Published in final edited form as:

Free Radic Biol Med. 2015 December ; 89: 873–882. doi:10.1016/j.freeradbiomed.2015.10.411.

Zeaxanthin and α -tocopherol reduce the inhibitory effects of photodynamic stress on phagocytosis by ARPE-19 cells

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Abstract

Zeaxanthin and α -tocopherol have been previously shown to efficiently protect liposomal membrane lipids against photosensitized peroxidation, and to protect cultured RPE cells against photodynamic killing. Here the protective action of combined zeaxanthin and α -tocopherol was analyzed in ARPE-19 cells subjected to photodynamic (PD) stress mediated by rose Bengal (RB) or merocyanine-540 (MC-540) at sub-lethal levels. Stress-induced cytotoxicity was analyzed by the MTT assay. The peroxidation of membrane lipids was determined by HPLC-EC(Hg) measurements of cholesterol hydroperoxides using cholesterol as a mechanistic reporter molecule. The specific phagocytosis of FITC-labeled photoreceptor outer segments (POS) isolated from bovine retinas was measured by flow cytometry, and the levels of phagocytosis receptor proteins α_v integrin subunit, β_5 integrin subunit and MerTK were quantified by Western blot analysis. Cytotoxicity measures confirmed that PD stress levels used for phagocytosis analysis were sub-lethal and that antioxidant supplementation protected against higher, lethal PD doses. Sub-lethal PD stress mediated by both photosensitizers induced the accumulation of 5 α -OOH and 7 α / β -OOH cholesterol hydroperoxides and the addition of the antioxidants substantially inhibited their accumulation. Antioxidant delivery prior to PD stress also reduced the inhibitory effect of stress on POS phagocytosis and partially reduced the stress-induced diminution of phagocytosis receptor proteins. The use of a novel model system where oxidative stress was induced at sub-lethal levels enable observations that would not be detectable using lethal stress models. Moreover, novel observations about the protective effects of zeaxanthin and α -tocopherol on photodynamic damage to ARPE-19 cell membranes and against reductions in the abundance of receptor proteins involved in POS phagocytosis, a process essential for photoreceptor survival, supports the importance of the antioxidants in protecting of the retina against photooxidative injury.

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Keywords

Antioxidants; Zeaxanthin; α -Tocopherol; ARPE-19 cells; Phagocytosis; Photoreceptor outer segments; Lipid peroxidation; Cholesterol hydroperoxides; Phagocytosis receptors MerTK; α v β 5 integrin

Introduction

The accumulation of chronic sub-threshold oxidative damage to the retinal pigment epithelium (RPE) is believed to impair the function and long-term survival of photoreceptors and to predispose the retina to degenerative diseases such as age-related macular degeneration (ARMD) [4,15,45]. There is therefore considerable interest in identifying efficient antioxidant therapies to postpone disease onset or to slow its progression [1,61,62,64,67]. A synergistic enhancement of antioxidant protection against oxidative stress has been shown in several experimental systems by a combination of low-molecular-weight antioxidants such as carotenoids, vitamin C and vitamin E [51,54,62,63]. Supporting the value of antioxidant supplementation, the dietary intake of macular carotenoids zeaxanthin and lutein [15,57,58], and of zinc, vitamin E and C [1,15,41] has been shown to have beneficial, protective effects in epidemiological studies.

The use of combinations of antioxidants is significant because antioxidants differ in mechanisms of action, can have both pro- and antioxidant properties, and may require other antioxidants to sustain their antioxidant potential. Macular carotenoids can filter potentially damaging blue light [9,30] and, by virtue of their ability to quench singlet oxygen and excited triplet states of photosensitizing dye molecules [9,35], are also believed to protect the retina from photochemical damage by preventing the formation of reactive oxygen species (ROS) [9] or neutralizing already generated ROS. Conversely, carotenoids can also exhibit prooxidant activity [50,65]. This can occur if radical adducts or carbon-centered radicals, formed by the interaction of carotenoids with peroxy radicals [39,43], further interact with oxygen forming secondary peroxy radicals that are involved in the propagation of lipid peroxidation [50,62]. Due to their high oxidation potential, carotenoid radical cations may cause direct oxidative damage to various biological molecules [11] unless they are scavenged by other antioxidants such as vitamin E [44].

