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Mechanisms Involved in A2E Oxidation

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Abstract

A2E is one of the bis-retinoid pyridinium compounds that accumulate as lipofuscin pigments in retinal pigment epithelial (RPE) cells in association with aging and in some inherited forms of retinal degeneration. Here we observed that 430 nm irradiation of A2E in the presence of the spin trap DMPO, led to the appearance of a superoxide dismutase-inhibitable electron paramagnetic resonance (EPR) spectrum characteristic of DMPO-OH; this finding was indicative of hydroxyl radical (OH) formation following initial spin trapping of superoxide anion by DMPO. We also observed an increase in dihydroethidium (HET) fluorescence and luminol-based chemiluminescence that on the basis of inhibition by superoxide dismutase, was indicative of superoxide anion generation when A2E was irradiated at 430 nm in cell-free systems. Nevertheless, while A2E was readily oxidized in the presence of a singlet oxygen generator, superoxide anion did not serve to oxidize A2E. Specifically, by HPLC quantitation and FAB-mass spectroscopy, there was no evidence of A2E oxidation when A2E was incubated with a superoxide anion generator (xanthine/xanthine oxidase) in a variety of solvents (100% PBS, 30% DMSO in PBS, 100% MeOH and CHCl₃) or in the presence of detergent. On the other hand, however, peroxy-A2E, an oxidized form of A2E with an endoperoxide moiety on the short-arm of the molecule, readily underwent further oxygen addition when incubated with xanthine/xanthine oxidase. Superoxide anion may be generated by irradiation of A2E but is not involved in the early events that oxidize A2E. Superoxide can contribute to the further oxidation of already-oxidized A2E.

Keywords

A2E; bisretinoid; lipofuscin; photooxidation; retinal pigment epithelium; singlet oxygen; superoxide anion

Introduction

Fluorescent bis-retinoid pyridinium pigments accumulate as lipofuscin constituents in retinal pigment epithelial (RPE) cells in association with aging and in some inherited forms of retinal degeneration. The excessive formation of these compounds in Stargardt macular degeneration, is considered to be the cause of a loss of RPE cells in recessive Stargardt disease, a blinding

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macular disorder of juvenile onset and probably also contributes to the etiology of age-related macular degeneration (AMD), a common cause of blindness in elderly people (Delori et al., 2001; Holz et al., 2001; Radu et al., 2005; Schmitz-Valckenberg et al., 2004; Scholl et al., 2004). Photochemical reactions initiated by excitation from the blue region of the spectrum may contribute to the adverse effects of lipofuscin accumulation. Of particular importance is the finding that the photo-damage to cells induced by A2E, one of the lipofuscin constituents that have been characterized, involves the formation of A2E photooxidation products. The oxygen-containing moieties formed by the photoexcitation of A2E include epoxides, furanoid oxide structures and cyclic peroxides that are expected to be reactive (Ben-Shabat et al., 2002; Dillon et al., 2004; Jang et al., 2005a).

Involvement of singlet oxygen in A2E photooxidation is indicated by the detection of a characteristic 1270 nm phosphorescence upon 430 nm irradiation of A2E (Ben-Shabat et al., 2002), by the potentiation of A2E photooxidation in deuterium oxide (D₂O) (Ben-Shabat et al., 2002; Jang et al., 2005a), a solvent that extends the lifetime of singlet oxygen, and by the ability of the singlet oxygen quencher 1,2,2,6,6-pentamethyl-4-piperidinol to protect against A2E photooxidation (Sparrow et al., 2002). Additionally, singlet oxygen generated by thermal decomposition of the 1, 4-endoperoxide of 1,4-dimethylnaphthalene can substitute for blue light in mediating A2E oxidation (Ben-Shabat et al., 2002; Jang et al., 2005a; Kim et al., 2006). Support for the involvement of singlet oxygen in A2E photooxidation is further provided by the observation that azide, histidine and DABCO, all of which are quenchers/scavengers of singlet oxygen, suppress irradiation-induced death of cells that have accumulated A2E in culture; conversely, the effect is potentiated in deuterium oxide-based medium (Sparrow et al., 2002). Additionally, however, investigations employing a cholesterol peroxidation assay or electron paramagnetic resonance (EPR), suggest that aerobic irradiation of A2E or whole lipofuscin, with short-wavelength visible light, brings about the generation of reactive oxygen species other than singlet oxygen including superoxide anion (O₂^{•-}) and perhaps hydroxyl radical (•OH) (Gaillard et al., 2004; Godley et al., 2005; Kanofsky et al., 2003; Pawlak et al., 2003; Rozanowska et al., 1995). An important question that we address here, is the extent to which superoxide anion can add to carbon-carbon double bonds of A2E to form oxidized A2E species.

