

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. SPARROW^{1,3}

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-

dants. Glutathione (GSH) is a tripeptide-antioxidant that is present at millimolar concentrations in cells and plays a major role in protecting cells against electrophiles and reactive oxygen species. Adequate levels of reduced glutathione are maintained by synthesis from glutamic acid, cysteine and glycine, a process that is rate-limited by γ -glutamylcysteine synthetase and reduction of oxidized glutathione

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.

(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.² The enzymes in this class include glutathione-S-transferases (GSTs) NAD(P)H:quinone reductase (NQO1), epoxide hydrolase, γ -glutamylcysteine synthetase, UDP-glucuronosyl transferases.³ One particularly potent inducer of phase 2 enzymes is sulforaphane, an isothiocyanate that is abundant in cruciferous plants such as broccoli.⁴ 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.⁵

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.⁶ 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.⁷ As of now, the lipofuscin fluorophores that have been characterized are A2E and its isomers and all-*trans*-retinal dimer--phosphatidylethanolamine conjugate.⁸⁻¹¹ A2E and ATR-dimer-PE have absorbance maxima in the visible range at \sim 440 nm and 500 nm, respectively. The proposed biosynthetic pathways for both of these bis-retinoid pigments involve all-*trans*-retinals as precursors.¹¹⁻¹³ 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.¹⁴⁻¹⁶ 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.¹⁶

A number of antioxidants including the singlet oxygen quenchers histidine, DABCO (diazabicyclooctane), and azide¹⁵ and naturally occurring compounds such as vitamins E and C¹⁶ have been shown to confer resistance to blue light-induced death of A2E-containing RPE. In addition, bilberry-derived anthocyanins¹⁷ 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 diene 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 confluence from a 20- μ M concentration delivered in the culture medium, and were irradiated as previously described.^{15,18,19} In some experiments cells were treated with BSO [buthionine-R,S-sulfoximine] (Sigma-Aldrich, St. Louis, MO) at 100 μ 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 μ 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 μ L/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 μ L/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-yl)-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 β 2-microglobulin (β 2MG) as internal amplification control. NQO1-specific primers were designed to generate a 217-bp amplicon. For β 2MG, 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 μ M) 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

