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A2E-epoxides Damage DNA in Retinal Pigment Epithelial Cells

VITAMIN E AND OTHER ANTIOXIDANTS INHIBIT A2E-EPOXIDE FORMATION*

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The autofluorescent pigments that accumulate in retinal pigment epithelial cells with aging and in some retinal disorders have been implicated in the etiology of macular degeneration. The major constituent is the fluorophore A2E, a pyridinium bisretinoid. Light-exposed A2E-laden retinal pigment epithelium exhibits a propensity for apoptosis with light in the blue region of the spectrum being most damaging. Efforts to understand the events precipitating the death of the cells have revealed that during irradiation (430 nm), A2E self-generates singlet oxygen with the singlet oxygen in turn reacting with A2E to generate epoxides at carbon-carbon double bonds. Here we demonstrate that A2E-epoxides, independent of singlet oxygen, exhibit reactivity toward DNA with oxidative base changes being at least one of these lesions. Mass spectrometry revealed that the antioxidants vitamins E and C, butylated hydroxytoluene, resveratrol, a trolox analogue (PNU-83836-E), and bilberry extract reduce A2E-epoxidation, whereas single cell gel electrophoresis and cell viability studies revealed a corresponding reduction in the incidence of DNA damage and cell death. Vitamin E, a lipophilic antioxidant, produced a more pronounced decrease in A2E-epoxidation than vitamin C, and treatment with both vitamins simultaneously did not confer additional benefit. Studies in which singlet oxygen was generated by endoperoxide in the presence of A2E revealed that vitamin E, butylated hydroxytoluene, resveratrol, the trolox analogue, and bilberry reduced A2E-epoxidation by quenching singlet oxygen. Conversely, vitamin C and ginkgolide B were not efficient quenchers of singlet oxygen under these conditions.

The di-retinal conjugate A2E forms as a consequence of light related vitamin A cycling in the retina. This orange-emitting fluorophore is formed synthetically as the condensation product of all-*trans*-retinal and ethanolamine (1–3). NMR and corroborative total chemical synthesis revealed A2E to be a pyridinium bisretinoid consisting of an unprecedented pyridinium polar head group and two hydrophobic retinoid tails (4, 5). A2E, its slightly less polar photoisomer, iso-A2E, and other minor *cis*-isomers together constitute the most prominent age-related hydrophobic pigments (lipofuscin) in retinal pigment epithelial (RPE)[1] cell extracts assayed by reverse phase HPLC (3, 6). *In vivo*, A2E is generated by phosphate hydrolysis of the fluorophore phosphatidylpyridinium bisretinoid, the latter precursor forming from reactions between all-*trans*-retinal and phosphatidylethanolamine in the photoreceptor outer segment membrane (3, 7, 8).

Although certain levels of A2E are clearly tolerated by RPE cells, adverse effects of its accumulation have also been reported. Thus, not only can A2E mediate detergent-like effects on cell membranes (9), its accumulation can also lead to the alkalinization of lysosomes (10) and to the detachment of pro-apoptotic proteins from mitochondria (11). A2E also bestows a sensitivity to blue light damage (11–13) that is proportional to the A2E content of the cells that is not exhibited by cells devoid of A2E and that exhibits a wavelength dependence that reflects the excitation spectrum of A2E (12). Evidence indicates that the generation of oxygen reactive species upon photoexcitation of A2E is integral to the death of the cells. For instance, an enhancer (D_2O) and quenchers (histidine, DABCO, and azide) of singlet oxygen modulate the incidence of nonviable A2E-laden RPE following blue light illumination (14). Importantly, A2E itself undergoes photoxidation during irradiation with blue light to produce a series of epoxide rings along the retinoid side arms of the molecule (A2E-epoxides) (14, 15). The extent of epoxidation is dependent on the intensity and duration of illumination with irradiated A2E forming a mixture of compounds exhibiting epoxides of varying numbers, including an unprecedented nonaoxirane. The involvement of singlet oxygen in the photoxidation of A2E is indicated by the phosphorescence detection of singlet oxygen upon 430-nm irradiation of A2E by deuterium solvent potentiation, by inhibitory effects of singlet oxygen quenchers, and by experiments demonstrating that endoperoxide-derived singlet oxygen can substitute for blue light irradiation (14, 15).

We recently demonstrated that DNA is one of the subcellular targets of the photodynamic events initiated by the interaction of blue light and A2E (16). Although the exact mechanism by which the DNA damage occurs is not known, chemical reac-

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[1] The abbreviations used are: RPE, retinal pigment epithelial cells; 8-oxo-dG, 8-oxo-deoxyguanosine; A2E, pyridinium bisretinoid; AMD, age-related macular degeneration; BHT, 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene); D_2O, deuterium oxide; DABCO, 1,4-diazabicyclooctane; DPBS, Dulbecco's phosphate buffered saline; FAB-MS, fast atom bombardment ionization mass spectrometry; HPLC, high performance liquid chromatography; ANOVA, analysis of variance.

tions between DNA and the epoxides of photooxidized A2E may be a critical step. Because of the large ring strain and electrophilicity of these carbon- and oxygen- (2:1) containing three-membered epoxide rings, nucleophilic macromolecules such as proteins and DNA tend to react with them spontaneously (17, 18). In fact, several classes of carcinogens that induce structural changes in DNA are known to react with guanosine and to a lesser extent adenosine and cytosine through epoxide moieties (19–26). In some cases, these reactions generate bulky adducts. Intrastrand and interstrand cross-links can also form when two or more epoxides are available for reaction (23, 27). Moreover, modifications associated with skeletal aromatic nitrogens of DNA bases can be destabilizing. Consequently, secondary transformations such as facile depurination or imidazole ring opening at *N*-7-alkyl-deoxyguanosines (17) can occur.