Vitamin E in the form of α -tocopherol is the most effective free radical scavenger and the predominant form of vitamin E present in human blood plasma, photoreceptor outer segments and RPE [4,31]. This lipophilic vitamin efficiently reduces alkoxy and peroxy radicals [26] correspondingly to alcohols or hydroperoxides and is converted to a secondary chromanoxyl radical [62,63], which is a relatively nonreactive species that can be reduced back to α -tocopherol by ascorbate [19]. Vitamin E also functions as a quencher of photogenerated singlet oxygen; a single molecule of α -tocopherol can deactivate 120 molecules of singlet oxygen before it is oxidized [52]. The retinal concentration of α -tocopherol is highly sensitive to dietary intake [4]; diets deficient in vitamin E have been shown to enhance the accumulation of lipofuscin-like granules in the RPE of monkeys and rats, especially when the diet was high in polyunsaturated fatty acids [53].

The aim of this work was to determine whether a combination of two important retinal antioxidants, zeaxanthin and α -tocopherol, confer protection against sub-lethal oxidative stress induced in ARPE-19 cells by photodynamic treatment mediated by MC-540 or RB. The results showed significant inhibition by the antioxidants of the accumulation of 5 α -OOH and 7 α / β -OOH cholesterol hydroperoxides, which are produced by PD treatment. The antioxidants also produced a small but detectable preservation of RPE phagocytic function; the phagocytosis of photoreceptor outer segments (POS) is an essential RPE process that was previously shown to be sensitive to PD stress [48]. Accompanying the partial protection of phagocytosis was a partial preservation of the abundance of POS receptor proteins MerTK or α v and β 5 integrin subunits, which are known to exhibit reductions with PD stress [49]. Overall, the results suggest that adequate levels of zeaxanthin and α -tocopherol in RPE cells may over time help protect against the photodynamic impairment of key biological functions of the cells.

Material and Methods

Chemical and reagents

The following were obtained from Sigma-Aldrich (Steinheim, Germany or St. Louis, MO, USA): merocyanine-540 (MC-540), rose Bengal (RB), 3 - (4,5- dimethylthiazol - 2 - yl) - 2,5 - diphenyltetrazolium bromide (MTT), minimum essential medium (MEM), trypsin, streptomycin, penicillin, gentamicin, Amphotericin B, bovine serum albumin (BSA), 2,6-di-*tert*-butyl-4-methylphenol (BHT), chelating resins Chelex 100, Triton X-100, $\pm$ - α -tocopherol, deferoxamine mesylate (DFO), protease inhibitor cocktail, sodium dodecyl sulfate (SDS), ethyleneglycol-O, O'-bis(2-aminoethyl)-N, N, N', N'-tetraacetic acid (EGTA), glycine, glycerol, cholesterol and trizma hydrochloride (Tris HCl). LiChrosolv grade solvents for liquid chromatography (chloroform, methanol, isopropanol, acetonitrile and tetrahydrofuran [THF]) were obtained from Merck (Darmstadt, Germany). Zeaxanthin was a gift from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Hank's Balanced Salt Solution (HBSS) and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen (Carlsbad, CA, USA or Auckland, New Zealand). Fluorescein-5-isothiocyanate (FITC) was obtained from Molecular Probes (Eugene, OR, USA). The following were purchased from Polskie Odczynniki Chemiczne (Gliwice, Poland): disodium hydrogen phosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$), potassium dihydrogen phosphate (KH_2PO_4), tris (hydroxymethyl) aminomethane, hydrochloric acid (HCl), sucrose, magnesium chloride hexahydrate ($\text{MgCl}_2 \times 6\text{H}_2\text{O}$), potassium chloride (KCl), sodium chloride (NaCl), calcium chloride (CaCl_2), ethylen diaminetetraacetic acid disodium salt (EDTA-Na_2), sodium hydrogen carbonate (NaHCO_3), ethanol, methanol and dimethyl sulfoxide (DMSO). All chemicals were high analytical grade of purity. All working solutions were prepared with double distilled water and stored at 4°C or at room temperature.