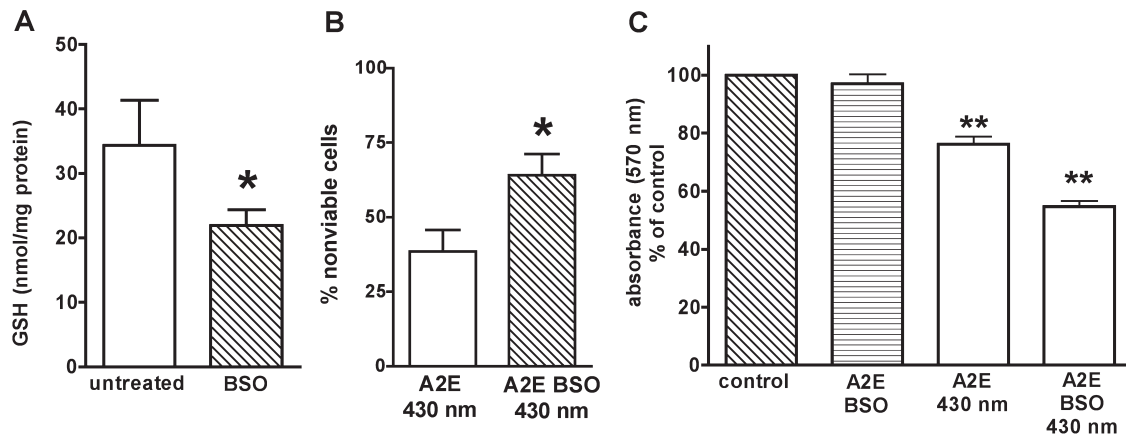


FIG. 1. Treatment with buthionine sulfoximine (BSO) depresses cellular GSH levels and accentuates blue light-related death of A2E-laden RPE. **A.** GSH levels in cells treated with BSO (100 μ M) for 2 days. Mean \pm standard deviation (SD) of four experiments. **B,C.** Cell viability. A2E-laden RPE cells were treated with BSO (100 μ M) for 48 hours before exposure to 430 nm light. Cell viability was assayed after 8 hours by exclusion of a cell impermeable dye (**B**) or after 24 hours by MTT assay (**C**), a decrease in the absorbance (570 nm) of reduced MTT being indicative of diminished cellular viability. Mean \pm SD, three to four experiments. * $p < 0.01$, unpaired *t*-test; ** $p < 0.01$ as compared with A2E/430 nm, one-way analysis of variance (ANOVA) and Newman-Keul Multiple Comparison test.

treated with sulforaphane, a dietary inducer of phase 2 proteins that at a concentration of 5 μ M has been shown previously to be protective for ARPE-19 cells.²² 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 irra-

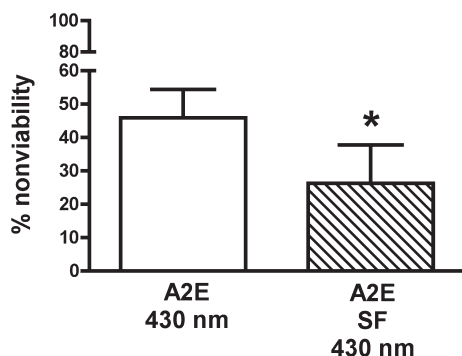


FIG. 2. Protective effect of the phase 2 enzyme-inducer sulforaphane on photoinduced damage to A2E-laden RPE. ARPE-19 cells were allowed to accumulate A2E with and without prior treatment with sulforaphane (5 μ M) (SF) for 24 hours and were irradiated at 430 nm. Percent nonviable cells; mean \pm SD of 3 experiments; * $p < 0.01$, unpaired *t*-test.

diation only or sulforaphane treatment and 430 nm irradiation (without A2E accumulation) was 0.10 ± 0.2 (mean \pm SD) and 0.16 ± 0.3 (mean \pm 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 β 2MG. Accordingly, treatment of ARPE-19 cells with sulforaphane (5 μ M) 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),

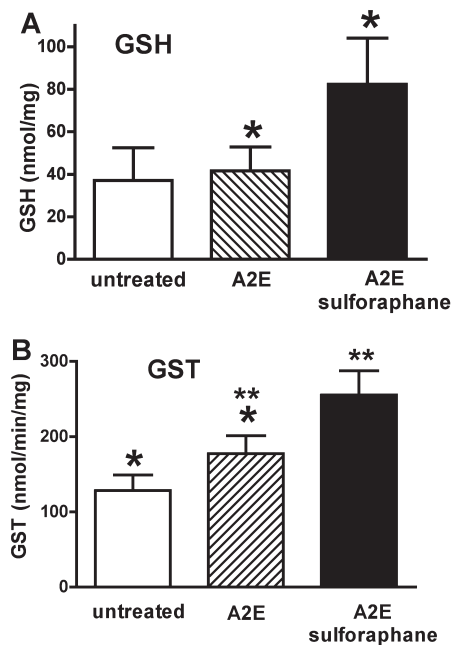


FIG. 3. Elevation of GSH levels and induction of glutathione S transferase in ARPE-19 cells exposed to sulforaphane for 24 hours. **A.** Total GSH expressed as nanomoles per milligram of cytosolic protein. **B.** GST activity was determined as the amount of enzyme producing 1 nanomole of CDNB-GSH conjugate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

some studies have employed relatively high concentrations of the oxidants tert-butyl hydroperoxide and hydrogen peroxide.^{23–26} The approach the authors have taken is to study a

naturally occurring constituent of the lipofuscin of RPE, a compound that mediates photooxidative events. This pigment is one of a group of lipofuscin fluorophores that are considered to be responsible for RPE cell death in Stargardt disease^{27–29} and also have been implicated in atrophic age-related macular degeneration.^{30–34} Whether the photooxidative 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 function³⁵ is not yet clear. Here a relationship between cellular GSH levels and the phototoxicity of A2E has been demonstrated. Specifically, the attenuation 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.²² 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.⁵ The induction of phase 2 enzymes by