Here we report that A2E-epoxides can generate DNA lesions independent of direct damage by singlet oxygen. By mass spectrometry and quantitative HPLC, we have also screened some antioxidants for their ability to inhibit A2E-epoxide formation.

EXPERIMENTAL PROCEDURES

Reagents—Resveratrol and PNU-83836-E were obtained from Pharmacia. (*L*)-Ascorbic acid (vitamin C), BHT, m-chloroperoxybenzoic acid, ethanolamine, and trifluoroacetic acid were purchased from Aldrich. HEPES, α-tocopherol acetate, α-tocopherol, and dehydroascorbic acid were obtained from Sigma. Bilberry extract was from Nature's Resource Premium Herb (Mission Hills, CA). Acetonitrile was purchased from Fisher, and Dulbecco's phosphate-buffered saline (DPBS) was from Invitrogen. Glass-backed TLC were purchased from Merck (Darmstadt, Germany). All of the other chemicals were from Sigma. A2E was synthesized as described previously (3).

Cell Culture and Illumination—Human adult RPE cells (ARPE-19, American Type Culture Collection, Manassas, VA) lacking endogenous A2E (9) were grown as described previously (9, 12). To generate A2E-laden RPE, nonconfluent cultures were allowed to accumulate A2E from a 20 μM concentration in medium (9). For some experiments, cultures were treated prior to blue light illumination with α-tocopherol (100 μM) or α-tocopherol acetate (100 μM) prepared as a 1/1000 dilution of stock solution in ethanol for 24 h. For the accumulation of ascorbic acid, cells were pretreated for 30 min with dehydroascorbic acid (100 μM), the latter being reduced intracellularly to ascorbate (28). Dehydroascorbic acid was diluted in medium from a 10 mM stock solution in DPBS. Cells were subsequently exposed to 430-nm illumination (0.36 milliwatt/mm^2) as described previously (12–14). To assess cell viability, cultures were exposed to a 430-nm light line (0.8-mm wide, 0.34 milliwatt/mm^2).

A2E-epoxides—A2E-epoxides were generated by illuminating (430 nm, 10-min exposure, 0.36 milliwatt/mm^2) 200 μM A2E (3) in DPBS with calcium, magnesium, and glucose. Under these conditions, >50% A2E in the sample undergoes epoxidation with 1–7 epoxides (out of a possible maximum of nine) forming on the parent A2E molecule (14). A2E-bisepoxide, a compound containing a single epoxide on each of the two side arms of A2E (positions 7,8 and 7′,8′), was also generated by oxidation with m-chloroperoxybenzoic acid as described previously (15). To determine whether DNA damage can be induced by epoxidized A2E, A2E-epoxides and A2E-bisepoxide were subsequently incubated with ARPE-19 cells (specifically, A2E-free cells). To ensure that epoxidized A2E could permeate the cell membrane to access the nuclear material in these experiments, two modifications of the protocol were used. First, the cells were permeabilized on ice (for 1 h) before incubation with A2E-epoxides (for 3 h) or A2E-bisepoxide (for 5 h). As a second approach, a modified comet assay was performed in which the agarose-embedded cells were incubated with A2E-epoxides or A2E-bisepoxide (at 1 h) before lysis (29). The results were similar with these two approaches, and the data were pooled.

For analysis by FAB-MS, preparations of A2E (200 μM) in DPBS with and without the antioxidants (100 μM) vitamin E, vitamin C, BHT, ginkgolide B, bilberry, resveratrol, or PNU-83836E were exposed to 430-nm light (0.36 milliwatt/mm^2). In other experiments, A2E (500 μM) was epoxidized by incubation with a singlet oxygen generator, the endoperoxide of 1,4-dimethylnaphthalene (20 mM in 75 μl of methanol). Stock solutions of the antioxidants vitamin E, vitamin C, BHT, ginkgolide B, bilberry, or resveratrol were prepared in Me_2SO and added (25 μl) to a final concentration of 40 mM. The mixture was stirred for 14–15 h in the dark at room temperature, and afterward, the sample was stored at −80 °C. The samples either underwent HPLC quantitation (3, 9, 12) using A2E as external standard or were analyzed by FAB-MS.

Single Cell Gel Electrophoresis—DNA damage was probed using the comet assay (Trevigen, Gaithersburg, MD). After treatment, cells were released from the well by brief trypsinization, and after being added to low melting point agarose (1×10^5 cells/ml, 1/10 dilution, 37 °C), 75-μl aliquots of the mixture were pipetted onto comet slides and allowed to gel. After incubating in lysis solution at 4 °C for 90 min followed by washing and denaturation in an alkali buffer for 45 min (room temperature and darkness), the slides were transferred to a horizontal chamber for electrophoresis (1 V/cm, 30 min) in alkali solution (0.3 M NaOH, 1 mM EDTA, pH >13) on ice. Finally, the slides were immersed in ethanol (5 min), air-dried, and stained with SYBR Green. Because comet tail moment departs from normality (30), the means were compared using the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test (Prism, GraphPad Software, San Diego, CA). The level of significance was 0.05.