Enrichment of ARPE-19 cell cultures with zeaxanthin and α -tocopherol

The human RPE cell line ARPE-19 (American Type Culture Collection, Rockville, MD) was propagated under standard culture conditions with twice-weekly feedings with Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 150 U/mL, streptomycin 100 $\mu\text{g}/\text{ml}$, amphotericin B 2.5 $\mu\text{g}/\text{ml}$, and gentamicin

0,01mg/ml). Cells were plated in 96 well plates, 24 well plates or 100 mm polystyrene dishes (depending upon the experiment) at a density 10^5 cells/cm².

A stock solution of zeaxanthin (5 mM) was prepared from the solid, using tetrahydrofuran (THF) as solvent and its concentration was determined spectrophotometrically from the zeaxanthin molar absorption coefficient of $144300 \text{ M}^{-1} \text{ cm}^{-1}$ at 450 nm in ethanol [2] as described previously [63]. A stock solution of α -tocopherol (40 mM) was prepared in ethanol. Both solutions were divided into small portions, aspirated under argon and stored in the dark at -80°C. Prior to each experiment, a fresh aliquot of each antioxidant solution was diluted in FBS and incubated at 37°C for 1 hr. Then a measured amount of each antioxidant solution in FBS was aspirated and quickly injected into the culture medium, giving the following final concentrations: 10 μM zeaxanthin, 100 μM α -tocopherol and 3% FBS. The selected concentrations were based upon previous studies from our group testing the efficiency of zeaxanthin and α -tocopherol in protecting against photosensitized lipid peroxidation [62] and photodynamic cell damage [63], and related protocols used to analyze antioxidant protection against light-induced toxicity in ARPE-19 cells [7,54]. Higher concentrations (especially for zeaxanthin) were avoided for empirical reasons, to prevent precipitation in culture medium and thus inefficient incorporation into cell membranes as detected after Folch's extraction. The final concentration of THF in the culture medium was 0.2% and of ethanol was 0.25%; no toxic effects of THF or ethanol were observed up to the highest concentrations tested (0.5%).

A confluent monolayer of ARPE-19 cells was enriched with a combination of the antioxidants at 6 hr after plating. Parallel control cultures were exposed to medium containing equivalent amounts of THF, ethanol and FBS. After 18 hours at 37°C, the medium was removed, cells were rinsed three times with serum-free MEM and subjected to photodynamic treatment using MC-540 or RB and green light as described below.

Induction of oxidative stress and cytotoxicity assay

Oxidative stress was induced by photodynamic treatment as previously described [48,49]. Briefly, control cultures or cultures supplemented with a combination of zeaxanthin and α -tocopherol were treated with a range of concentrations of MC-540 (0-30 μM) or RB (0-1000 nM) in serum-free medium for 0.5 hr at 37°C. After incubation, the cells were washed three times in Hank's Balanced Salt Solution containing calcium and magnesium ions (HBSS) and irradiated for 30 min (for MC-540) or 15 min (for RB) with green light derived from six 36W / 830 fluorescent lamps (LUMILUX Warm White, Osram, Italy) covered with a green film filter (Lee Filters, Central Way Walworth Industrial Estate Andover, Hampshire, England). The fluence rate in the spectral region where the photosensitizers absorb light (520-570 nm) was 0.40 mW/cm².

To quantify photodynamic stress-induced cytotoxicity, a previously-described MTT assay for mitochondrial redox function was used [48]. Briefly, a stock solution of MTT (5.0 mg/ml in PBS) was diluted with MEM supplemented with 10% FBS to a final concentration of 0.5 mg/ml and added to all culture wells. After incubation for 2 hr at 37°C [29], the resulting blue precipitate was solubilized in DMSO:ethanol (1:1). The absorbance was read at 560 nm

in a plate reader (GENios Plus, Tecan, Austria GMBH) [66] and results are reported as a percent of untreated controls.

Phagocytosis assay

The effect of photodynamic stress on POS phagocytosis by control cultures or cells supplemented with a combination of zeaxanthin and α -tocopherol was analyzed by the previously-described protocol [48,49]. Briefly, following irradiation of control and photodynamically-treated cells, cultures were challenged with FITC-labeled POS (3.2×10^8 POS/ml) and incubated for 5.5 hr at 37°C. After incubation, cultures were washed three times in PBS, then the cells were detached by trypsinization and suspended in PBS containing 10% FBS. The percentage of fluorescence-positive events ($\lambda_{\text{excitation}} = 488$ nm, $\lambda_{\text{emission}} = 525$ nm) from 10,000 unfixed cells per sample was analyzed on a FACScan® flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software. Data were expressed as normalized phagocytosis ($\pm$ SD) for paired control (untreated) and photosensitized cultures. The rate of phagocytosis by untreated cells was taken as 100%.