Materials and Methods

Reagents

Dulbecco's phosphate-buffered saline (PBS) was purchased from Gibco (Grand Island, NY). HPLC grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ). All-*trans*-retinal, dihydroethidium (HET), 5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO), ethanolamine, phosphatidylethanolamine, superoxide dismutase, trifluoroacetic acid (TFA), and all other chemicals were purchased from Sigma (St Louis, MO). A2E (Parish et al., 1998), A1E (Jockusch et al., 2004), and 1,4-endoperoxide of 1,4-dimethylnaphthalene (Turro et al., 1981) were synthesized as previously described. A lumiMax® superoxide anion detection kit was purchased from Stratagene®.

Electron spin resonance (EPR) spectroscopy

A 35 μM solution of A2E, with 90 mM DMPO in a 0.3 mm quartz cell (Wilma Glass, Buena, NJ) was irradiated at 430 nm. Electron spin resonance (EPR) spectra were obtained on an EMX EPR spectrometer (Bruker BioSpin Corporation, Billerica, MA) operating at X band (9.8 GHz) equipped with a 4102ST resonator with the following instrumental settings: microwave power 20 mW, modulation amplitude 1 G, receiver gain 5.02×10^5 , time constant 82 msec, conversion time 82 msec, and scan rate of 100G/84 sec. Spectra shown are an average of 15 scans at a

temperature of 22 °C. DMPO was purified using activated charcoal before use with EPR spectroscopy.

HEt fluorescence assay and luminol-based chemiluminescence

A mixture of HEt (50 μ M) and 200 μ M A2E (prepared from 20 mM stock in DMSO) in 0.2 ml PBS was irradiated at 430 nm (430 ± 20 ; 2.6 mW/cm²; 10 min; tungsten halogen source) and the change in HEt fluorescence was measured by spectrofluorometry (excitation, 485 nm; emission, 565 nm). Control samples included HEt (50 μ M) incubated for 30 minutes in PBS with the superoxide anion generator xanthine/xanthine oxidase (0.4 mM xanthine/100 mUnit/ml xanthine oxidase), or the singlet oxygen generator endoperoxide of 1, 4-dimethylnaphthalene (1 mM). HEt fluorescence was also used to test superoxide anion production by xanthine/xanthine oxidase as a function of time. In these experiments xanthine/xanthine oxidase (xanthine, 0.4 mM; xanthine oxidase, 100 mUnits/mL) was incubated for 0.5, 1, 3 and 6 hours; 30 minutes before stopping the incubation, 50 μ M HEt was added and HEt fluorescence was measured spectrofluorometrically.

Superoxide anion generation was also measured with LumiMax® superoxide anion detection kit (Stratagene, CA). Luminol (100 μ M) and 125 μ M enhancer were added to 188 μ l of assay medium. Then, 200 μ M A2E or 0.4 mM xanthine/100 mUnit/mL xanthine oxidase (superoxide anion generator as control) was added to the luminol/enhancer mixture. The sample containing A2E was exposed to 430-nm illumination (0.36 milliwatt/mm²). Chemiluminescence was recorded at 30-second intervals for 2 minutes. All the provided solutions were freshly made and maintained on ice before assaying.

Conditions for A2E oxidation

A2E (200 μ M) prepared in PBS with 0.01% DMSO or in methanol was irradiated at 430 nm (430 ± 20 nm; 2.6 mW/cm²; tungsten halogen source) for 2, 5, or 30 minutes. A2E has an absorbance maximum in the visible spectrum at $\sim$ 439 nm (range $\sim$ 400 to 480 nm). A2E (200 μ M) was also incubated with the singlet oxygen generator endoperoxide of 1, 4-dimethylnaphthalene (1 mM) in CD₃OD and the mixture was stirred for 6 hours at room temperature in the dark. A2E (200 μ M) was also incubated with the superoxide anion generator xanthine/xanthine oxidase at the concentrations and for the durations indicated and in a variety of solvents (100% PBS, 30% DMSO in PBS, 100% DMSO, 100% MeOH, 100% CHCl₃) or in the presence of detergent (0.4% Tween-80 with 68 mM citric acid, 136 mM sodium phosphate and 0.03% EDTA). The reaction mixtures were subjected to quantitative HPLC and mass spectral analysis, as described below.