Immunostaining for 8-Oxodeoxyguanosine—Detection of 8-oxodeoxyguanosine (8-oxo-dG), the oxidized derivative of the parent nucleoside deoxyguanosine, was performed by immunostaining with monoclonal antibody to 8-oxo-dG (Trevigen Inc.). After the cells were fixed in 70% ethanol (−20 °C, 10 min) and DNA was denatured (4 N HCl, 7 min), the cells were incubated in blocking serum (10% fetal bovine serum in 10 mM Tris-HCl, pH 7.5, for 1 h at 37 °C) followed by anti-8-oxo-dG diluted 1/300 in blocking serum (4 °C, overnight). Visualization was done by an alkaline phosphatase detection system (ABC-AP, Vector Laboratories, Burlingame, CA) with Vector Red (Vector Laboratories) as substrate.

HPLC—A Waters 600E HPLC equipped with Waters 996 photodiode array detector was used with a reverse phase C18 column (250 × 4.6 mm, Cosmosil 5C18, Nacalai Tesque, Osaka, Japan). A2E was eluted with the following gradient of acetonitrile in water (containing 0.1% trifluoroacetic acid): 84–96% (10 min), 96% (5 min), 96–100% (2 min), and 100% (3 min) and a flow rate of 1 ml/min with monitoring at 430 nm. For A2E quantitation, an external standard of A2E was used.

Mass Spectrometry—FAB-MS was performed on a JEOL JMS-HX110A/110A tandem MS (Akishima, Tokyo, Japan) using 10-kV acceleration voltage and fitted with a Xe beam FAB gun (6 kV) on the MS-1 ion source. 3-Nitrobenzyl alcohol was used as matrix.

Assay of Cell Viability—The nuclei of nonviable cells were labeled with the membrane-impermeant dye Dead Red (Molecular Probes, Eugene OR), and the nuclei of all of the cells were labeled with DAPI (4′,6-diamidino-2-phenylindole) as described previously (14). Counting was performed from digital images, nonviable cells were expressed as a proportion of the total number of cells in an illuminated field, and means were compared by ANOVA followed by the Student's-Newman Keul multiple comparison test. The level of significance was 0.05.

RESULTS

A2E-epoxides Can Induce Cellular Damage—To test the reactivity of A2E-epoxides with cellular macromolecules, A2E-epoxides (1–7 epoxides on each A2E molecule) were incubated with permeabilized ARPE-19 cells. To detect direct strand breaks and alkali labile sites (31, 32), single cell gel electrophoresis was performed under alkaline conditions that allow DNA to be drawn out into a comet tail when subjected to an electrical field. As shown in Fig. 1, the presence of strand breaks was visualized by the emergence of comet tails from the nuclei of A2E-epoxide-treated cells. The nuclei of A2E- and vehicle-treated cells remained spherical. The presence of comet tails in A2E-epoxides-treated cells corresponded to significantly elevated ($p < 0.01$) measures of tail moment, a parameter whose magnitude reflects the frequency of DNA strand breaks per nucleus (Fig. 2*A*) (30, 33, 34). Moreover, 83% nuclei were associated with values of mean tail moment that were 2 S.D. greater than the mean of vehicle-treated cells (range 48–98%, five experiments). Conversely, the values for mean tail moment obtained for the A2E-treated and vehicle-treated cells were consistent with that of intact nuclei. In the experiments described above, the cells were permeabilized on ice for 1 h before incubation with A2E-epoxides. Nevertheless, less pronounced (60% of that observed with permeabilized cells) measures of tail moment were also observed when nonpermeabilized cells were incubated with A2E-epoxides. Elevated mean tail

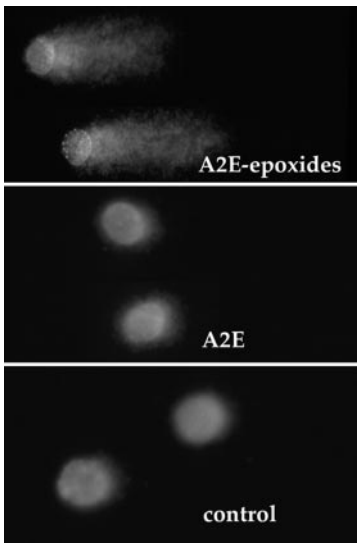

FIG. 1. **A2E-epoxides induce DNA damage.** Cells were processed by alkaline comet assay. RPE cells were incubated with A2E after it was irradiated at 430 nm (*A2E-epoxides*) or with nonirradiated A2E (*A2E*) and PBS (*control*). Comets induced with A2E-epoxides are indicative of DNA damage.

moment, indicative of DNA damage, was also observed when A2E-bisepoxide was incubated with ice-permeabilized cells or with nonlysed cells embedded in agarose (Fig. 2*B*). The detection of comets after cells are incubated with A2E-epoxides indicates that the latter can injure DNA independent of singlet oxygen. Under the current experimental design, singlet oxygen that was generated by blue light irradiation of A2E (15) but which has a short life time would have been unavailable to react directly with DNA.

A2E-epoxides Induce the Formation of 8-Oxo-dG—The epoxide moiety of aflatoxin B1 (35, 36) among several other agents (37) has been shown to oxidize the C8 position of guanine to form 8-oxo-dG. Therefore, we sought to determine whether cells treated with A2E-epoxides exhibited the same base modification. Immunocytochemical labeling with monoclonal antibody to 8-oxo-dG revealed specific nuclear staining in cells incubated with A2E-epoxides, whereas staining was absent in control cells incubated with vehicle (DPBS) (Fig. 3).