Western blotting for POS receptor proteins

Extracts of ARPE-19 cells were prepared from replicate wells of control and antioxidant-supplemented cultures subjected to photodynamic treatment by published protocols [36,49]. Protein was quantified by the Bradford dye method and protein-equivalent samples from replicate cultures were loaded onto gels and were separated by electrophoresis under reducing conditions and transferred to PVDF membranes (Millipore, Bedford, MA) by previously published protocols [36,49]. Immunoblots were probed for MerTK (abcam, Cambridge, MA), α_v integrin (BD Bioscience, San Jose, CA), β_5 integrin (Santa Cruz Biotechnology, Santa Cruz, CA) and β -actin (Sigma-Aldrich, St. Louis, MO) and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or secondary antibodies from LI-COR (LI-COR Biosciences, Lincoln, NE; 1:20,000 dilution) followed by ECL enhanced chemiluminescence detection (GE Healthcare, Buckinghamshire, UK) or the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Quantitative densitometry of the immunoblots was performed and band density was given in arbitrary units and expressed as the mean density ($\pm$ SD) from replicate culture wells within an experiment. All experiments were performed a minimum of three times and representative experiments are shown.

Cholesterol hydroperoxide analysis by HPLC-EC(Hg)

Cholesterol hydroperoxides in lipid extracts from ARPE-19 cells subjected to photodynamic treatment after supplementation with zeaxanthin and α -tocopherol or with organic solvents (controls) were determined by reverse-phase high-performance liquid chromatography with reductive (mercury cathode) electrochemical detection (HPLC-EC[Hg]) as described previously [32,62,63]. Samples were chromatographed using a C18 Ultrasphere column (Beckman Instruments, San Ramon, CA, USA) and deoxygenated methanol/acetonitrile/isopropanol/1mM aqueous sodium perchlorate (NaClO_4) (72:11:8:9 by vol) as the mobile phase. Peroxides were analyzed using an analytical system consisting of an HPLC unit (Star 9100, Varian, USA) integrated with an absorption detector (HP 1100 diode array detector,

Hewlett Packard) interfaced with an EG&G Princeton Model EC 420 electrochemical detector (Princeton, NJ, USA) with a renewable hanging mercury drop electrode set at -150 mV vs. Ag/AgCl. Eluent flow was set to 1.0 ml/min. Identification and quantitation of cholesterol hydroperoxides formed in 5×10^6 ARPE-19 cells subjected to photodynamic treatment were based on the retention times and electrochemical characteristics of authentic standards (5α -OOH and $7\alpha/\beta$ -OOH) [32,33]. Cholesterol was used as an internal standard, which was monitored by its absorbance at 212 nm to correct for any sampling discrepancies.

Results

Photodynamic stress and antioxidant supplementation: cytotoxicity measurements

In separate previous investigations, zeaxanthin and α -tocopherol at the doses used here were shown to provide efficient protection against photosensitized lipid peroxidation in a liposomal system [62], *lethal* levels of photodynamic stress were shown to damage membrane lipids in ARPE-19 cells [63], and *sub-lethal* levels of photodynamic stress were shown to inhibit the specific phagocytosis of POS in ARPE-19 cells [48,49]. The goals of this investigation were to determine whether sub-lethal photodynamic stress damages membrane lipids in living cells, whether the combination of antioxidants reduces lipid peroxidation, and whether the antioxidants help maintain phagocytic function. As indicated above (Materials and Methods), a combination of 10 μ M zeaxanthin and 100 μ M α -tocopherol was used in the present work. The cellular concentration of zeaxanthin was assessed after 18 h of antioxidant supplementation and was determined to be 2.2 nmol/ 10^6 cells. The incubation time of 18 h was based on preliminary experiments showing that the time was sufficient for incorporation of zeaxanthin into cell membranes and that longer incubations produced no detectable increase in the cellular zeaxanthin concentration after Folch's extraction (data not shown).