The reactivity of A2E, *all-trans*-retinal, A1E, peroxy-A2E and furano-A2E with superoxide anion was determined by incubating the compounds with xanthine/xanthine oxidase at increasing concentrations (0.2 mM xanthine/50 mUnits/ml xanthine oxidase; 0.4 mM xanthine/100 mUnits/ml xanthine oxidase; 0.8 mM xanthine/200 mUnits/ml xanthine oxidase) and for increasing intervals of time (15, 30 and 60 minutes). The reaction mixtures were analyzed by quantitative high performance liquid chromatography (HPLC) and by fast atom bombardment mass spectrometry (FAB-MS spectroscopy) as described below.

HPLC analysis

For quantification of A2E, a Waters™ 2695 HPLC equipped with photodiode array detector (Model 2996) and operating with Empower® software was used with a dC18 column (4 $\times$ 150 mm, 3 μ m) and an acetonitrile/water (containing 0.1% trifluoroacetic acid) gradient: 90–100% (0–10 min), 100% acetonitrile (10–20 min), with a flow rate of 0.8 ml/min and monitoring at 430 nm. Injection volumes were 10 μ l and each sample was injected three times for reliability. Peaks areas in the HPLC profile were determined using Empower® software. A2E was

identified by UV-visible absorbance and by a retention time corresponding to that of authentic A2E standard.

Mass spectrometry

Fast atom bombardment ionization mass spectrometry (FAB-MS) of oxidized A2E was performed on a JMS-HX110A/110A tandem mass spectrometer (JEOL, Akishima, Tokyo, Japan) fitted with a Xe beam FAB gun (6 kV) on the MS-1 ion source. A 10 kV acceleration voltage was used. The matrix was 3-nitrobenzyl alcohol.

Statistical analysis

Data were analyzed by one-way ANOVA, and the Newman Keuls Multiple Comparison test (Prism, GraphPad Software, San Diego, CA).

RESULTS

1. Oxidant generation accompanies A2E irradiation

We used multiple approaches to probe for evidence of superoxide anion production by A2E irradiation. First, A2E with the spin trap DMPO (5,5'-dimethyl-1-pyrroline-*N*-oxide) followed by 430 nm irradiation, lead to the appearance of an electron paramagnetic resonance (EPR) spectrum characteristic of a DMPO-OH adduct (Fig. 1A). Superoxide dismutase, an enzyme that converts superoxide anion to molecular oxygen and hydrogen peroxide (Kahl et al., 2004), abolished the EPR signal indicating that the hydroxyl radical (OH) adduct to DMPO probably formed following initial spin trapping of superoxide anion by DMPO. It cannot be ruled out, however, that superoxide anion formed as a result of decomposition of A2E-OOH, that latter being generated by interaction of A2E with singlet oxygen.

To probe for evidence of oxidant generation under our conditions, we also used a fluorescence assay based on production of the fluorescent ethidium cation upon oxidation of dihydroethidium (HET) (Budd et al., 1997). Accordingly, A2E (200 μ M) in PBS with 50 μ M HET was illuminated at 430nm for 10 min and fluorescence was measured in a spectrofluorometer (Ex, 485 nm; Em, 565nm). HET fluorescence was significantly increased after 430 nm irradiation (Fig. 1B). Since HET fluorescence was inhibited by exogenous superoxide dismutase in a concentration-dependent manner, the conversion to a fluorescent product could be interpreted as evidence of superoxide anion generation.

Oxidant generation was further assayed using a luminol-based chemiluminescence detection system (Fig. 1C). Non-irradiated A2E did not exhibit chemiluminescence but upon 430 irradiation the chemiluminescence associated with the oxidation of luminol was readily measurable for 30–120 seconds. In these experiments, the superoxide anion generator xanthine-xanthine oxidase was used as positive control. Addition of superoxide dismutase reduced the chemiluminescent emission by 80–96% (Fig. 1C).