Vitamins E and C Protect against Blue Light-mediated DNA Damage to A2E-laden RPE—We have previously established that singlet oxygen is involved in the epoxidation of A2E (15). Accordingly, because vitamins E and C are naturally occurring compounds with antioxidant capability, we tested their efficacy in preventing DNA damage when A2E-laden RPE are blue light-irradiated. When analyzed by alkaline comet assay with the measurement of tail moment, pretreatment with vitamin E resulted in a dose-dependent decrease in the formation of single strand breaks with significant decreases ($p < 0.01$) being obtained at 1, 10, and 100 μM concentrations (Fig. 4). Preincubation with α-tocopherol (100 μM) and α-tocopherol acetate (100 μM), a hydrophilic analog of α-tocopherol, attenuated mean tail moment to a similar extent (44–58 and 52–66%, respectively), and thus, the data were pooled. Pretreatment with dehydroascorbic acid, the oxidized and readily accumulated form of ascorbic acid (vitamin C) also provided protection against DNA damage in blue light-illuminated A2E-laden RPE, although vitamin E was more effective than vitamin C at lower doses (1 and 10 μM). Specifically, only vitamin E generated statistically significant differences ($p < 0.01$) at the dose of 1 μM. Although the data at the 10 μM concentration were statistically significant for both vitamins E and C ($p < 0.01$ and $p < 0.05$, vitamins E and C, respectively), the effect was greater with vitamin E (47 *versus* 19% reduction, vitamins E and C, respectively). When treatment with vitamins E and C was combined, the difference in tail moment as compared with vitamin E alone was not statistically significant (Fig. 4). Conversely, combined treatment produced a 40% decrease over that with vitamin C alone, a decrease that was statistically significant ($p < 0.01$). Taken together, these findings indicate that vitamin E is more effective than vitamin C in resisting blue light-mediated DNA damage to A2E-laden RPE.

The ability of vitamins E and C to confer a resistance to blue light damage in the context of intracellular A2E was further exemplified in experiments in which the viability of A2E-containing RPE was assayed after blue light illumination (Fig. 4). Again, pre-incubation with vitamin E yielded a more pronounced protective effect than that of vitamin C with the additive effects of vitamins E and C being small and not significantly different.

Vitamins E and C Reduce Blue Light-induced A2E Epoxidation—To establish whether the protective effects of vitamins E and C were mediated, at least in part, by inhibiting light-induced A2E-epoxidation, A2E in aqueous medium was exposed to 430-nm illumination followed by FAB-MS analysis (Fig. 5). The FAB-MS spectra of blue light-irradiated A2E disclosed a molecular ion peak at *m/z* 592, corresponding to the mass of A2E, and an additional series of higher molecular mass peaks representing the sequential insertion of oxygens at carbon-carbon double bonds. We have previously shown that these additional peaks correspond to A2E-epoxides (15). When A2E was irradiated in the presence of vitamin E or C, the higher mass peaks arising from the epoxides were suppressed as evidenced by the overall reduction in epoxide peak intensity and by the loss of the peaks at *m/z* 688 and 704. Moreover, vitamin E appeared to be more effective than vitamin C at reducing the generation of molecular ion peaks corresponding to A2E-epoxides. Not only were the molecular ion peaks of lower intensity, only three epoxides were detectable (*m/z* 608, 624, and 640) in the presence of vitamin E as compared with six with vitamin C.

Vitamin E but Not Vitamin C Protects against A2E-epoxidation by Quenching Singlet Oxygen—To determine whether vitamins E and C inhibit A2E-epoxidation by quenching singlet oxygen, we generated singlet oxygen from the decomposition of the endoperoxide of 1,4-dimethylnaphthalene, which decomposes with a half-life of ~5 h at 25 °C into 1,4 dimethylnapthalene and singlet oxygen. We previously showed that for the generation of A2E-epoxides, the endoperoxide can substitute for blue light irradiation (15). Methanol solutions of A2E (500 μM), the endoperoxide of 1,4-dimethylnaphthalene (20 mM), and antioxidant (40 mM) were incubated for 14–15 h at room temperature. The extent of A2E-epoxidation in the presence and absence of vitamin E or vitamin C was monitored by quantifying the consumption of A2E by HPLC (14). As shown in Fig. 6, the incubation of A2E in the presence of the singlet oxygen generator but without antioxidant resulted in approximately a 50% decrease in measurable levels of A2E. The addition of vitamin E (α-tocopherol, 40 mM) conserved A2E at levels comparable to control samples not exposed to the endoperoxide ($p < 0.01$). The same concentration of vitamin C (L-ascorbic acid), however, did not attenuate the loss of A2E ($p > 0.05$). This finding was consistent with our results, indicating that vitamin E was more effective than vitamin C in suppressing A2E-epoxidation. Moreover, it appears that whereas vitamin E reduces A2E-epoxidation by singlet oxygen quenching, the protective effect of vitamin C depends, to at least some extent, on mechanisms other than singlet oxygen quenching.

Antioxidants Vary in Their Ability to Inhibit A2E-epoxidation by Quenching Singlet Oxygen—In addition to vitamins E

