E-containing RPE. In addition, bilberry-derived anthocyanins were shown to be potent antioxidants that suppressed photooxidative processes initiated in RPE cells by A2E. The protective effect of anthocyanins was likely afforded by the unsaturated diener conjugation in the C (pyrene) ring that allows for singlet oxygen quenching and the presence of hydrogen-donating hydroxyl groups on the B ring. The authors have probed for evidence of protection by yet another phytochemical, the phase 2 inducer sulforaphane.

**METHODS**

**Cell cultures**

Human adult RPE cells (ARPE-19 cells; American Type Culture Collection) without endogenous lipofuscin, accumulated A2E at concentration delivered in the culture medium, and were irradiated as previously described. In some experiments cells were treated with BSO [buthionine-R,S-sulfoximine] (Sigma-Aldrich, St. Louis, MO) at 100 \( \mu \)M for 48 hours. For experiments involving treatment with sulforaphane [1-isothiocyanato-4-(methyl sulfinyl)butane], ARPE-19 cells were cultured in DMEM and Ham’s F-12 medium (1:1) with 10% FBS that had been heat (90 min at 55°C) and charcoal-treated (1% wt/vol) to reduce the presence of endogenous NQO1 inducers. Cells were subsequently treated with 5 \( \mu \)M sulforaphane for 48 hours.

**Glutathione analysis**

Total glutathione (GSH+GSSG) in supernatant fractions of cell lysates was determined
by a colorimetric assay based on DTNB (2-nitro-5-thiobenzoic acid) as substrate and the generation of GSH from GSSG by glutathione reductase (BioVision Research Products, Mountain View, CA). The absorbance of the reaction product was read at 405 nm and GSH concentration was determined by reference to a glutathione calibration curve after measurement of protein (Bio-Rad Laboratories, Hercules, CA).

**Enzyme assays**

GST activity in cell lysates was determined as a colorimetric measure of the GST-catalyzed reaction between GSH and the substrate CDNB (1-chloro-2,3-dinitrobenzene) (BioVision). The change in absorbance at 340 nm was recorded after a 2-minute interval, samples were assayed in duplicate, and background values were subtracted from that of the samples. GST activity was expressed as nanomoles of CDNB-GSH conjugate formed per minute per milligram protein at 25°C.

NQO1 (QR) activity was measured in sulforaphane-treated cells that had been maintained in either serum-free (3 days prior to sulforaphane treatment) or serum-containing (10%) medium. ARPE-19 cells were lysed by addition of 0.08% digitonin (200 μL/well of a 24-well plate), at 37°C for 15 min, agitated gently at 150 rpm (15 min) and centrifuged at 1500 g for 15 min. The activity of NQO1 in the supernatant (15 μL/well) was then assayed by measuring the formation of the blue-brown formazan as an absorbance at 610 nm following the addition of menadione, an NADPH-generating system and the tetrazolium dye MTT.4,20 Specific activity was obtained by adjusting the measured absorbance with respect to protein concentration. The assay was performed as two sets of triplicates.

**Cell viability assay**

Percent of nonviable cells was determined by labeling the nuclei of nonviable cells with Dead Red (Molecular Probes, Eugene, OR) and nuclei of all cells with DAPI.15 Values in each experiment were based on the sampling of 10 fields. Cytotoxicity also was assayed by MTT (4,5-dimethylthiazol-2-yI)-2,5-diphenyltetrazolium bromide) assay (Roche Diagnostics, Basel, Switzerland), a measure of the ability of healthy cells to cleave the yellow tetrazolium salt MTT to purple formazan crystals. A decrease in the absorbance (570 nm) of reduced MTT is indicative of diminished cellular viability.

**Real-time RT-PCR**

Total RNA was extracted using RNeasy Mini Kit (Qiagen Science, Valencia, CA). Real-time RT-PCR was performed on a LightCycler system (Roche Molecular Biochemicals, Indianapolis, IN) using the LightCycler RNA amplification SYBR Green I one-step kit (Roche Diagnostics, GmbH, Germany) and β2-microglobulin (β2MG) as internal amplification control. NQO1-specific primers were designed to generate a 217-bp amplicon. For β2MG, the expected size was 295-bp. Amplifications reactions were performed in triplicate and the relative amount of mRNA in a sample was determined in relation to a standard curve constructed using total RNA from untreated cells.