2. Conditions favoring photooxidation of A2E

A2E is an amphiphilic molecule consisting of a hydrophilic pyridinium head group and two hydrophobic side-arms. In aqueous milieu this duality can be expected to lead to self-organizing behaviours whereby individual A2E molecules form aggregates with their non-polar side-arms oriented inwards to form a hydrophobic core. This behavior in water differs from that in a solvent such as methanol wherein the A2E molecules are dispersed. Thus we compared A2E photooxidation in an aqueous versus methanolic milieu using HPLC to monitor the loss of A2E due to photooxidation (Fig. 2 A,B). A2E photooxidation was also monitored by mass spectrometry (Fig 2C). As shown in Figure 2, when A2E was placed in an aqueous milieu (PBS with 0.01% DMSO), irradiation for 2 and 5 minutes resulted in a substantial reduction in peak

height (Fig. 2B). Furthermore, by FAB-MS, A2E photooxidation in PBS was evidenced by the presence of a series of $m/z +16$ peaks extending from the m/z 592 peak attributable to A2E (Fig 2C, middle panel). Conversely with methanol as solvent, irradiation under our photolysis conditions was not associated with a change in absorbance of the A2E HPLC peak, even after 30 minutes of exposure (Fig 2A). The resistance of A2E photooxidation in methanol was confirmed by mass spectrometry, the mass profiles in the unirradiated and irradiated samples being the same (Fig. 3C, upper and lower panels), even after irradiation for 30 minutes.

3. Superoxide anion does not generate oxidized-A2E

To test reactive forms of oxygen for the ability to generate oxidized A2E (oxo-A2E), we began by incubating A2E (200 μ M) with the singlet oxygen generator, endoperoxide of 1,4-dimethylnaphthalene (1 mM in CD_3OD) for 6 hours, the half-life of this generator being approximately 5 hours at room temperature. The reaction mixtures were subsequently subjected to FAB-MS and quantitative HPLC analysis (Figs. 3 and 4). As reported previously, incubation with the singlet oxygen generator endoperoxide of 1,4-dimethylnaphthalene under hydrophobic conditions, resulted in A2E oxidation. The latter was evidenced by the presence in the FAB-MS spectra, of not only the m/z peak attributable to A2E (m/z 593 in deuterium solvent) but also a series of higher mass peaks (m/z 609, 625, 641, 657 and 673) reflecting the addition of oxygens to the A2E polyene structure (Fig. 3B). We have previously shown that A2E oxidation can be monitored by consumption of the compound when analyzed by quantitative HPLC. Accordingly, incubation with the singlet oxygen generator endoperoxide of 1,4- dimethylnaphthalene resulted in an appreciable decrease in the chromatographic peak corresponding to A2E (Fig 4).

A2E was also incubated with the superoxide generator xanthine-xanthine oxidase [xanthine (0.4 mM)/ xanthine oxidase (100 mUnits/ml)] for 30 minutes. However with analysis by FAB-MS, additional higher mass peaks indicative of A2E oxidation were not observed, except for a low intensity mass peak at m/z 608 that was present in the starting sample (Fig. 3 D, E). There was also no appreciable change in the area of the A2E chromatographic peak after incubating A2E with the superoxide anion generator (Figs 5 and 6A), at a range of concentrations (xanthine/xanthine oxidase: 0.2 mM/50mUnits/ml, 0.4 mM/100mUnits/ml, 0.8 mM/200mUnits/ml) and for up to 60 minutes. Moreover, A2E failed to oxidize in the presence of xanthine-xanthine oxidase despite the use of a variety of solvents: 100% PBS, 30% DMSO in PBS, 100% DMSO, 100% MeOH, 100% $CHCl_3$. (data not shown). Extending the time of incubation with xanthine/xanthine oxidase (0.4 mM xanthine/100mUnit/mL xanthine oxidase or 4 mM xanthine/1 Uint/mL xanthine oxidase) to 6 hours and carrying out the incubation in the presence of detergent (0.4% Tween-80), did not result in a decrease in A2E peak area (Fig. 4). In these experiments with xanthine/xanthine oxidase (0.4 mM xanthine/100mUnit/mL xanthine oxidase or 4 mM xanthine/1 Uint/mL xanthine oxidase) we confirmed by measuring the intensity of HET fluorescence, that the levels of superoxide anion generated were only slightly less at the end of a 6 hour-incubation as compared to a 1 hour-incubation (HET fluorescence: after 1 hour incubation, 784 a.u.; after 3 hours, 792 a.u., after 6 hours, 678 a.u.).

