Involvement of Oxidative Mechanisms in Blue-Light–Induced Damage to A2E-Laden RPE

Janet R. Sparrow,[1] Jilin Zhou,[1] Shimon Ben-Shabat,[2] Heidi Vollmer,[2] Yasuhiro Itagaki,[2] and Koji Nakanishi[2]

Purpose. The lipofuscin fluorophore A2E is known to be an initiator of blue-light–induced apoptosis in retinal pigment epithelial cells (RPE). The purpose of this study was to evaluate the role of oxidative mechanisms in mediating the cellular damage.

Methods. Human RPE (ARPE-19) cells that had accumulated A2E were exposed to blue light in the presence and absence of oxygen, and nonviable cells were quantified. Potential suppressors (histidine, azide, 1,4-diazabicyclooctane [DABCO], and 1,3-dimethyl-2-thiourea [DMTU]) and enhancers (deuterium oxide [D_2O] and 3-aminotriazole [3-AT]) of oxidative damage, were also screened for their ability to modulate the frequency of nonviable cells. A2E in PBS, with and without an oxygen-depleter or singlet-oxygen quencher and A2E-laden RPE, were exposed to 430-nm light and examined by reversed-phase high performance liquid chromatography (HPLC) and fast atom bombardment mass spectrometry (FAB-MS).

Results. The death of blue-light–illuminated A2E-laden RPE was blocked in oxygen-depleted media. When A2E-laden RPE were transferred to D_2O-based media and then irradiated (480 nm), the number of nonviable cells was increased, whereas the latter was decreased in the presence of histidine, DABCO, and azide. Conversely, no affect was observed with 3-AT and DMTU. When A2E, in either acellular or cellular environments, was irradiated at 430 nm, FAB-MS revealed the generation of a series of higher molecular mass derivatives of A2E. The sizes of these species increased by increments of mass 16. The generation of these photo-products was accompanied by the consumption of A2E, the latter being diminished, however, when illumination was performed after oxygen depletion and in the presence of a singlet-oxygen quencher.

Conclusions. The augmentation of cell death in the presence of D_2O and the protection afforded by quenchers and scavengers of singlet oxygen, indicates that the generation of singlet oxygen may be involved in the mechanisms leading to the death of A2E-containing RPE cells after blue light illumination. The finding that irradiation also produces oxygen-dependent photochemical changes in A2E, indicates that the effects of singlet oxygen may be mediated either directly or through the generation of reactive photo-products of A2E. (*Invest Ophthalmol Vis Sci.* 2002;43:1222–1227)

The propensity for retinal pigment epithelial cells to be damaged or destroyed by excessive exposure to visible light may be of significance to retinal disorders characterized by enhanced accumulation of the autofluorescent pigments that constitute lipofuscin. Lipofuscin has long been considered capable of eliciting photodynamic damage.[1] Indeed, the major hydrophobic fluorophore of RPE lipofuscin, the diretinal adduct A2E, when accumulated within the lysosomal compartment of cultured RPE,[2] has been shown to confer a susceptibility to blue-light–mediated death,[3,4] which is proportional to the A2E concentration in the cultures and which is not manifest in cells devoid of A2E.[3] The wavelength dependency of the insult is consistent with the excitation spectra of A2E[3] and with the known susceptibility of RPE cells to blue light exposure in animal models.[5–10] Moreover, the photochemical events triggered by blue light in the context of intracellular A2E initiate a cell death pathway[3] that involves the activation of caspases and is modulated by the mitochondrial protein Bcl-2.[11]

A chromophore such as A2E mediates light damage, because it is capable of absorbing photons of specific energy by electron excitation. The photoexcited molecule can then either react directly with a substrate, or it can transfer the excitation energy onto an adjacent oxygen molecule, converting it to the singlet state, while the photosensitizer itself is returned to the ground state.[12] Subsequently, the singlet oxygen produced on illumination has the option of attacking the photosensitizer (photobleaching) and/or of reacting with other molecules present. Although less efficiently, some photosensitizers can also transfer electrons to oxygen to produce superoxide ($O^{\bullet}_2$) and then hydroxyl radical ($OH^{\bullet}$). Thus, all told, photoinduced damage can arise either through a direct reaction of the photoactivated molecule with cellular constitutents and/or through the formation of reactive oxygen species.

Previous studies have shown that isolated human RPE cells exhibit substantial light-dependent uptake of oxygen that appears to be related to their content of lipofuscin.[13] Photophysical studies have demonstrated that intact lipofuscin granules or chloroform-methanol extracts of lipofuscin can serve as photosensitizers for the generation of singlet oxygen and possibly superoxide and hydrogen peroxide.[13–16] In addition, in the presence of light and isolated lipofuscin, suspensions of RPE cells and rod outer segments undergo lipid peroxidation by mechanisms that can be inhibited by the antioxidants 1,4-diazabicyclooctane (DABCO) and superoxide dismutase.[17] Nevertheless, in these studies the identity of the reacting fluorophore was not known.