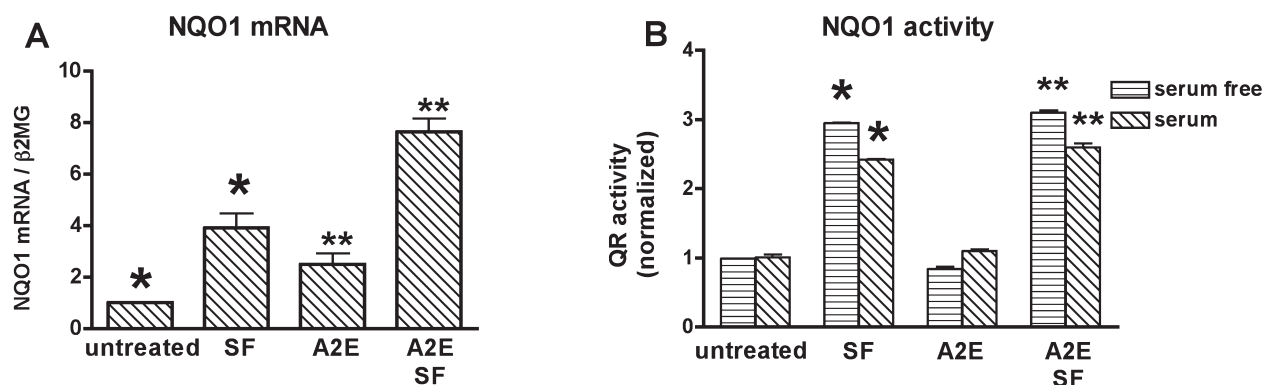


FIG. 4. Induction of quinone reductase by sulforaphane. **A.** NQO1 mRNA was quantified by real time RT-PCR expressed as a ratio of β 2-microglobulin (β 2MG) and normalized to untreated controls; 5 μ M sulforaphane for 48 hours, three independent experiments. *, ** $p < 0.01$ as compared to the corresponding value without SF; one-way analysis of variance (ANOVA) and Newman Keul Multiple Comparison test. Values are mean \pm 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 μ M). Values are mean \pm 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.

sulforaphane 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 sulforaphane 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 sulforaphane, the phase 2 inducer dimethyl fumarate (DMF), was shown to provide cultured RPE cells with an increased resistance to t-BHP oxidative damage.⁴¹ 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

1. Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine*, 3rd ed. Oxford, UK: Oxford University Press, 1999.