To test our oxidation conditions, we also examined A2E in parallel with several other compounds having structural features similar to that of A2E. For instance, A1E, a non-physiological single side-arm counterpart of A2E, was resistant to oxidation with lower concentrations/shorter duration exposure to xanthine/xanthine-oxidase, but succumbed to oxidation when exposed to the highest concentration (0.8 mM/200mUnits/ml) for 15, 30 and 60 minutes (Fig. 6B). Oxidation of A1E was accompanied by a ~ 30 nm blue-shift in absorbance (Fig. 6B), that was indicative of a loss of conjugation. Conversely, A2E exhibited little evidence of a decline in absorbance peak area that would be indicative of oxidation, when incubated under the same conditions (Fig. 6A).

Since each side-arm of A2E is derived from a molecule of all-*trans*-retinal, we also incubated the latter compound with xanthine/xanthine oxidase for various times (15, 30 and 60 minutes) and at 2 different concentrations (xanthine/xanthine-oxidase: 0.2 mM/50 mUnits/mL, 0.4 mM/100mUnits/mL) and analyzed the samples by HPLC. As shown in Figures 5 and 6, a progressive decrease in the absorbance of the all-*trans*-retinal peak was observed, the decrease being greater as the incubation in xanthine/xanthine-oxidase was prolonged (Figs. 6C) and as the concentration of xanthine-xanthine oxidase was increased (Fig. 5A, B). The decrease in 380 nm-absorbance is indicative of a loss of compound as oxidation proceeded. The UV-visible absorbance spectrum of all-*trans*-retinal was also blue-shifted by ~ 30 nm (Fig. 6 C, bottom panel), a change indicative of a loss of conjugation. Under the same conditions, there was no or little change in A2E peak area and no change in absorbance (Figs. 5B and 6A).

4. Peroxy-A2E undergoes further oxidation in the presence of superoxide anion

Given the resistance of A2E to oxidation in the presence of the superoxide anion generators, we subsequently examined already-oxidized species of A2E for a tendency to undergo further oxidation by superoxide anion. For instance, by incubating A2E with endoperoxide of 1,4-dimethylnaphthalene we generated peroxy-A2E, an oxidized species of A2E containing an endoperoxide at the 5, 8 position on the short arm of A2E (Jang et al., 2005a). The loss of conjugation that accompanies peroxy-A2E formation was evidenced by a 30 nm hypsochromic shift in the shorter-wavelength absorbance band (compare insets in Fig. 6A, top panel and Fig. 7B, top panel). Interestingly, when peroxy-A2E was incubated with xanthine /xanthine oxidase at even the lowest concentration (0.2 mM xanthine/50 mUnits/ml xanthine oxidase; 15 minutes), the compound exhibited considerable tendency toward further oxidation as indicated by a decrease in chromatographic peak area (Fig. 5A). The change in peak absorbance became more pronounced as the concentration of xanthine / xanthine-oxidase was increased (Fig. 5B). A hypsochromic shift (~30 nm) in absorbance was further indicative of oxidation and since the shift occurred in the longer wavelength band (λ 437 to 403; Fig. 7B, top and bottom panel), oxidation could be attributed to the long arm of the molecule (Jang et al., 2005a). The results obtained with peroxy-A2E contrasted with the behavior of monofurano-A2E, an oxidized compound produced by oxidation of A2E with *m*-chloroperoxybenzoic acid (mCPBA) and that contains a furanoid ring at the 5, 8 position (Jang et al., 2005a). Monofurano-A2E showed only little change in absorbance peak area even when incubated with xanthine/xanthine-oxidase at the highest concentration (0.8 mM/200mUnits/mL) for 60 minutes (Fig. 5C). The latter result may indicate a difference between photooxidation and oxidation with superoxide anion in the dark, since we previously observed that 5, 8-monofuran-A2E can undergo further oxidation under 430 nm-irradiation (Jang et al., 2005a).