To begin to track the events that occur from the time that blue light is absorbed by intracellular A2E until the cell death program is initiated, we used a cell culture model that provides for populations of cells with and without intracellular A2E.[2] The results of our search for the intermediaries involved in the cellular damage not only implicate singlet oxygen, they also indicate that A2E undergoes photochemical changes that are

From the Departments of [1]Ophthalmology and [2]Chemistry, Columbia University, New York, New York.

Supported by National Eye Institute Grants EY-12951 (JRS) and GM-36564 (KN), Fight for Sight, the Charina Foundation, and Macula Vision Research Foundation. JRS is the recipient of a Research to Prevent Blindness Lew R. Wasserman Merit Award.

Submitted for publication September 24, 2001; revised December 11, 2001; accepted December 21, 2001.

Commercial relationships policy: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Janet R. Sparrow, Department of Ophthalmology, Columbia University, 630 W. 168th Street, New York, NY 10032; jrs88@columbia.edu.

Investigative Ophthalmology & Visual Science, April 2002, Vol. 43, No. 4

novel and that may be significant to our understanding of A2E-associated injury.

Materials and Methods

Deuterium oxide (D_2O; 99.96%) was obtained from ICN Biomedicals, (Aurora, OH); 3-aminotriazole (3-AT), L-histidine, and DABCO were obtained from Sigma Chemical Co. (St. Louis, MO); 1,3-dimethyl-2-thiourea (DMTU) was purchased from Aldrich Chemical Co. (Milwaukee WI) and 1,2,2,6,6-pentamethyl-4-piperidinol (NMP) was from Acros Organics (Geel, Belgium). Sodium azide was obtained from Fisher Scientific (Fairlawn, NJ).

Preparation of A2E-Laden RPE Cultures

Human adult RPE cells (ARPE-19; American Type Culture Collection, Manassas, VA), which are devoid of endogenous A2E,[2] were grown in Dulbecco's modified Eagle's medium (DMEM) and at confluence were incubated with A2E to allow for intracellular accumulation of the fluorophore, as previously documented by microscopy and HPLC.[2,3]

Illumination and Treatment

Confluent cultures were transferred to phosphate-buffered saline with calcium, magnesium and glucose (PBS-CMG) and were exposed either to a single spot of blue light delivered from a 100-W mercury lamp (480 ± 20 nm; 35 mW/mm^2; 60 seconds) or to a light line delivered from a tungsten halogen source (470 ± 20 nm; 0.4 mW/mm^2; 20-minute exposure),[3,11] as indicated.

For illumination in the presence of heavy water, D_2O was prepared at a final concentration of 90% in PBS-CMG. The D_2O-based solution was substituted for culture media just before light exposure, and the cells were returned to culture media immediately afterward. Blue-light–exposed, A2E-laden RPE incubated in H_2O-based PBS-CMG served as the control. To screen potential suppressors (histidine, azide, DABCO, and DMTU), and an enhancer (3-AT) of oxidative damage, stock solutions of histidine (200 mM), sodium azide (1 M), DMTU (1 M), and 3-AT (1 M) were prepared in PBS-CMG. A stock solution of DABCO (0.5 M) was prepared in 95% ethanol. Before illumination, cells were incubated for 1 hour (37°C) with each reagent diluted in culture media to the final concentrations indicated. Illuminations were subsequently performed in the presence of the reagent diluted in PBS-CMG. Control cultures were blue-light–exposed, vehicle-treated, A2E-laden RPE.

Oxygen-Depleted Medium

To remove oxygen from the media before illumination, cultures were preincubated at 37°C for 20 minutes with an oxygen depleter (Oxyrase; Oxyrase Inc., Mansfield, OH), diluted 1:50 in media with the addition of 20 mM sodium lactate. After light exposure, Oxygen-depleter–containing media were replaced with fresh media, and the cells were returned to the incubator.

Assaying Cell Viability

For illuminations performed in the presence of D_2O or oxygen depleter, cell viability was determined either 6 (D_2O) or 18 (oxygen depleter) hours after blue light exposure by labeling the nuclei of dead cells with a membrane-impermeable dye (Dead Red, 1:500 dilution, 15-minute incubation; Molecular Probes, Eugene, OR). Cell death was determined as labeled nuclei per zone of illumination (0.5-mm diameter). In all other experiments, the nonviable cells were assayed 8 hours after blue light exposure by labeling the nuclei of nonviable cells with the red dye, together with 4′6′-diamidino-2-phenylindole (DAPI) labeling of all nuclei. Counting was performed from digital images, and nonviable cells were expressed as a proportion of the total number of cells in a photographic field. In all experiments, replicates were assayed as indicated in the figure legends. Mean results were compared using the unpaired *t*-test, the Mann-Whitney nonparametric test, and ANOVA followed by the Student-Newman-Keuls multiple-comparison test.