A key observation from earlier studies was that the protocol used for PD-treatment was sub-lethal. Sub-lethality was previously confirmed by multiple measures of cell death showing that PD treatment induced no significant changes in cell number or trypan blue exclusion [48], and no increase in nuclear staining with the membrane impermeant fluorescent dye propidium iodide [49]. Morphologic analyses also confirmed no significant changes with PD-treatment in the distribution of proteins known to be sensitive to re-distribution by oxidative stress [49]. Since the experiments performed here required combining protocols from previous investigations, several preliminary studies using the MTT assay were conducted to confirm that the modified and melded protocols were also not cytotoxic and therefore sub-lethal. MTT analyses showed no cytotoxicity associated with the addition of the organic solvents (THF, ethanol) or the antioxidants to ARPE-19 cultures (not shown). Additionally, MTT experiments testing the PD treatment protocol confirmed that doses of the photosensitizers slated for use were, as planned, minimally cytotoxic in the presence of the antioxidants and their solvents. As shown and consistent with previous investigations [48,49], cytotoxicity was minimal after irradiation of ARPE-19 cultures pre-loaded with low doses of MC-540 (Fig. 1A) or RB (Fig. 1B), and did not differ for cells without or with antioxidant supplementation. At higher PD doses, however, moderate lethality was observed and a modest but significant cytoprotective effect was seen with antioxidant addition, similar

to what has been previously reported [63]. For example, cell viability increased from 76% to 89% in cultures preloaded with 15 μ M MC-540 (Fig. 1A), and from 84% to 96% in cultures with 800 nM RB (Fig. 1B).

For subsequent measurement of cholesterol hydroperoxides, a range of doses of the photosensitizers was used from sub-lethal to borderline lethal (Figs. 2-3). For phagocytosis analysis, only sub-lethal concentrations were used (Fig. 4).

Photodynamic stress and antioxidant supplementation: formation of cholesterol hydroperoxides

To determine whether sub-lethal PD treatment peroxidizes membrane lipids and leads to detectable accumulation of cholesterol hydroperoxides, similar to the accumulation we previously observed with lethal levels of PD mediated by MC-540 [63], we treated ARPE-19 cells with a range of concentrations of MC-540 or RB (Figs. 2-3). Initial experiments showed that the two most prominent products of the interaction of membrane cholesterol with singlet oxygen (5α -OOH) or free radicals ($7\alpha/\beta$ -OOH) were photogenerated by both photosensitizers in a dose-dependent manner (Fig. 2).

Kinetic analysis revealed that the rate of accumulation of singlet oxygen-specific 5α -OOH was about five times higher for 8 μ M MC-540 (Fig. 2A) and four times higher for 600 nM RB (Fig. 2B) than that of free radical-dependent $7\alpha/\beta$ -OOH. RB is a more efficient photosensitizer than MC-540 under the conditions used (Fig. 2B and Fig. 3B) and photogenerated higher fluxes of both singlet oxygen and free radicals than did M-540 (Fig. 2A and Fig 3A). Control experiments showed no measureable accumulation of lipid hydroperoxides in cells incubated in the presence of photosensitizers in the dark, or in cells irradiated in the absence of the photosensitizers (not shown).

To determine whether zeaxanthin and α -tocopherol protect against photodynamic damage to membrane lipids, the antioxidants were added to cultures prior to PD treatment followed by analysis of cellular lipids by HPLC-EC(Hg). Antioxidant supplementation substantially reduced the accumulation of singlet oxygen-specific and free radical-dependent cholesterol hydroperoxides in cells subjected to oxidative stress mediated by both photosensitizers (Fig. 3). Relative to cultures without antioxidant supplementation, the photosensitized formation of 5α -OOH was reduced by 60% - 70% (Fig. 3A) or 25% - 45% (Fig. 3B) when mediated by MC-540 or RB, respectively. Respective outcomes for $7\alpha/\beta$ -OOH were 65%-95% reduction with MC-540 (Fig. 3C) or 70% reduction with RB (Fig. 3D). Interestingly, the antioxidant combination inhibited the formation of $7\alpha/\beta$ -OOH with higher efficiency than 5α -OOH (compare Figs. 3A, B and 3C, D).