Discussion

Questions related to the reactive oxygen species responsible for A2E oxidation are significant, since photooxidation products of A2E have been implicated as the damaging agents that account, at least in part, for the adverse effects of lipofuscin accumulation in RPE cells. For instance, cellular damage occurs in the presence of oxidized-A2E even under conditions that eliminate singlet oxygen or other reactive forms of oxygen as the immediate agent of damage (Sparrow et al., 2003a). Moreover, cleavage of oxidized-A2E, for instance at the O-O bonds of endoperoxide moieties, followed by diffusion of fragments may explain the observation that cellular damage can be observed at sites other than the lysosomal compartment in which A2E is housed (Sparrow et al., 2003b). The finding that complement can be activated in serum overlying irradiated-A2E-laden RPE is also consistent with the view that reactive cleavage products of A2E are generated by photooxidation (Zhou et al., 2006). Not only have oxidized forms of A2E been detected in human and mouse RPE (Jang et al., 2005a), but comparison of the levels of the A2PE precursor that forms versus A2E that accumulates (Kim et al., 2006),

suggests that a portion of A2E is lost through light-dependent conditions involving photooxidative processes.

Thus far A2E oxidation has been shown to occur under three different conditions - irradiation with light from the blue region of the spectrum ($\lambda_{\text{max}} \sim 430 \text{ nm}$) (Ben-Shabat et al., 2002; Jang et al., 2005a; Sparrow et al., 2002), exposure to a singlet oxygen generator (e.g. endoperoxide of 1, 4-dimethylnaphthalene) (Ben-Shabat et al., 2002; Jang et al., 2005a; Kim et al., 2006), and exposure to mCPBA (*m*-chloroperoxybenzoic acid) (Ben-Shabat et al., 2002; Jang et al., 2005a). As an oxidizing agent, mCPBA serves as a source of an electrophilic oxygen, adding one oxygen per double bond, in sequence, whereas molecular singlet oxygen reacts in a step-wise fashion adding to an olefin to form a dioxetane or perepoxide intermediate that rearranges to a cyclic peroxide product.

The photoreactivity of RPE lipofuscin has been demonstrated on several fronts. Uptake of oxygen has been measured in both suspensions of RPE cells and in lipofuscin granules with efficiency being greatest using short-wavelength visible light (Pawlak et al., 2002; Rozanowska et al., 1995; Rozanowska et al., 2002; Rozanowska et al., 2004). It is reported that photoexcited pigments in lipofuscin granules form triplet states with both singlet oxygen and superoxide anion being produced (Gaillard et al., 1995; Reszka et al., 1995; Rozanowska et al., 1995; Rozanowska et al., 1998). Using ESR (electron spin resonance) with DMPO as a spin trapping agent, superoxide anion production by irradiated A2E has previously been detected (Pawlak et al., 2002) while photosensitization studies have shown that photoexcitation of A2E also leads to singlet oxygen production (Ben-Shabat et al., 2002; Cantrell et al., 2001; Gaillard et al., 2004; Kanofsky et al., 2003; Lamb et al., 2001; Pawlak et al., 2003; Ragauskaitė et al., 2001; Reszka et al., 1995). Measurements of the quantum yield of singlet-oxygen generation by A2E or quantum yields of intersystem crossing of A2E indicate relatively modest singlet-oxygen production when measured in polar solvents and when compared to the performance of photosensitizers used in photodynamic therapy. These findings are consistent with the concept that the destructive capacity of the photoreactive process is compounded by the photoproducts generated when A2E is oxidized (Bunting, 1992; Delaey et al., 2000; Krieg et al., 1993). Also of significance to this discussion however, are spectroscopic studies indicating that the retinoid-derived side-arms of intracellular A2E are located in an apolar milieu, most likely at the hydrophobic interior of intracellular membranes (Sparrow et al., 1999). The latter environment, would be akin to the hydrophobic interior of aggregate molecular structures. Thus it is of interest that in the current experiments we noted that A2E is more readily photooxidized under conditions (aqueous environment), that allow A2E molecules to aggregate with retinaldehyde-derived side-arms oriented toward a hydrophobic milieu as opposed to conditions (methanol) favoring full solvation and thus greater separation of neighbouring side-arms.