Photo-Derivatives of A2E

Preparations of A2E in PBS (200 μM) were exposed to 430-nm light at intensities of 0.075, 0.095, 0.15, and 0.19 mW/mm^2 for 10 minutes After extraction in chloroform-methanol (2:1), separation of the chloroform layer, and evaporation, the samples were examined by fast atom bombardment mass spectrometry (FAB-MS). In some experiments, illumination was also performed in the presence of oxygen depleter (as described earlier) or the singlet-oxygen quencher NMP (100 mM). Subsequently, A2E was quantitated by reversed-phase HPLC (C18 column, 250 mm × 4.6 mm; Cosmosil 5C18; Nacalai Tesque, Kyoto, Japan) using the area of the A2E peak normalized to the internal standard, A2-propylamine, the latter being synthesized from all-*trans*-retinal and propylamine. To examine for photooxidation of intracellular A2E, A2E-laden ARPE-19 cells were irradiated (430 nm) at an intensity of 0.095 mW/mm^2 for 6 minutes. The pelleted cells were homogenized in chloroform-methanol (2:1) and water (vol/vol 3:1), filtered through a column (RP-C18 Sep-Pak; Millipore, Bedford, MA) followed by washing with methanol and 0.1% trifluoroacetic acid (TFA). After drying, the sample was redissolved in methanol and analyzed by FAB-MS.

Results

To determine whether the death of blue-light–illuminated, A2E-containing RPE occurs through oxygen-associated mechanisms, oxygen was removed from the media before illumination using an enzyme system (oxygen depleter) derived from *Escherichia coli*, which reduces dissolved oxygen to water in the presence of sodium lactate as hydrogen donor. Subsequent blue light illumination revealed that cell death was blocked by an average of 98% in the oxygen-depleted media (0.6 nuclei/illumination zone versus 40 nuclei/illumination zone, oxygen depleter–treated and control respectively; mean of 4 experiments, five to eight replicates/experiment). The numbers of nonviable cells in nonilluminated zones of oxygen-depleted A2E-laden cultures were not different from those in non-oxygen depleted cultures.

Given the observed oxygen-dependence of blue-light–induced apoptosis in our model, we began to evaluate the role of oxygen-derived species in mediating apoptosis by examining the capacity of enhancers (D_2O) and quenchers (histidine, DABCO, azide) of singlet oxygen to affect the frequency of nonviable cells after blue light illumination.[12] In D_2O, the lifetime of singlet oxygen is significantly prolonged; thus, when cells are placed in D_2O-based media, cellular responses to intracellularly generated singlet oxygen are potentiated.[18–24] Correspondingly, when A2E-laden cultures were blue light illuminated in the presence of D_2O-based buffered salts, we observed an increase of 66% in the numbers of nonviable cells compared with control cultures illuminated in H_2O-based buffered solution ($P = 0.001$; Fig. 1).

In the presence of sodium azide,[18–20,25,26] histidine,[21,24,26] and DABCO,[27–29] all of which act as efficient quenchers and scavengers of intracellularly generated singlet oxygen, consistent decreases in the numbers of nonviable cells were observed after blue light illumination (Fig. 1). Thus, azide at a concentration of 1 mM had no effect, whereas 5 mM azide reduced cell death by 43% ($P < 0.01$). Inclusion of 1 mM DABCO produced a difference of 25% compared with the control ($P = 0.06$). At higher concentrations, DABCO was toxic to the cells. A dose-dependent attenuation of cell death was also observed in the presence of histidine, with no effects observed at 0.1 and 1 mM concentrations ($P > 0.05$), but a reduction of 23% being observed in the presence of 10 mM