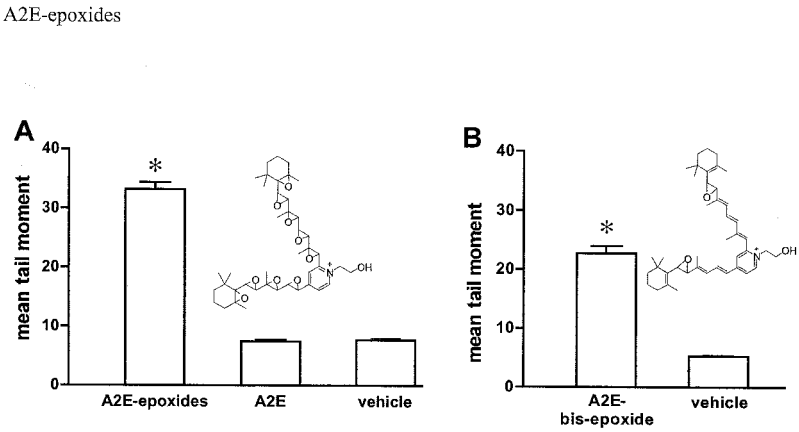

FIG. 2. **Quantitation of DNA damage induced by epoxidized A2E.** *A*, cells were incubated with A2E-epoxides generated by irradiation at 430 nm, nonirradiated A2E (*A2E*), or PBS (*vehicle*). *B*, incubation with A2E-bisepoxide produced by oxidation with m-chloroperoxybenzoic acid. Comet assay performed with quantification by tail moment. Mean ± S.E. of 2–5 experiments. *, $p < 0.01$ as compared with A2E and vehicle. *Insets* in *A* and *B* illustrate structures of A2E-nonaepoxide (the species within the mixture of A2E-epoxides that has the maximum number of epoxides) and A2E-bisepoxide (two epoxides), respectively.

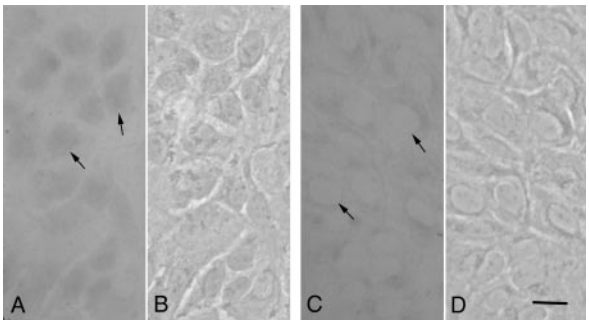

FIG. 3. **A2E-epoxides mediate base-specific DNA damage.** Immunocytochemical staining with antibody to 8-oxo-dG is shown. Cells were incubated with A2E that had been previously irradiated at 430 nm (A2E-epoxides) (*A*) or with PBS (*C*). *B* and *D*, phase-contrast images of fields shown in *A* and *C*, respectively. *Arrows*, stained (*A*) and unstained (*C*) nuclei. Scale bar, 10 μm.

and C, we compared several other antioxidants in terms of their ability to inhibit the epoxidation of A2E. From the FAB-MS spectra obtained after samples of A2E were irradiated at 430 nm in the absence of antioxidant, it was apparent that as many as seven epoxides (*m/z* 608, 624, 640, 656, 672, 688, and 704) formed along the retinal-derived side arms of A2E (Fig. 7). However, only the monoepoxides and bisepoxides (*m/z* 608 and 624) formed in the presence of BHT, a well known antioxidant. Similarly, only the monoepoxides, bisepoxides, and triepoxides (*m/z* 608, 624, and 640) formed in the presence of resveratrol, an antioxidant derived from grapes (38) or PNU-83836E, a water-soluble trolox analogue and chroman derivative, which is related to vitamin E and which acts to restore endogenous vitamin E (39). Up to four epoxides (monoepoxides through tetraepoxides; *m/z* 608, 624, 640, and 656) were generated when irradiation was performed in the presence of bilberry (*Vaccinium myrtillus*), a widely used nutritional supplement exhibiting antioxidant activity (40, 41). On the other hand, ginkgolide B, a diterpenoid trilatone (from Ginkgo biloba), which has demonstrated antioxidant effects under other conditions (42), showed only a small effect in reducing A2E-epoxidation when evaluated by FAB-MS (Fig. 7).

To establish whether the effect of these antioxidants on A2E-epoxidation occurred through the quenching of singlet oxygen, the latter was generated from the decomposition of endoperoxide and the A2E consumption that accompanies epoxidation was quantified by HPLC. In the presence of BHT, the loss of A2E was reduced from 50 to 4%, such that the amount of A2E in the sample was not significantly different from that in the absence of the endoperoxide (Fig. 6). Similar protection was mediated by bilberry and resveratrol with bilberry, reducing the loss of A2E from 50 to 3% and resveratrol diminishing the loss to 5%. Just as with vitamin C, however, ginkgolide B did not protect against A2E-epoxidation and consumption of A2E when the latter was exposed to the singlet oxygen generated from endoperoxide. Thus, at least under these conditions, ginkgolide B appears not to quench singlet oxygen.

DISCUSSION

We previously observed that the double bond structure of A2E predisposes it to reaction with the singlet oxygen that is autogenerated by irradiation with blue light, the oxidation of the double bonds leading to epoxide formation (14, 15). Thus, at the outset of this work, we reasoned that as alkylating agents, these epoxides would exhibit reactivity toward cellular macromolecules. Accordingly, we have shown that A2E-epoxides, both a mixture with varying numbers of epoxides on the A2E side arms and bisepoxide, can induce DNA lesions within cultured RPE cells. Moreover, by immunoperoxidase labeling with monoclonal antibodies that recognize 8-oxo-dG, we demonstrated in individual cells that at least one of these lesions is an oxidatively modified guanine base. Although cells, including ARPE-19 cells (16), have developed mechanisms for repairing oxidative lesions to DNA bases, a decline in repair activity with age has been recognized (43, 44). Indeed, for cells that do not turnover as is the case for RPE, there is a greater accumulation of such lesions (45). The persistence of DNA damage can result in altered gene expression with the transcription of some genes being arrested, while the transcription of other genes is induced (46). DNA is not the only macromolecule in RPE cells toward which A2E-epoxides are likely to react because epoxides can react with a large range of nucleophiles, the latter attacking the electrophilic carbons of the epoxide, causing it to open. Examples of common cellular nucleophiles include the sulfhydryl (SH) groups of proteins, the electron-rich nitrogen atoms of the amino groups (NH_2) of proteins and DNA, and the oxygen atom in a hydroxyl ion (OH).