Here we observed that incubation of A2E with the spin trap DMPO followed by 430 nm irradiation, lead to the appearance of an electron paramagnetic resonance (EPR) spectrum characteristic of DMPO-OH and indicative of hydroxyl radical (OH) formation either directly or following initial spin trapping of superoxide anion by DMPO (Britigan et al., 1986). We also observed an increase in H₂O₂ fluorescence and luminol-based chemiluminescence that on the basis of inhibition by superoxide dismutase, is indicative of superoxide anion generation when A2E is irradiated at 430 nm in both cell-free systems. With respect to the luminal assay, it should be noted that the luminal radical can be generated by oxidants other than superoxide anion and given that the luminal radical can spontaneously reduce molecular oxygen to superoxide anion, the specificity of this assay is not certain (Fridovich, 1997).

Nevertheless, despite the likelihood that A2E irradiation generates superoxide anion in addition to singlet oxygen, superoxide anion does not oxidize A2E as does singlet oxygen. Specifically,

by HPLC quantitation and FAB-mass spectroscopy, there was no evidence of A2E oxidation when A2E was incubated with a superoxide anion generator (xanthine/xanthine oxidase) in a variety of solvents. Conversely, peroxy-A2E, an oxidized species bearing an endoperoxide moiety at the 5,8 position, readily underwent oxygen addition when incubated with xanthine/xanthine oxidase. Compounds related to A2E, specifically, all-*trans*-retinal and A1E a single-side arm counterpart to A2E, were also oxidized in the presence of superoxide anion. Thus we conclude, that whereas superoxide anion may be generated by irradiation of A2E, this reactive form of oxygen is not involved in the early events that oxidize A2E leading to the generation of photooxidized A2E products. Superoxide can however, contribute to the further oxidation of already-oxidized A2E.

The specific species of reactive oxygen that are generated by photoexcitation of A2E and that photooxidize A2E could be significant to the design of antioxidants used therapeutically in AMD. The report from the AREDS group (Group, 2001) found that patients taking supplements of vitamin C, vitamin E, beta carotene and zinc had reduced odds for progression to advanced AMD. Besides metabolic sources of reactive forms of oxygen, photooxidative processes elicited by RPE lipofuscin likely add to the oxidative stress. Indeed, a number of antioxidants including the singlet oxygen quenchers histidine, DABCO (diazabicyclooctane) and azide (Sparrow et al., 2002) and naturally occurring compounds such as vitamins E and C (Sparrow et al., 2003a) have been shown to confer resistance to blue light-induced death of A2E-containing RPE. Two phytochemicals, bilberry-derived anthocyanins (Jang et al., 2005b) and the phase 2 inducer sulforaphane were also shown to be potent antioxidants that suppressed photooxidative processes initiated in RPE cells by A2E. Sulforaphane, itself, does not directly participate in antioxidant or prooxidant reactions but instead 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 and UDP-glucuronosyl-transferases (Dinkova-Kostova et al., 2001).

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Figure 1.

Oxidant production associated with 430 nm irradiation of A2E. **A.** EPR spectrum of an aqueous buffer solution (pH 7.2) of DMPO (90 mM in DMSO) and A2E (35 μ M) after 430 nm irradiation for 10 minutes (0.36 mWatt/mm²). The four EPR signal peaks (arrows) correspond to the formation of an adduct of OH-radical with DMPO; typical coupling constants g_N and g_H are presented. DMPO structure, upper left. **B.** Superoxide dismutase inhibits dihydroethidium (H2O₂) fluorescence in the presence of A2E and 430 nm irradiation. H2O₂ (50 μ M) was incubated at the indicated concentrations of A2E and the mixture was irradiated at 430nm. The H2O₂ fluorescence was measured by spectrofluorometry (Ex, 485 nm; Em, 565 nm). Values plotted are mean $\pm$ S.E.M. of 3 experiments, three replicates per experiment. $p <$

0.01, A2E + 430nm versus A2E + 430nm + SOD (30 Units/ml); $p < 0.001$, A2E + 430nm versus A2E + 430nm + SOD (100 Units/ml). C. Luminol chemiluminescence in the presence of 430 nm-irradiated A2E. A2E (200 μM) or xanthine (0.4 mM)/xanthine oxidase (100 mUnits/ml), a superoxide anion generator was added to the mixture of luminol and enhancer. The mixture containing A2E was exposed to 430-nm illumination (0.36 milliwatt/ mm^2). Emission was recorded at 30-second intervals (beginning from the time the reactants were combined or beginning at onset of 430 nm irradiation) for 2 minutes. Values are mean $\pm$ S.E.M. of 3 experiments, duplicates per experiment.