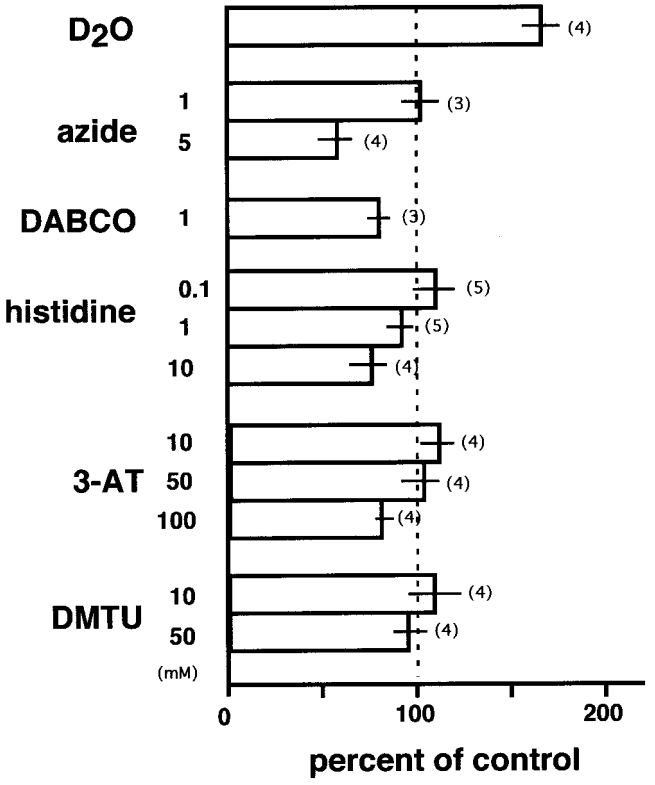

FIGURE 1. Agents capable of modulating oxygen derivatives: effects on the incidence of nonviable A2E-laden RPE after blue light illumination in culture. Concentrations (in millimolar) of the compounds are presented. Cultures were incubated with the compounds. Cell death was assayed in 5 to 10 fields of illumination per condition in each experiment. Data are presented as a percentage in relation to the control (blue-light–exposed, vehicle-treated, A2E-laden RPE) and are the mean ± SEM of the number of experiments indicated in parentheses.

histidine ($P < 0.01$) compared with the level of cell death in its absence.

To assay for participation of other oxygen-derived species in mediating blue light damage to A2E-containing cells, we also pretreated cultures with 3-AT, an inhibitor of the cellular antioxidant enzyme catalase, that can be expected to increase the susceptibility of cells to potential H_2O_2-induced damage.[24,30–32] However, the frequency of nonviable A2E-laden cells after blue light exposure was not increased when cultures were preincubated with 10, 50, and 100 mM concentrations of 3-AT for 1 hour ($P > 0.05$; Fig. 1), nor when pretreatment was extended to 6 hours (not shown). Indeed, at 100 mM 3-AT, there appeared to be a decrease in nonviable cells, although the difference between this level and the control level was not significant ($P > 0.05$). Similarly, DMTU, a strong hydroxyl radical (OH^-) scavenger with additional capability for eliminating superoxide anion radical ($O_2^{\bullet -}$) and H_2O_2,[12,33–35], had no effect on the incidence of cell death when used at concentrations of 10 and 50 mM ($P > 0.05$; Fig. 1).

Although these results suggest that the modulation of singlet oxygen has an impact on the frequency of nonviable, A2E-laden RPE after blue light illumination, we were also intrigued by the proclivity for intracellular A2E to undergo fluorescence quenching under blue light (data not shown). To begin to investigate this observation, A2E in aqueous media was exposed to 430 nm illumination (0.095 mW/mm^2, 10 minutes), and A2E was quantified by HPLC. The HPLC profile revealed a decrease in the absorbance of the A2E peak, denoting a loss of A2E after blue light illumination, compared with the control nonilluminated sample (Fig. 2). This decrement in A2E was attenuated, however, when the sample was incubated in oxygen depleter to reduce oxygen before 430-nm illumination (Fig. 2). The FAB-MS spectra of blue-light–illuminated A2E revealed not only a molecular ion peak at a mass-to-charge ratio (m/z) of 592, corresponding to the molecular mass of A2E ($C_{42}H_{58}ON$),[36] but also a series of additional molecular ion peaks (e.g., m/z 608, 624, 640, 656, 672, 688), each of which differed from its neighbors by mass 16 (Fig. 3). Moreover, the extent to which these derivatives of A2E were formed was dependent on the intensity of illumination. Thus, when the spectra derived from irradiances of 0.075, 0.095, and 0.15 mW/mm^2 were compared, it was apparent that as the irradiance increased, the higher-mass peaks became prominent, and additional peaks at m/z 656, 672, and 688 appeared. Concomitant with these spectral differences, the intensity of the A2E peak at m/z 592 was diminished (Figs. 3C, 3D), consistent with the light associated reduction of A2E observed by quantitative HPLC (Fig. 2) and fluorescence microscopy (not shown).

These photochemical changes in A2E were not limited, however, to preparation of A2E in aqueous media. When A2E-containing RPE cells were irradiated at an intensity of 0.095 mW/mm^2, extracted in chloroform-methanol as for the detection of A2E,[36] and analyzed by FAB-MS, prominent molecular ion peaks at m/z 608, 624, and 640 were observed in addition to the peak at m/z 592 attributable to A2E (Fig. 4). That these higher m/z peaks correspond to the photoproducts generated in aqueous media is supported by the observation that they were not present in unirradiated cells and by the detection of not just one, but the three peaks in series with A2E.