Sub-lethal photodynamic stress and antioxidant supplementation: phagocytic activity and phagocytosis receptor protein abundance

Sub-lethal PD treatment was previously shown to inhibit the specific phagocytosis by ARPE-19 cells of photoreceptor outer segments in a PD dose-dependent manner [48,49]. Here, using the same protocol, combined antioxidant supplementation with zeaxanthin and α -tocopherol produced a modest but consistent and significant restoration of phagocytic function (Fig. 4). The restoration was similar (12-14%) for both MC-540 (Fig. 4A) and RB

(Fig. 4B) across the range of photosensitizer dosages that were tested. Control experiments of non-irradiated cultures confirmed that supplementation of the cells with antioxidants had no effect on phagocytosis (data not shown).

Under conditions in which sub-lethal PD treatment impaired POS phagocytosis by ARPE-19 cells, the treatment was also shown to reduce the abundance of receptor proteins known to mediate phagocytosis [49]. Measurements of protein abundance were repeated here, comparing PD-treated cultures that were or were not supplemented with zeaxanthin and α -tocopherol (Figs. 6, 7). As shown, and consistent with the previous report [49], sub-lethal PD-treatment mediated by either MC-540 or RB diminished the quantitative immunoblotting signals for MerTK (Fig. 6), α v integrin or β 5 integrin subunits (Fig. 7) by a similar amount (approximately 50%). Control experiments were initially performed to confirm that supplementation of the cells with antioxidants did not affect the receptor proteins or the cytoskeletal protein actin (Fig. 5).

Supplementation of the cultures with the combination of antioxidants prior to PD-treatment partially prevented the receptor protein loss, reducing it from a loss of approximately 50% to about 20-25%. The protective effect of the antioxidants was similar for both photosensitizers and for all proteins.

In addition to quantifying phagocytosis receptor proteins, in our previous study we also measured the effects of PD-treatment on β -actin, a cytoskeletal protein known to participate in RPE phagocytosis [13,14], and found that actin was reduced by the protocol using MC-540 [49]. We confirmed that outcome here and additionally tested the effects of antioxidant supplementation (Fig. 8). In contrast to the membrane-bound phagocytosis proteins, the combination of antioxidants did not restore actin protein levels (Fig. 8).

Discussion

We previously showed that the specific phagocytosis of POS by ARPE-19 cells was significantly inhibited by sub-lethal photic stress induced by photodynamic treatment using MC-540 or RB as photosensitizers [48]. We also reported that proteins known to mediate POS binding and internalization, the Mer tyrosine kinase receptor MerTK [21] and the α v β 5 integrin [25,40,42,46], were affected by sub-lethal photodynamic stress showing a transient reduction and recovery after PD-treatment that coincided with a transient reduction and recovery in phagocytic function [49]. The investigation here was aimed at determining whether zeaxanthin and vitamin E could partially protect ARPE-19 cells from the stress-induced impairment of phagocytic activity and the reduced abundance of the proteins that mediate phagocytosis. Another goal was to determine whether sub-lethal PD-stress leads to the accumulation of cholesterol hydroperoxides, used as a measure of stress-induced membrane damage, and whether the combined antioxidants reduce the accumulation. This question is of interest because, as discussed below, stress-induced membrane damage could, like loss of receptor proteins, underlie phagocytosis impairment.

The rationale for analyzing antioxidant protection of stress-induced phagocytosis is the well-accepted hypothesis that antioxidants protect the retina from damage due to oxidative stress

[4,5]. This hypothesis is supported by many lines of investigation employing cell free models, culture systems and epidemiological approaches and clinical trials [3,47], but sources of stress and types of antioxidants are numerous and the mechanisms whereby antioxidant protection can occur are also many. Of particular relevance to this study are prior investigations in which stress was induced using photic sources, and antioxidant supplementation included the use of macular xanthophylls, especially zeaxanthin, in combination with vitamin E. This combination of antioxidants has been previously shown to synergistically protect against photosensitized (RB-mediated) lipid peroxidation in liposomal model systems [62]. In cellular systems using the ARPE-19 cell line, zeaxanthin plus vitamin E (or vitamin C) was shown to confer protection against photodynamic damage mediated by MC-540 [63], and the zeaxanthin-vitamin E combination also enhanced the protective effects of vitamin C against RB-mediated PD-stress [54].