2. Talalay P. Chemoprotection against cancer by induction by Phase 2 enzymes. *BioFactors* 2000;12:5–11.
3. Dinkova-Kostova AT, Massiah MA, Bozak RE, Hicks RJ, Talalay P. Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulphydryl groups. *Proc Natl Acad Sci USA* 2001;98:3404–3409.
4. Fahey JW, Zhang Y, Talalay P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc Natl Acad Sci USA* 1997;94:10367–10372.
5. Zhang Y, Talalay P. Mechanism of differential potencies of isothiocyanates as inducers of anticarcinogenic Phase 2 enzymes. *Cancer Res* 1998;58:4632–4639.
6. Liang FQ, Godley BF. Oxidative stress-induced mitochondrial DNA damage in human retinal pigment epithelial cells: a possible mechanism for RPE aging and age-related macular degeneration. *Exp Eye Res* 2003;76:397–403.
7. Sparrow JR, Boulton M. RPE lipofuscin and its role in retinal photobiology. *Exp Eye Res* 2005;80:595–606.
8. Sakai N, Decatur J, Nakanishi K, Eldred GE. Ocular age pigment “A2E”: an unprecedented pyridinium bis-retinoid. *J Am Chem Soc* 1996;118:1559–1560.
9. Eldred GE, Lasky MR. Retinal age pigments generated by self-assembling lysosomotropic detergents. *Nature* 1993;361:724–726.
10. Parish CA, Hashimoto M, Nakanishi K, Dillon J, Sparrow JR. Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. *Proc Natl Acad Sci USA* 1998;95:14609–14613.
11. Fishkin N, Sparrow JR, Allikmets R, Nakanishi K. Isolation and characterization of a retinal pigment epithelial cell fluorophore: an all-trans-retinal dimer conjugate. *Proc Natl Acad Sci USA* 2005;102:7091–7096.
12. Ben-Shabat S, Parish CA, Vollmer HR, Itagaki Y, Fishkin N, Nakanishi K, Sparrow JR. Biosynthetic studies of A2E, a major fluorophore of RPE lipofuscin. *J Biol Chem* 2002;277:7183–7190.
13. Liu J, Itagaki Y, Ben-Shabat S, Nakanishi K, Sparrow JR. The biosynthesis of A2E, a fluorophore of aging retina, involves the formation of the precursor, A2-PE, in the photoreceptor outer segment membrane. *J Biol Chem* 2000;275:29354–29360.
14. Ben-Shabat S, Itagaki Y, Jockusch S, Sparrow JR, Turro NJ, Nakanishi K. Formation of a nona-oxirane from A2E, a lipofuscin fluorophore related to macular degeneration, and evidence of singlet oxygen involvement. *Angew Chem Int Ed* 2002;41:814–817.
15. Sparrow JR, Zhou J, Ben-Shabat S, Vollmer H, Itagaki Y, Nakanishi K. Involvement of oxidative mechanisms in blue light induced damage to A2E-laden RPE. *Invest Ophthalmol Vis Sci* 2002;43:1222–1227.
16. Sparrow JR, Vollmer-Snarr HR, Zhou J, Jang YP, Jockusch S, Itagaki Y, Nakanishi K. A2E-epoxides damage DNA in retinal pigment epithelial cells. Vitamin E and other antioxidants inhibit A2E-epoxide formation. *J Biol Chem* 2003;278:18207–18213.