Finally, to investigate whether singlet oxygen was involved in the photooxidation and consumption of A2E, samples of A2E were irradiated (430 nm) in the presence of the singlet-oxygen quencher NMP. The latter compound is efficiently oxidized by singlet oxygen to an N-demethylated product.[37] Accordingly, although the amount of A2E in samples irradiated at 0.19 mW/mm^2 for 10 minutes was decreased by 97%, NMP (100 mM) reduced this loss to 75% (Fig. 5).

DISCUSSION

In this study, we demonstrated that the illumination of A2E with blue light leads to the formation of a specific pattern of

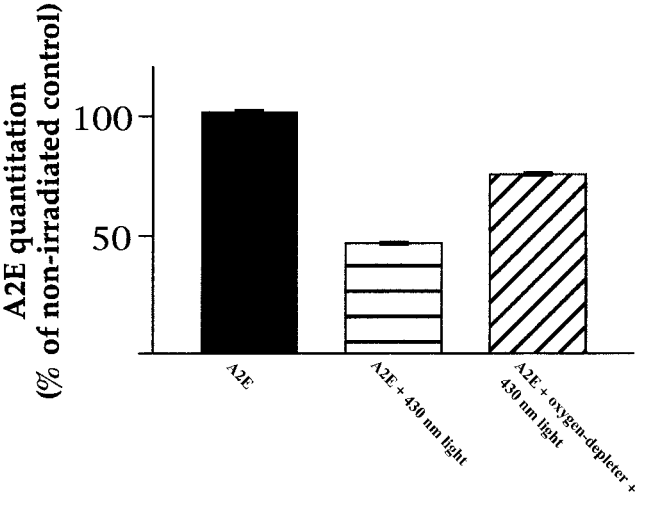

FIGURE 2. HPLC quantitation of A2E after blue light irradiation. The content of A2E in a sample was reduced after 430-nm illumination. The loss of A2E was diminished in the presence of an oxygen-depleter. Data are the mean ± SEM of three experiments.

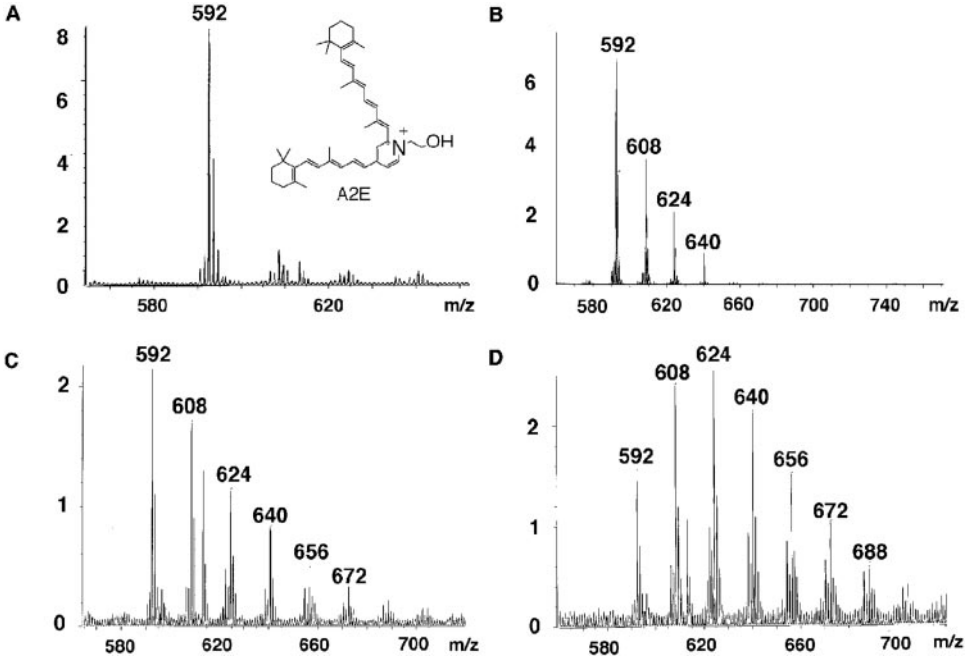

FIGURE 3. FAB-MS of blue-light–illuminated A2E. For nonirradiated A2E (**A**), the molecular ion peak at m/z 592 corresponded to the molecular mass of A2E. Illumination (430 nm) of A2E in PBS (200 μM) for 10 minutes at irradiances of 0.075 mW/mm^2 (**B**), 0.095 mW/mm^2 (**C**), and 0.15 mW/mm^2 (**D**) generated a series of molecular ion peaks, each of which differed from the previous peak by mass 16. (**A**, *inset*) Structure of A2E.