It is well established that RPE cells that have accumulated A2E are subject to blue light-mediated injury (12–14, 16), and we showed that at least one of the cellular macromolecules damaged is DNA. Although we report here that A2E-epoxides can damage DNA and probably other cellular constituents, the singlet oxygen generated by the photosensitization of A2E is an additional potentially important cytotoxic agent. Within polar

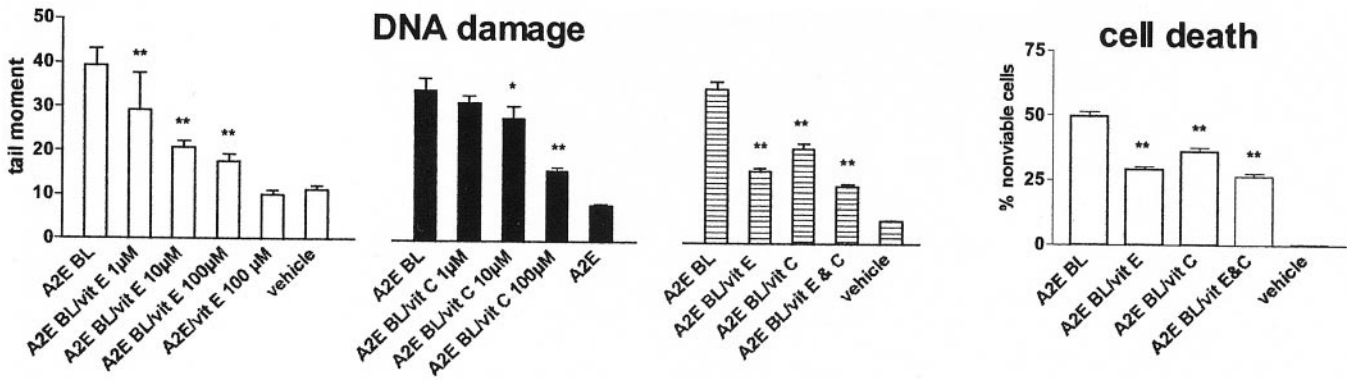

FIG. 4. **Modulation of DNA damage and cell death in the presence of vitamin E, vitamin C, and the latter antioxidants in combination.** DNA damage was detected by single cell gel electrophoresis (comet assay) and was quantified by tail moment (50 nuclei/experiment). Percentage of nonviable cells was determined by labeling all of the nuclei with DAPI and nuclei of nonviable cells with a membrane-impermeable dye. Concentrations of 100 μM were used for combined treatment and cell death assay. Values are the mean ± S.E. of 2–5 experiments. *, $p < 0.05$; **, $p < 0.01$ as compared with conditions of A2E and blue light (*A2E BL*).

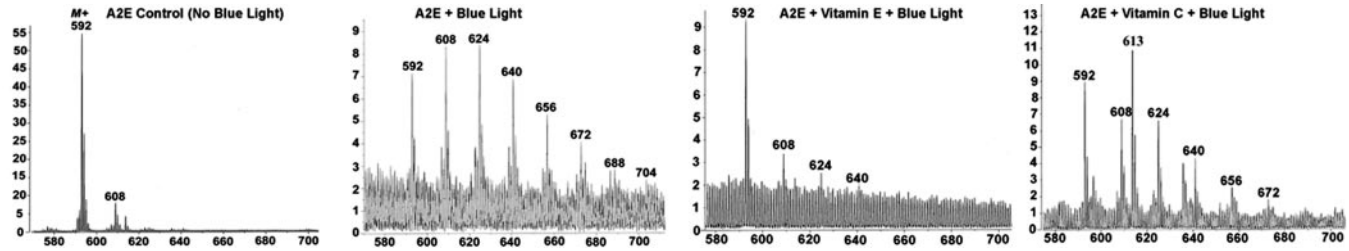

FIG. 5. **Vitamins E and C reduce the formation of A2E-epoxides.** FAB-MS of nonirradiated A2E (*A2E control*), A2E exposed to blue light (*A2E + Blue Light*), and A2E irradiated with blue light in the presence of vitamin E (200 μM) or vitamin C (200 μM) is shown. The molecular ion peak at *m/z* 592 corresponds to the molecular mass of A2E. The formation of A2E-epoxides by illumination is indicated by the presence of additional peaks that differ by mass 16. Illumination in the presence of vitamin E or C reduces the formation of these epoxides. The peak at 613 is a matrix peak.