17. Jang YP, Zhou J, Nakanishi K, Sparrow JR. Anthocyanins protect against A2E photooxidation and membrane permeabilization in retinal pigment epithelial cells. *Photochem Photobiol* 2005;81:529–536.
18. Sparrow JR, Nakanishi K, Parish CA. The lipofuscin fluorophore A2E mediates blue light-induced damage to retinal pigmented epithelial cells. *Invest Ophthalmol Vis Sci* 2000;41:1981–1989.
19. Sparrow JR, Parish CA, Hashimoto M, Nakanishi K. A2E, a lipofuscin fluorophore, in human retinal pigmented epithelial cells in culture. *Invest Ophthalmol Vis Sci* 1999;40:2988–2995.
20. Prochaska HJ, Santamaria AB, Talalay P. Rapid detection of inducers of enzymes that protect against carcinogens. *Proc Natl Acad Sci USA* 1992;89:2394–2398.
21. Meister A. Glutathione deficiency produced by inhibition of its synthesis, and its reversal: applications in research and therapy. *Pharmacol Ther* 1991;51:155–194.
22. Gao X, Dinkova-Kostova AT, Talalay P. Powerful and prolonged protection of human retinal pigment epithelial cells, keratinocytes, and mouse leukemia cells against oxidative damage: the indirect antioxidant effects of sulforaphane. *Proc Natl Acad Sci USA* 2001;98:15221–15226.
23. Cai J, Wu M, Nelson KC, Sternberg P, Jones DP. Oxidant-induced apoptosis in cultured human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 1999;40:959–966.
24. Ballinger SW, Van Houten B, Jin GF, Conklin CA, Godley BF. Hydrogen peroxide causes significant mitochondrial DNA damage in human RPE cells. *Exp Eye Res* 1999;68:765–772.
25. Jiang S, Moriarty SE, Grossniklaus H, Nelson KC, Jones DP, Sternberg P. Increased oxidant-induced apoptosis in cultured nondividing human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 2002;43:2546–2553.
26. Godley BF, Jin GF, Guo YS, Hurst JS. Bcl-2 overexpression increases survival in human retinal pigment epithelial cells exposed to H₂O₂. *Exp Eye Res* 2002;74:663–669.
27. Eagle RC, Lucier AC, Bernardino VB, Yanoff M. Retinal pigment epithelial abnormalities in fundus flavimaculatus. *Ophthalmology* 1980;87:1189–1200.
28. Lopez PF, Maumenee IH, de la Cruz Z, Green WR. Autosomal-dominant fundus flavimaculatus. Clinicopathologic correlation. *Ophthalmology* 1990;97:798–809.
29. Lois N, Holder GE, Fitzke FW, Plant C, Bird AC. Intrafamilial variation of phenotype in Stargardt macular dystrophy-fundus flavimaculatus. *Invest Ophthalmol Vis Sci* 1999;40:2668–2675.
30. Feeney-Burns L, Hilderbrand ES, Eldridge S. Aging human RPE: morphometric analysis of macular, equatorial, and peripheral cells. *Invest Ophthalmol Vis Sci* 1984;25:195–200.
31. Dorey CK, Wu G, Ebenstein D, Garsd A, Weiter JJ. Cell loss in the aging retina. Relationship to lipofuscin accumulation and macular degeneration. *Invest Ophthalmol Vis Sci* 1989;30:1691–1699.