A2E-derived products and is accompanied by fluorescence quenching and consumption of A2E. That these photochemical changes in A2E involve the acceptance of oxygen, is supported by the finding that the sizes of the A2E-derived species exhibit mass increments of 16 and by the observation that the loss of A2E is decreased when oxygen is withdrawn from the media and when a singlet-oxygen quencher is added. The insertion of oxygen molecules into A2E is additive, because both the mass of the light-derived species and the abundance of these products progress with the intensity of the light exposure. Because an A2E molecule bears two chains of conjugated carbon-carbon double bonds (Fig. 3, insert), it is likely that oxygen molecules are inserted at these double bonds. We are currently performing structural studies to confirm this. It is important to note that these photooxidative changes in A2E also occur in a cellular environment.

The enhancement of cell death in the presence of D_2O and the protection afforded by quenchers and scavengers of singlet oxygen are both consistent with singlet-oxygen generation's being a contributing factor in blue-light–mediated death of A2E-containing RPE cells. In the presence of the singlet-oxygen quencher NMP, the photoxidative changes in A2E were diminished. Thus, singlet oxygen may not only mediate the cellular damage directly, it may also serve in the photooxidation of A2E with the products generated from the photochemical changes in A2E, being the ravaging agents. This is an important issue and one we are currently investigating.

Although our observations with respect to D_2O and quenchers and scavengers of singlet oxygen do not exclude the possibility that other reactive oxygen species are involved in the photooxidation of A2E, we did not obtain evidence for this prospect. Neither an inhibitor of catalase (3-AT) nor a scavenger of hydroxyl radical and superoxide (DMTU) had an effect on the frequency of cell death after blue light illumination of A2E. Conversely, DMTU has been shown to reduce the loss of 22:6 fatty acids from photoreceptor outer segments of light-exposed rats.[38]

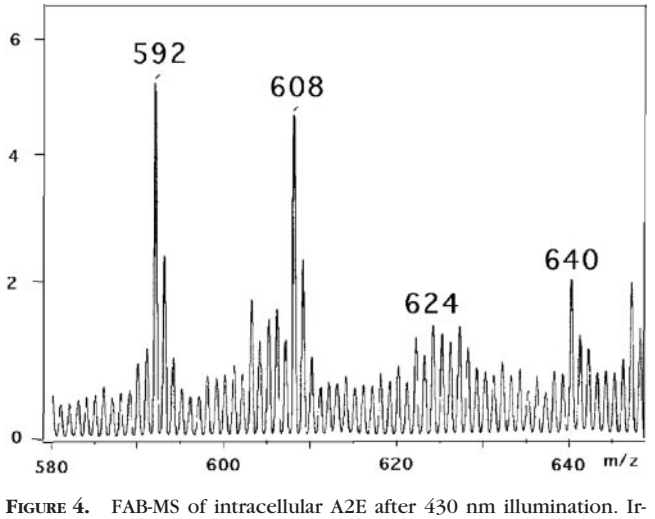

FIGURE 4. FAB-MS of intracellular A2E after 430 nm illumination. Irradiation at 0.095 mW/mm$_2$ yielded, in addition to the peak at m/z 592 attributable to A2E, a series of molecular ion peaks that differed by increments of 16.

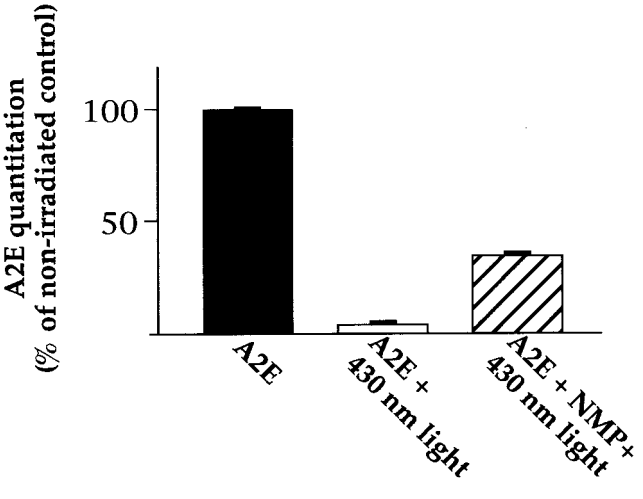

FIGURE 5. HPLC quantitation of blue-light–illuminated A2E in the presence of a singlet-oxygen quencher. The amount of A2E was reduced after 430-nm illumination. This loss of A2E was diminished in the presence of the singlet-oxygen quencher NMP (100 mM). Data are the mean ± SEM of three experiments.