**RESULTS**

GSH is a tripeptide cellular thiol whose abundance in the reduced state is important for cellular defense against reactive electrophiles and oxidants. To address the role that GSH may play in the resistance of A2E-laden RPE to blue light damage, the authors treated cells with BSO, an analogue of methionine that depletes GSH levels by inhibiting glutamylcysteine, the rate-limiting step in GSH synthesis.21 When ARPE-19 cells were treated with BSO (100 μM) for 2 days, a 36% reduction in cellular GSH levels was observed (Fig. 1A). Parallel studies performed to test for a concomitant effect on the viability of A2E-laden RPE following irradiation at 430 nm, revealed that prior treatment with BSO for 48 hours to deplete GSH produced a 1.6-fold increase in the proportion of nuclear stained-nonviable cells (see Fig. 1B). When tested by MTT assay, absorbance of the formazan product reflected a 22% reduction in cell viability (see Fig. 1C).

To determine whether phase 2 enzyme induction confers resistance to light induced death of A2E-laden ARPE-19 cells, the authors
treated with sulforaphane, a dietary inducer of phase 2 proteins that at a concentration of 5 μM has been shown previously to be protective for ARPE-19 cells. Accordingly, it was observed that pretreatment with sulforaphane protected RPE cells that had accumulated A2E and were light-exposed. Thus, in the absence of sulforaphane, 46% of the A2E-laden cells became nonviable after blue light illumination, whereas treatment with sulforaphane reduced the nonviability to 26% (Fig. 2). In these experiments, percent nonviability with 430 nm irradiation only or sulforaphane treatment and 430 nm irradiation (without A2E accumulation) was 0.10 ± 0.2 (mean ± SD) and 0.16 ± 0.3 (mean ± SD), respectively.

Because the protection afforded by sulforaphane is considered to depend on the induction of phase 2 enzymes such as NQO1 and glutathione-S-transferases, the authors also assayed for changes in these activities. In cells that had accumulated A2E and were treated with sulforaphane, GST activity was increased by 50%. Furthermore, the cellular content of GSH was increased twofold (Fig. 3). Additionally, NQO1 enzyme activity reached levels that were threefold higher (Fig. 4B). To examine for effects of sulforaphane on NQO1 mRNA expression, real time RT-PCR was performed and expression levels were normalized to β2MG. Accordingly, treatment of ARPE-19 cells with sulforaphane (5 μM) for 2 days resulted in a fourfold increase in NQO1 mRNA (see Fig. 4A). Interestingly, cells that had accumulated A2E in the absence of sulforaphane-treatment also exhibited increases in NQO1 mRNA; however, A2E-associated changes in NQO1 enzyme activity were not observed.

**DISCUSSION**

To investigate the role of oxidative damage in age-related macular degeneration (AMD),
some studies have employed relatively high concentrations of the oxidants tert-butyl hydroperoxide and hydrogen peroxide. The approach the authors have taken is to study a naturally occurring constituent of the lipofuscin of RPE, a compound that mediates phototoxicative events. This pigment is one of a group of lipofuscin fluorophores that are considered to be responsible for RPE cell death in Stargardt disease and also have been implicated in atrophic age-related macular degeneration. Whether the phototoxicative events mediated by A2E are an immediate threat to the RPE or whether they elicit sustained perturbation that could ultimately alter neighboring tissues and impact cell function is not yet clear. Here a relationship between cellular GSH levels and the phototoxicity of A2E has been demonstrated. Specifically, the accentuation of cell death that accompanied BSO-induced reduction in GSH indicated that GSH shields the cell against A2E-mediated phototoxicity. In addition, the phase 2 enzyme-inducer sulforaphane protected against A2E-associated phototoxicity to an extent that was similar to the protection previously described against the toxicity of other oxidants, such as menadione, tert-butyl hydroperoxide (t-BHP), 4-hydroxynonenal (HNE), and peroxynitrite. The improvement in cell survival was accompanied by and was likely attributable to the observed increases in phase 2 enzymes GST and NQO1. The level of QR induction by sulforaphane was greater than the induction of GST, a difference that has been observed previously. The induction of phase 2 enzymes by

FIG. 3. Elevation of GSH levels and induction of glutathione S transferase in ARPE-19 cells exposed to sulforaphane for 24 hours. A. Total GSH expressed as nanomoles per milligram of cytosolic protein. B. GST activity was determined as the amount of enzyme producing 1 nanomole of CDNB-GSH conjugate per minute per milligram protein. Means ± SEM of three experiments with samples assayed in duplicate. **p < 0.05. One way ANOVA and Newman Keul Multiple Comparison test.