32. Weiter JJ, Delori FC, Wing GL, Fitch KA. Retinal pigment epithelial lipofuscin and melanin and choroidal melanin in human eyes. *Invest Ophthalmol Vis Sci* 1986;27:145–151.
33. Holz FG, Bellmann C, Margaritidis M, Schutt F, Otto TP, Volcker HE. Patterns of increased in vivo fundus autofluorescence in the junctional zone of geographic atrophy of the retinal pigment epithelium associated with age-related macular degeneration. *Graefe's Arch Clin Exp Ophthalmol* 1999;237:145–152.
34. Solbach U, Keilhauer C, Knabben H, Wolf S. Imaging of retinal autofluorescence in patients with age-related macular degeneration. *Retina* 1997;17:385–389.
35. Zhou J, Cai B, Jang YP, Pachydaki S, Schmidt AM, Sparrow JR. Mechanisms for the induction of HNE-MDA- and AGE-adducts, RAGE and VEGF in retinal pigment epithelial cells. *Exp Eye Res* 2005;80:567–580.
36. Mulcahy RT, Wartman MA, Bailey HH, Gipp JJ. Constitutive and naphthoflavone-induced expression of the human-glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. *J Biol Chem* 1997;272:7445–7454.
37. Winkler BS, Boulton ME, Gottsch JD, Sternberg P. Oxidative damage and age-related macular degeneration. *Mol Vision* 1999;5:32.
38. Devasagayam TP, Sundquist AR, Di Mascio P, Kaiser S, Sies H. Activity of thiols as singlet molecular oxygen quenchers. *J Photochem Photobiol B* 1991;9:105–116.
39. Ayalasomayajula SP, Kompella UB. Induction of vascular endothelial growth factor by 4-hydroxynonenal and its prevention by glutathione precursors in retinal pigment epithelial cells. *Eur J Pharmacol* 2002;449:213–220.
40. Sternberg PJ, Davidson PC, Jones DP, Hagen TM, Reed RL, Drews-Botsch C. Protection of retinal pigment epithelium from oxidative injury by glutathione and precursors. *Invest Ophthalmol Vis Sci* 1993;34:3661–3668.
41. Cai J, Nelson KC, Wu M, Sternberg P, Jones DP. Oxidative damage and protection of the RPE. *Prog Ret Eye Res* 2000;19:205–221.
42. Talalay P, Fahey JW. Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. *J Nutr* 2001;131:3027S–3033S.
43. Nelson KC, Armstrong JS, Moriarty S, Cai J, Wu MW, Sternberg P, Jones DP. Protection of retinal pigment epithelial cells from oxidative damage by oltipraz, a cancer chemopreventive agent. *Invest Ophthalmol Vis Sci* 2002;43:3550–3554.
44. Smith W, Mitchell P, Leeder SR. Smoking and age-related maculopathy. *Arch Ophthalmol* 1996;114:1518–1523.
45. AREDS Study Group. Risk factors associated with age-related macular degeneration. A case-control study in the age-related eye disease study: age-related eye disease study report number 3. *Ophthalmology* 2000;107:2224–2232.
46. Hyman LG, Lilienfeld AM, Ferris FL, Fine SL. Senile macular degeneration: a case-control study. *Am J Epidemiol* 1983;118:213–227.
47. Church DF, Pryor WA. Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ Health Perspect* 1985;64:111–126.
48. Cruickshanks KJ, Klein R, Klein BEK, Nondahl DM. Sunlight and the 5-year incidence of early age-related maculopathy: the Beaver Dam Eye Study. *Arch Ophthalmol* 2001;119:246–250.
49. Taylor HR, West S, Munoz B, Rosenthal FS, Bressler SB, Bressler NM. The long-term effects of visible light on the eye [see comments]. *Arch Ophthalmol* 1992;110:99–104.
50. Tomany SC, Cruickshanks KJ, Klein R, Klein BEK, Knudtson MD. Sunlight and the 10-year incidence of age-related maculopathy. The Beaver Dam Eye Study. *Arch Ophthalmol* 2004;122:750–757.
51. Darzins P, Mitchell P, Heller RF. Sun exposure and age-related macular degeneration. An Australian case-control study. *Ophthalmology* 1997;104:770–776.
52. Delori FC, Goger DG, Dorey CK. Age-related accumulation and spatial distribution of lipofuscin in RPE of normal subjects. *Invest Ophthalmol Vis Sci* 2001;42:1855–1866.
53. Sparrow JR, Zhou J, Cai B. DNA is a target of the photodynamic effects elicited in A2E-laden RPE by blue light illumination. *Invest Ophthalmol Vis Sci* 2003;44:2245–2251.
54. Singhal SS, Godley BF, Chandra A, Pandya U, Jin GF, Saini MK, Awasthi S, Awasthi YC. Induction of glutathione S-transferase hGST 5.8 is an early response to oxidative stress in RPE cells. *Invest Ophthalmol Vis Sci* 1999;40:2652–2659.
55. Nelson KC, JL C, ML N, P S, DP J, TJ K, D D, J C, M W. Effect of dietary inducer dimethylfumarate on glutathione in cultured human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 1999;40:1927–1935.
56. Ham WT, Mueller HA, Ruffolo JJ, Millen JE, Cleary SF, Guerry RK, Guerry D. Basic mechanisms underlying the production of photochemical lesions in the mammalian retina. *Curr Eye Res* 1984;3:165–174.
57. Ham WTJ, Allen RG, Feeney-Burns L, Marmor MF, Parver LM, Proctor PH, Sliney DH, Wolbarsht ML. The involvement of the retinal pigment epithelium. In: Waxler M, Hitchins VM, eds. *CRC Optical Radiation and Visual Health*. Boca Raton, FL: CRC Press, 1986, pp. 43–67.
58. Busch EM, Gorgels TGMF, Roberts JE, van Norren D. The effects of two stereoisomers of N-acetylcysteine on photochemical damage by UVA and blue light in rat retina. *Photochem Photobiol* 1999;70:353–358.

Address reprint requests to:
Janet R. Sparrow, M.D.
Department of Ophthalmology
Columbia University
630 W. 168th Street
New York, NY 10032

E-mail: jrs88@columbia.edu