Figure 2.

Photooxidation of A2E in aqueous and methanolic environment. **A.** Overlay of HPLC chromatographs generated with unirradiated A2E (black trace) or after A2E in methanol is irradiated (430 nm) for 5 mins (blue) and 30 mins (red). **B.** Chromatographic overlay of unirradiated A2E (black) and A2E in PBS with 0.1% DMSO after irradiation for 2 mins (blue) and 5 (red) mins. Reversed phase C18 column with monitoring at 430 nm. **C.** FAB-mass spectrometry. Unirradiated A2E (upper), A2E in methanol (MeOH) irradiated at 430 nm for 30 mins (middle) and A2E in phosphate buffered saline (PBS; with 0.01% DMSO) irradiated for 5 minutes (lower). Peaks differing in m/z by 16 (e.g. 608, 624, 640 etc) are indicative of A2E photooxidation.

Figure 3.

A2E is oxidized in the presence of a generator of singlet oxygen but not in the presence of a superoxide anion generator. Analysis by FAB-MS. A2E was incubated without (A) and with (B) 1 mM endoperoxide of 1, 4-dimethylnaphthalene, a singlet oxygen generator. In deuterium solvent, the mass of A2E (m/z 592) is shifted by 1 mass unit to 593, due to hydrogen-deuterium exchange. A2E oxidation in B is indicated by the appearance of m/z 593 + n (16). Samples of A2E incubated in the absence (C) and presence (D) of the superoxide anion generator xanthine/100 mUnits/ml xanthine oxidase (0.4 mM).

Figure 4.

Chromatographic overlay of HPLC profiles generated with starting sample of A2E (black) and A2E incubated for 6 hours with either the singlet oxygen generator, endoperoxide of 1,4-dimethylnaphthalene (1 mM) (blue trace) or the superoxide anion generator, xanthine/xanthine oxidase (xanthine, 0.4 mM; xanthine oxidase, 100 mUnits/mL; with 0.4% Tween-80) (red trace). Black and red traces overlap; blue trace exhibits reduced height relative to black trace. Reversed phase C18 column with monitoring at 430 nm.

Figure 5. The reactivity of A2E, A1E, furano-A2E, peroxy-A2E and all-*trans*-retinal with superoxide anion. HPLC quantitation determined as integrated peak areas and expressed as percent of control (untreated sample). Compounds were incubated with three different conditions of xanthine/xanthine oxidase, 0.2 mM xanthine/50 mUnits/ml xanthine oxidase (A); 0.4 mM xanthine/100 mUnits/ml xanthine oxidase (B); 0.8 mM xanthine/200 mUnits/ml xanthine oxidase (C) and for 15, 30 and 60 minutes (A–C). Structures of A2E, A1E, furano-A2E, peroxy-A2E and all-*trans*-retinal.

Figure 6.

Representative HPLC chromatograms of A2E, A1E and *all-trans*-retinal (ATR) incubated with the superoxide anion generator xanthine/xanthine oxidase (X/XOD). Starting samples (top) and samples incubated with X/XOD for 15, 30 and 60 mins at 25°C were analyzed by HPLC. Inset in top panels, UV-visible absorbance of A2E, A1E and ATR. Oxidation is indicated by a reduction in peak height and by a blue shift in UV-visible absorbance; compare insets in top and bottom panels of B and C.

Figure 7.

Representative HPLC chromatograms of furano-A2E and peroxy-A2E, that were incubated with the superoxide anion generator xanthine/xanthine oxidase (X/XOD). Starting samples (top panels) and samples incubated with X/XOD for 15, 30 and 60 mins at 25°C were analyzed by HPLC. Inset in top panels, UV-visible absorbance of furano-A2E and peroxy-A2E. Oxidation is indicated by a reduction in peak height and by a blue shift in UV-visible absorbance; compare insets in top and bottom panels of B.