The results of the present study are consistent with reports of blue light damage in animal models. Several investigators have observed that in monkeys[5,6] and rats[10] exposed to damaging levels of blue light the primary injury occurs in the RPE cells and is severe. Moreover, blue light damage was accentuated as arterial partial oxygen tensions were increased, whereas a diet that increased plasma levels of β-carotene, a singlet-oxygen scavenger, was protective.[5,6] Repeated illumination of monkey retina leads to RPE atrophy that investigators have described as being similar to that occurring in atrophic AMD.[39] Photochemical damage may emanate from a given amount of light, regardless of whether that amount of light is absorbed over a brief or extended period. In other words, over a range of exposure durations, the product of irradiance and exposure duration is constant.[1,40] Indeed, it is only cellular repair mechanisms that permit a deviation from this relationship, with long-duration exposure at low irradiances allowing repair processes to neutralize damage.[40]

In vivo fundus spectrophotometry,[41] laser scanning ophthalmoscopy,[42] and counts of lipofuscin granules[43] and measurements of fluorescence in histologic sections[44,45] all support the concept that the content of lipofuscin in RPE cells increases up to approximately age 70 years. An additional finding of some of these studies was, however, that lipofuscin fluorescence declines[41] or reaches a plateau[45] within the eighth decade. Although the death of lipofuscin-containing RPE cells is suggested to be a cause of the declining fluorescence in this older age group,[41] perhaps the change in fluorescence associated with photooxidation of A2E is an additional contributing factor.

References

1. Young RW. Solar radiation and age-related macular degeneration. *Surv Ophthalmol.* 1988;32:252–269.
2. 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.
3. 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.
4. Schutt F, Davies S, Kopitz J, Holz FG, Boulton ME. Photodamage to human RPE cells by A2-E, a retinoid component of lipofuscin. *Invest Ophthalmol Vis Sci.* 2000;41:2303–2308.
5. Ham WTJ, Allen RG, Feeney-Burns L, et al. 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.
6. Ham WTJ, Mueller HA, Ruffolo JJJ, et al. Basic mechanisms underlying the production of photochemical lesions in the mammalian retina. *Curr Eye Res.* 1984;3:165–174.
7. Ham WTJ, Ruffolo JJJ, Mueller HA, Clarke AM, Moon ME. Histologic analysis of photochemical lesions produced in rhesus retina by short-wavelength light. *Invest Ophthalmol Vis Sci.* 1978;17:1029–1035.
8. Putting BJ, Van Best JA, Vrensen GFJM, Oosterhuis JA. Blue-light-induced dysfunction of the blood-retinal barrier at the pigment epithelium in albino versus pigmented rabbits. *Exp Eye Res.* 1994;58:31–40.
9. Busch EM, Gorgels TGMF, van Norren D. Temporal sequences of changes in rat retina after UV-A and blue light exposure. *Vision Res.* 1999;39:1233–1247.
10. 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.
11. Sparrow JR, Cai B. Blue light-induced apoptosis of A2E-containing RPE: involvement of caspase-3 and protection by Bcl-2. *Invest Ophthalmol Vis Sci.* 2001;42:1356–1362.
12. Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine.* Oxford, UK: Oxford University Press; 1999.
13. Rozanowska M, Jarvis-Evans J, Korytowski W, Boulton ME, Burke JM, Sarna T. Blue light-induced reactivity of retinal age pigment: in vitro generation of oxygen-reactive species. *J Biol Chem.* 1995;270:18825–18830.
14. Gaillard ER, Atherton SJ, Eldred G, Dillon J. Photophysical studies on human retinal lipofuscin. *Photochem Photobiol.* 1995;61:448–453.
15. Reszka K, Eldred GE, Wang RH, Chignell C, Dillon J. The photochemistry of human retinal lipofuscin as studied by EPR. *Photochem Photobiol.* 1995;62:1005–1008.
16. Rozanowska M, Wessels J, Boulton M, et al. Blue light-induced singlet oxygen generation by retinal lipofuscin in non-polar media. *Free Radic Biol Med.* 1998;24:1107–1112.
17. Wassell J, Davies S, Bardsley W, Boulton M. The photoreactivity of the retinal age pigment lipofuscin. *J Biol Chem.* 1999;274:23828–23832.
18. Wlaschek M, Wenk J, Brenneisen P, et al. Singlet oxygen is an early intermediate in cytokine-dependent ultraviolet-A induction of interstitial collagenase in human dermal fibroblasts in vitro. *FEBS Lett.* 1997;413:239–242.
19. Wlaschek M, Briviba K, Stricklin GP, Sies H, Scharffetter-Kochanek K. Singlet oxygen may mediate the ultraviolet A-induced synthesis of interstitial collagenase. *J Invest Dermatol.* 1995;104:194–198.
20. Berneburg M, Grether-Beck S, Kurten V, et al. Singlet oxygen mediates the UVA-induced generation of the photoaging-associated mitochondrial common deletion. *J Biol Chem.* 1999;274:15345–15349.
21. Polo L, Presti F, Schindl A, Schindl L, Jori G, Bertoloni G. Role of ground and excited singlet state oxygen in the red light-induced stimulation of *Escherichia coli* cell growth. *Biochem Biophys Res Commun.* 1999;257:753–758.
22. Matroule JY, Carthy CM, Granville DJ, Jolois O, Hunt DW, Piette J. Mechanism of colon cancer cell apoptosis mediated by pyropheophorbide-a methylester photosensitization. *Oncogene.* 2001;20:4070–4084.
23. Runnels JM, Chen N, Ortel B, Kato D, Hasan T. BPD-MA-mediated photosensitization in vitro and in vivo: cellular adhesion and beta1 integrin expression in ovarian cancer cells. *Br J Cancer.* 1999;80:946–953.
24. Vile GF, Tyrrell RM. UVA radiation-induced oxidative damage to lipids and proteins in vitro and in human skin fibroblasts is dependent on iron and singlet oxygen. *Free Radic Biol Med.* 1995;18:721–730.
25. Chan W-H, Yu J-S. Inhibition of UV irradiation-induced oxidative stress and apoptotic biochemical changes in human epidermal carcinoma A431 cells by genistein. *J Cell Biochem.* 2000;78:73–84.
26. Grossman N, Schneid N, Reuveni H, Halevy S, Lubart R. 780 nm low power diode laser irradiation stimulates proliferation of keratinocyte cultures: involvement of reactive oxygen species. *Lasers Surg Med.* 1998;22:212–218.
27. Ohnishi Y, Murakami M, Wakeyama H. Effects of hematoporphyrin derivative and light on Y79 retinoblastoma cells in vitro. *Invest Ophthalmol Vis Sci.* 1990;31:792–797.
28. Mol de NJ, Beijersbergen von Henegouwen GMJ, Mohn GR, Glickman BW, van Kleef PM. On the involvement of singlet oxygen in mutation induction by 8-methoxypsoralen and UVA irradiation in *Escherichia coli* K-12. *Mutat Res.* 1981;82:23–30.
29. Hadjur C, Richard MJ, Parat MO, Favier A, Jardon P. Photodynamically induced cytotoxicity of hypericin dye on human fibroblast cell line MRC5. *J Photochem Photobiol B.* 1995;27:139–146.
30. Chuang S-M, Liou G-Y, Yang J-L. Activation of JNK, p38 and ERK mitogen-activated protein kinases by chromium (VI) is mediated through oxidative stress but does not affect cytotoxicity. *Carcinogenesis.* 2000;21:1491–1500.
31. Hiraishi H, Terano A, Ota S, et al. Antioxidant defenses of cultured gastric cells against oxygen metabolites: role of GSH redox cycle and endogenous catalase. *Am J Physiol.* 1991;261:G921–G928.
32. Masaki H, Okano Y, Sakurai H. Differential role of catalase and glutathione peroxidase in cultured human fibroblasts under exposure to H_2O_2 or ultraviolet B light. *Arch Dermatol Res.* 1998;290:113–118.