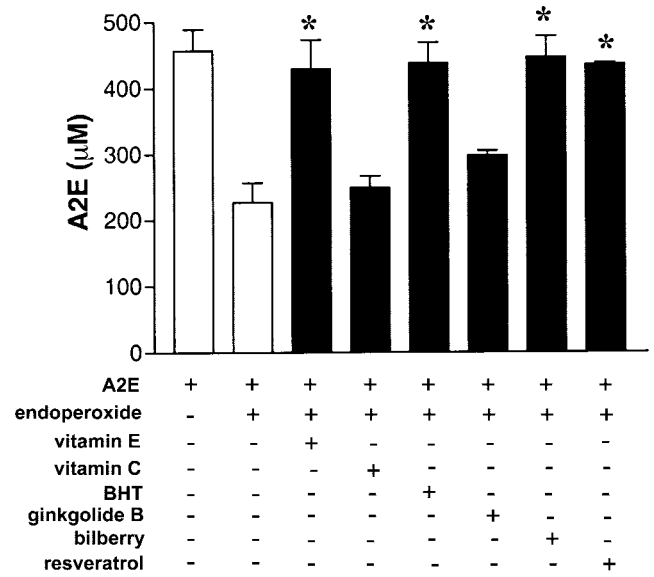

FIG. 6. **Antioxidants vary in their ability to inhibit A2E-epoxidation by quenching singlet oxygen.** The consumption of A2E, which accompanies A2E-epoxidation, was quantified by HPLC after A2E was exposed to singlet oxygen generated from the endoperoxide of 1,4-dimethylnaphthalene (20 mM) with and without the addition of various antioxidants (40 mM). The symbols (+) and (−) indicate the presence or absence, respectively, of a compound. *, values not different from levels of A2E present in the absence of singlet oxygen generation; ANOVA, $p > 0.05$. The absence of a difference is indicative of antioxidant protection.

solvents, A2E generates singlet oxygen with modest efficiency (0.03) (15, 47, 48), although the photosensitizing ability of a hydrophobic compound such as A2E is probably greater in nonpolar surroundings such as that can be found in the membrane environment of a cell (49, 50). Nevertheless, it is clear that much of the singlet oxygen generated under these conditions is quenched by A2E (15, 51), and in the process, A2E-epoxides are generated (15). Moreover, the ability of singlet oxygen to diffuse only a short distance (~10–20 nm) (52) within the cell may limit its ability to generate DNA damage. Thus, the potential for A2E-mediated photodamage in RPE cells in the eye may be very much dependent on the formation of A2E-epoxides. In keeping with this observation, it is important to note that some photosensitizers with low singlet oxygen quantum yields (0.005) exert a phototoxicity that is by orders of magnitude more potent than expected on the basis of their photophysical characteristics. The pronounced ability of these compounds to mediate light-induced cellular damage is explained by a conjugated double bond structure that serves to directly quench the singlet oxygen produced to form damaging oxidized intermediates (53–55). The proximity of the nucleus to the site of epoxide generation is likely to influence the ability of these compounds to interact with DNA. Thus, it is important to note that A2E accumulates intracellularly in lysosomal storage bodies that assume a perinuclear distribution (9). The access to the nucleus may be further facilitated if the A2E-epoxide fragment into readily diffusible compounds and/or if redistribution of the epoxides occurs because of lysosomal photodamage, the latter being the case for some other photosensitizers (56).

Based on analogy with other DNA alkylation reactions and the secondary chemical transformations they undergo, it is possible to conceive mechanisms by which A2E-epoxide can generate a carbonyl product at the C8 position of guanine. For instance, the dietary carcinogen aflatoxin-B_1 undergoes cytochrome P450-mediated oxidation to an aflatoxin-epoxide that can then form DNA adducts through covalent binding to the

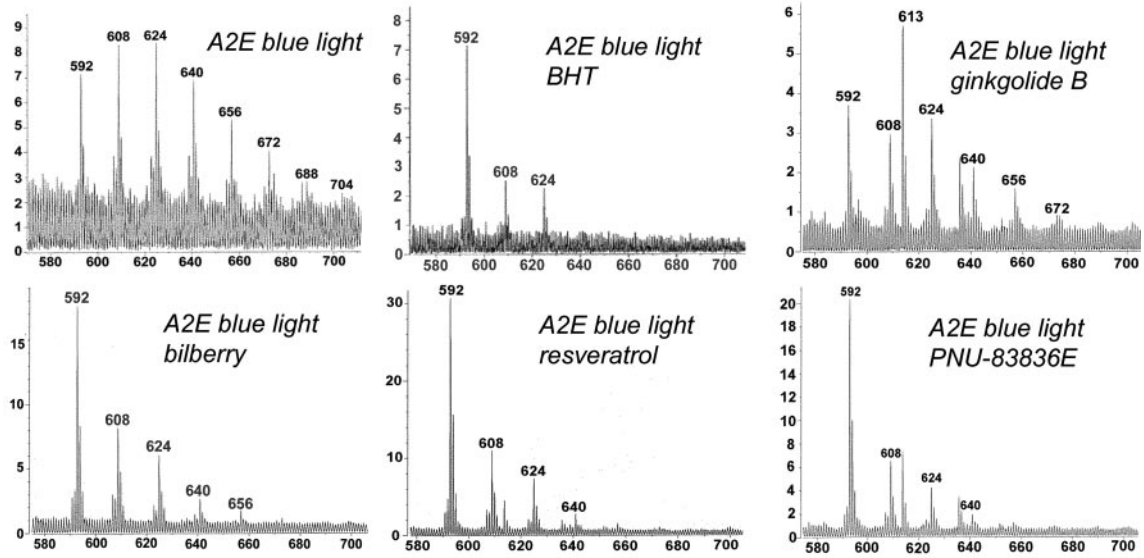

FIG. 7. **Effect of various antioxidants on A2E epoxidation.** FAB-MS of A2E (200 μM in PBS) exposed to blue light (*A2E blue light*) and A2E exposed to blue light in the presence of the antioxidants (100 μM) BHT, ginkgolide B, bilberry, resveratrol, and PNU-83836E. The molecular ion peak at *m/z* 592 correlates with the molecular mass of A2E. The higher molecular weight peaks correspond to A2E-epoxides with the exception of 613, which is a matrix peak.