FIG. 4. Induction of quinone reductase by sulforaphane. A. NQO1 mRNA was quantified by real time RT-PCR expressed as a ratio of β2-microglobulin (β2MG) and normalized to untreated controls; 5 μM sulforaphane for 48 hours, three independent experiments. **p < 0.01 as compared to the corresponding value without SF; one-way analysis of variance (ANOVA) and Newman Keul Multiple Comparison test. Values are mean ± standard deviation (SD). B. NQO1 activity measured in cells maintained in media with and without serum for 3 days before a 48-hour treatment with sulforaphane (5 μM). Values are mean ± SD, one experiment, six replicates. **p < 0.01 as compared with the corresponding value without SF; one-way ANOVA and Newman Keul Multiple Comparison test.
sulfuraphane also was accompanied by a cellular increase in GSH, a change that likely resulted from transcriptional up-regulation of γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH biosynthesis.36

Being a major water-soluble antioxidant, GSH can serve as a reductant of peroxides either by nonenzymatic reaction or a reaction catalyzed by glutathione peroxidase.37 GSH also is able to quench singlet oxygen38 and can detoxify reactive aldehydes that are generated from lipid peroxidation.39 Previous studies have shown that levels of reduced glutathione in cultured RPE are an important determinant of resistance to cell death induced by oxidants.40 Plasma GSH concentrations have been found to decrease with age, an observation that, along with age-related changes in other antioxidants,41 suggests a shift in the redox state to one that is more oxidizing. Importantly, comparison of AMD versus age-matched non-AMD subjects, revealed a tendency toward lower plasma GSH levels in individuals with AMD.41

There is considerable evidence that sulfuraphane itself does not directly participate in antioxidant or prooxidant reactions but rather that it acts indirectly to increase the antioxidant capacity of cells through the induction of enzymes such as glutathione-S-transferases (GST), NAD(P)H:quinone reductase (NQO1), epoxide hydrolase, γ-glutamylcysteine synthetase, UDP-glucuronosyl-transferases.3 The expression of phase 2 enzymes is under transcriptional control, at least partially, through the antioxidant response element (ARE) found in the regulatory regions of their genes.42 Nrf2, a member of the basic leucine zipper family of transcription factors, is at least one of the transcription factors that binds to ARE regions to activate phase 2 gene transcription. In addition to sulfuraphane, the phase 2 inducer dimethyl fumarate (DMF), was shown to provide cultured RPE cells with an increased resistance to t-BHP oxidative damage.43 Similar protection was afforded by Oltipraz (4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione), a cancer chemopreventive agent, the reduction in t-BHP-induced RPE cell death being accompanied by elevated GSH and the induction of glutathione-S-transferase and NQO1.43

The causes of AMD are not understood but multiple factors, including genetic, environmental, and dietary, are thought to be involved. Three known or suspected risk factors for AMD are considered to increase susceptibility because of oxidative processes. Of these, smoking is consistently recognized as increasing susceptibility to AMD.44–47 Light exposure has been suspected as a risk factor for sometime; however, not all epidemiologic studies have been able to establish a relationship.48–51 The view that there is a link between oxidative damage to RPE cells and the susceptibility to AMD is also supported by evidence showing that RPE lipofuscin, which accumulates with age and is of highest concentration at the posterior pole,52 can initiate photooxidative events.7 For instance, the photooxidative processes initiated in cultured RPE cells through blue light-induced sensitization of A2E can lead to DNA base lesions,53 modifications of protein, and changes in protein expression.35 Retinal pigment epithelial cells are equipped with a number of different enzymes to combat the potential deleterious effects of reactive oxygen intermediates, including superoxide dismutase (SOD), catalase, glutathione S-transferases, and glutathione peroxidase (GPX);43,54,55 nevertheless, the antioxidant capacity of the RPE decreases with age.41 The damage that is evoked by blue light irradiation of RPE cells that have accumulated A2E and other lipofuscin fluorophores also may explain, at least in part, the observation made in animal models that RPE cells are particularly susceptible to injury from short wavelength visible light (type 2 retinal light damage).56–58

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health Grant EY 12951 (JRS). JRS is a recipient of an Alcon Research Institute Award.

REFERENCES