In contrast to many investigations of oxidative stress in RPE culture models, our intent here (and in our previous related investigations [48,49]), was to titrate the photodynamic stress to levels that did not produce overt cell killing. The rationale for this strategy is that chronic stress of the type that is believed to occur in vivo is better modeled by sub-lethal stress than protocols involving acute, lethal stress. Sub-lethal stress protocols are also more likely to permit detection of deleterious but survivable stress effects on important cellular functions, in our case POS phagocytosis. As we showed previously and confirmed here, sub-lethal PD-stress impaired POS phagocytosis by ARPE-19 cells and reduced amounts of proteins known to participate in the process: MerTK and the $\alpha v \beta 5$ integrin. Here we additionally report that the stress-induced inhibition of phagocytosis is partially restored by antioxidant pre-treatment with a combination of zeaxanthin and α -tocopherol. We previously found a time-dependent partial restoration of phagocytic function that coincided with a partial restoration of the abundance of the phagocytosis receptor proteins and interpreted this outcome to suggest that one mechanism whereby PD stress could transiently impair POS phagocytosis is by reducing the receptor proteins that are required for POS binding and internalization [49]. The antioxidant effects seen here showing a coincident protection of both phagocytic function and receptor protein amounts lend additional support to this possibility. Additionally, the inability of antioxidant treatment to restore actin protein suggests that the antioxidant combination used here has some preferential protective effect on the membrane phagocytosis receptor proteins.

How PD-stress reduces phagocytosis receptor proteins and whether stress has other effects that could contribute to phagocytosis impairment are unanswered questions. Given the importance of membranes and their integral proteins in the phagocytic process, we sought here to determine whether sub-lethal PD-treatment oxidizes membrane lipids and whether the combined antioxidant treatment protects against membrane damage. Cholesterol was chosen as a molecular probe that undergoes oxidation to specific hydroperoxides when singlet oxygen or free radicals operate as the oxidants [10,27,32,62]. Cholesterol is not only a convenient probe, it is also relevant because cholesterol modulates important biophysical properties of membranes and participates in lipid and protein trafficking [8]. Cholesterol (and phospholipid) oxidation products can disturb the membrane bilayer structure, decrease membrane fluidity and impair key membrane functions such as transport and permeability [6,17,18,22,59]. Altered membrane fluidity can in turn affect the function of membrane

proteins [20,24,60] and certain biophysical parameters of the membrane lipid bilayer can regulate the localization of plasma membrane receptors [24]. Collectively these factors have the potential to directly or indirectly affect POS phagocytosis by ARPE-19 cells, and several lines of evidence suggest that POS phagocytosis is tightly coupled to RPE lipid and cholesterol metabolism [37].

Here we observed that sub-lethal PD-stress in ARPE-19 cells under conditions that impair phagocytosis leads to membrane lipid peroxidation, which was monitored as the accumulation of the singlet oxygen-specific cholesterol hydroperoxide 5α -OOH and the free radical-dependent cholesterol hydroperoxide $7\alpha/\beta$ -OOH. We further observed that supplementation of ARPE-19 cells with antioxidants prior to PD-treatment significantly inhibited the photosensitized generation of the cholesterol hydroperoxides, particularly $7\alpha/\beta$ -OOH. These observations raise the possibility that the peroxidation of unsaturated membrane lipids, including cholesterol, contributes to the reversible inhibition of phagocytic activity in ARPE-19 cells that follows sub-lethal photodynamic stress.