33. Collard CD, Agah A, Stahl GL. Complement activation following reoxygenation of hypoxic human endothelial cells: role of intracellular reactive oxygen species, NF-kappaB and new protein synthesis. *Immunopharmacology.* 1998;39:39–50.
34. Michael JR, Markewitz BA, Kohan DE. Oxidant stress regulates basal endothelin-1 production by cultured rat pulmonary endothelial cells. *Am J Physiol.* 1997;273:L758–L774.
35. Killilea DW, Hester R, Balczon R, Babal P, Gillespie MN. Free radical production in hypoxic pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol.* 2000;279:L408–L412.
36. 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.
37. Monroe BM. Quenching of singlet oxygen by aliphatic amines. *J Physical Chem.* 1977;81:1861–1864.
38. Organisciak DT, Darrow RA, Bicknell IR, Jiang Y-L, Pickford M, Blanks JC. Protection against retinal light damage by natural and synthetic antioxidants. In: Hollyfield JG, Anderson RE, La Vail MM, eds. *Retinal Degenerations.* Boca Raton, FL: CRC Press; 1991: 189–201.
39. Borges J, Li Z-Y, Tso MO. Effects of repeated photic exposures on the monkey macula. *Arch Ophthalmol.* 1990;108:727–733.
40. Mellerio J. Light effects on the retina. In: Albert DM, Jakobiec FA, eds. *Principles and Practice of Ophthalmology: Basic Science.* Philadelphia: WB Saunders; 1994:1326–1345.
41. 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.
42. von Rückmann A, Fitzke FW, Bird AC. Fundus autofluorescence in age-related macular disease imaged with a laser scanning ophthalmoscope. *Invest Ophthalmol Vis Sci.* 1997;38:478–486.
43. 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.
44. Wing GL, Blanchard GC, Weiter JJ. The topography and age relationship of lipofuscin concentration in the retinal pigment epithelium. *Invest Ophthalmol Vis Sci.* 1978;17:601–607.
45. Okubo A, Rosa RHJ, Bunce CV, et al. The relationships of age changes in retinal pigment epithelium and Bruch's membrane. *Invest Ophthalmol Vis Sci.* 1999;40:443–449.