ring nitrogen at position 7 of guanine (57). Aflatoxin-N(7)-guanine adducts, however, are very unstable and can spontaneously yield 8-oxo-dG (35, 36, 57). Other alkyl epoxides have also been shown to prefer the N(7) position of guanine, principally because it has high nucleophilicity and is sterically accessible (17) and some of these other N(7)-substituted guanines can also undergo hydrolytic rearrangement to form 8-oxo-dG (58). Thus, we propose that an epoxy-A2E may react at the position 7 of guanine to form an iminium salt. Reaction of the iminium salt with H_2O hydroxide ion would form a DNA adduct, which could subsequently eliminate its A2E moiety to give 8-oxo-dG. The foregoing mechanism is currently under investigation as is the potential for DNA cross-linking. The latter is feasible because A2E can undergo epoxidation at multiple double bonds and thus may be capable of reacting at two sites on DNA, leading to cross-link formation.

In addition to transforming to 8-oxo-dG, N(7)-guanine adducts derived from epoxides can also proceed to depurination (59). The resulting base loss can transpire under physiological conditions and occurs because alkylation at guanine N(7) introduces a positive charge on the nitrogen atom (quaternary nitrogen) that leads to cleavage of the glycosyl bond to regain ring stability (17). These abasic sites created by spontaneous fission of the base-sugar linkage can subsequently be transformed into DNA strand breaks by the alkaline unwinding solution of the comet assay (31, 32). Such alkali-labile abasic sites probably account, at least in part, for the single strand breaks we detected by comet assay. Some of the DNA strand breaks may have also resulted from excision of damaged bases by specific DNA glycosylases in the process of DNA repair (44).

We observed that vitamin E, vitamin C, and a combination of both vitamins reduced the number of A2E molecules undergoing epoxidation, diminished the number of epoxides formed on a given A2E-epoxide adduct, and decreased the incidence of associated DNA injury and cell death. However, a clear synergistic effect of these two vitamins was not observed. Vitamin E is considered to be a major lipophilic antioxidant, and in some (60) but not all (61) cases, its oxygen-scavenging efficiency can be increased by combined treatment with vitamin C. Our mass spectrometry and HPLC data indicate that vitamin E served to scavenge singlet oxygen after its generation by photosensitized A2E but before it was inserted into the hydrophobic side arms of A2E to form epoxides. The notion that the effect of vitamin E was to intercept singlet oxygen-induced A2E epoxidation rather than to block reactivity between A2E-epoxides and cellular targets is consistent with reports that treatment with vitamin E does not inhibit covalent binding of aflatoxin B_1-epoxide to DNA (36). A spectroscopic study (9) of A2E supports the assertion that the hydrophobic domains of this amphiphilic molecule are associated with the nonpolar lipid portions of intracellular membranes. With A2E positioned as such, vitamin E may function by neutralizing singlet oxygen at the site of its formation within the lipid bilayer of the membrane. This scenario may also account for the far more pronounced effect observed with vitamin E as compared with aqueous-soluble vitamin C and for the absence of additional benefit from combined treatment. It has been reported (62) that in addition to direct scavenging of reactive oxygen species, vitamin C can exert an antioxidant effect by maintaining vitamin E in its reduced functional form. Because under our experimental conditions significant quenching of singlet oxygen by vitamin C was not observed, we suggest that the protection afforded by vitamin C was related to its ability to recycle tocopheroxyl radicals.

The significance of our findings with respect to vitamins C and E and their suppression of A2E-epoxidation is underscored by studies suggesting that individuals with low plasma levels of the antioxidants vitamins C and E are at increased risk for AMD (63, 64). Moreover, the Age-related Eye Disease Study, a 9-year multicenter clinical trial, recently reported that supplementation with zinc, vitamins E and C, and β-carotene can reduce the risk of progression to advanced AMD (65). Although the Age-related Eye Disease Study did not attribute this finding to particular mechanisms, investigators have speculated for some time that antioxidant vitamins provide a shield against oxidative injury originating, at least partially, from light (64). Because the death of RPE is central to the etiology of atrophic AMD (66–69), defense against the damaging effects of light-induced A2E-epoxidation may be one means by which these vitamins afford protection.

Of the other presumed antioxidants we surveyed, bilberry and resveratrol are both plant-derived dietary constituents. Bilberry is widely used in the U. S. as a nutritional supplement to improve vision, although the mechanism by which this may occur is not clear (40, 41) and little work has been done in the

U. S. to elucidate indications for its use. The ability of bilberry to inhibit A2E-epoxidation in our studies may be related to its content of anthocyanosides (70, 71), the latter being reputed to have antioxidant activity (40). Resveratrol is a natural compound found in grapes (38). It is reported to have antioxidant properties, and its presence in wine in a soluble form that results in improved bioavailability is thought to be responsible for the cardioprotective effects of red wine. Both resveratrol and bilberry extract can inhibit A2E-epoxidation by quenching singlet oxygen. This appears not to be the case for ginkgolide B.

The oxidative damage to which all of the cells are subjected is considered to be a significant cause of an age-related decline in cell function (72–74). Nevertheless, as a nonreplicating cell that is exposed to wavelengths of light in the visible spectrum and accumulates a naturally occurring photoreactive chromophore with age, the RPE cell may be anomalous. The damage that A2E epoxidation bestows on the RPE cell may be the element linking the associations between RPE atrophy and lipofuscin accumulation (75–78) on the one hand and AMD and light exposure (79–82) on the other. Photoxidative mechanisms involving A2E may also underlie the known susceptibility of RPE to blue light damage *in vivo* (83–85).

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