The analyses of cholesterol hydroperoxide accumulation here also provides some additional useful information about the use of MC-540 and RB as photosensitizing dyes in the PD-treatment of ARPE-19 cells as a model of photic stress, and the testing of antioxidants in complex systems. It is known that the quantum yield of singlet oxygen photogeneration by MC-540 is very low compared to RB, making RB a more efficient photosensitizer [48,56]. Our results confirmed that RB was a more efficient photosensitizer than MC-540, and that 5α -OOH accumulated at a substantially higher rate than $7\alpha/\beta$ -OOH with both photosensitizing dyes, even though both photosensitizers were shown to be efficient mediators of free radical reactions [38,56]. The higher accumulation rate of 5α -OOH suggests that membrane damage results from the interaction of unsaturated lipids with singlet oxygen, or that the efficiency of enzymatic reduction of 5α -OOH in the cells was low [34]. We also found that antioxidant supplementation significantly inhibited the photosensitized generation of cholesterol hydroperoxides, particularly $7\alpha/\beta$ -OOH. Interestingly, the antioxidants were less effective inhibitors of 5α -OOH generation when RB rather than MC-540 was used as the photosensitizer. Since MC-540 mostly localizes in membranes and RB, being a polar and charged molecule, associates with membrane by localizing in the membrane interface, this outcome suggests that the antioxidants may be more efficient quenchers of singlet oxygen generated in this sub-cellular region [16]. There are likely several additional biological explanations for the limited, partial protection afforded by the antioxidants against both cholesterol hydroperoxide generation and inhibition of phagocytosis in response to PD-stress. Among them is the possibility that the antioxidants, especially zeaxanthin, exceeded their critical membrane concentration, which could induce local aggregation of zeaxanthin in the phospholipid membranes of living cells and reduce its effectiveness as a quencher of singlet oxygen [12,16,23,28]. This hypothesis is indirectly supported by the research of Burke and co-workers who found absorption spectra for higher concentrations of zeaxanthin in DPPC liposomal systems that were inconsistent with the monomeric form of zeaxanthin [12]. Although the formation of carotenoid aggregates in biological tissues, has not been observed directly, their presence was first suggested for light-harvesting complexes of higher plants [55]. Moreover, carotenoid aggregates are perhaps most likely to be formed in the aqueous environment of in

vitro experiments [23]. Aggregate formation may occur locally, once a critical carotenoid concentration is reached [16,23]. Indeed, zeaxanthin and lutein have a high tendency to form aggregates in lipid membranes even at moderate concentrations [16,28]. The high oxygen concentration typical of cell cultures also negatively impacts the antioxidant capacity of carotenoids [23]. Many aspects of the mechanisms of antioxidant action of zeaxanthin in vitro are currently unresolved and require further investigation.

Overall, we conclude that supplementation with antioxidants exerts protective effects against oxidative stress in ARPE-19 cells subjected to PD-treatment mediated by merocyanine-540 or rose Bengal. In the model used here, the antioxidants did not fully inhibit membrane damage or restore phagocytic activity and the proteins involved in phagocytosis. However, partial protection of the type seen here against chronic stress over long durations is likely to be significant for maintenance of tissue function and survival. The observations therefore support the hypothesis that one mechanism whereby natural low-molecular-weight antioxidants zeaxanthin and α -tocopherol (vitamin E) offer synergistic protection of the human retina against chronic oxidative stress is by protecting RPE cell membranes and components of phagocytic machinery.

Acknowledgements

The authors thank F. Hoffmann-La Roche Ltd. (Basel, Switzerland), for the generous gift of zeaxanthin. This work was supported by research grants 2661/B/P01/2010/39 and MAESTRO 4 2013/08/A/NZ1/00194 from the Poland National Science Centre (TS), grants R01EY019664 and P30EY01931 from the NEI (JMB).

Abbreviations

ARMD	age-related macular degeneration
BHT	2,6-di- <i>tert</i> -butyl-4-methylphenol
BSA	bovine serum albumine
Ch-OOHs	cholesterol hydroperoxides
5α-OOH	3 β -hydroxy-5 α -cholest-6-ene-5-hydroperoxide
7α-OOH	3 β -hydroxycholest-5-ene-7 α -hydroperoxide
7β-OOH	3 β -hydroxycholest-5-ene-7 β -hydroperoxide
DFO	deferoxamine mesylate
DMSO	dimethyl sulfoxide
EDTA- Na₂	ethylene diaminetetraacetic acid disodium salt
EGTA	ethyleneglycol-O, O'-bis(2-aminoethyl)-N, N, N', N'-tetraacetic acid
FBS	fetal bovine serum
FITC	fluorescei-5-isothiocyanate
HBSS	Hank's Balanced Salt Solution

HPLC-EC (Hg)	high-performance liquid chromatography with mercury cathode electrochemical detection
MC-540	merocyanine-540
MEM	minimum essential medium
MTT	3 - (4,5- dimethylthiazol - 2 - yl) - 2,5 - diphenyltetrazolium bromide
PBS	phosphate buffered saline
POS	photoreceptor outer segments
RB	rose Bengal
RPE	retinal pigment epithelium
SDS	sodium dodecyl sulfate
THF	tetrahydrofuran

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Figure 1.

Effect of antioxidants on cytotoxicity in ARPE-19 cells subjected to photodynamic stress mediated by merocyanine-540 (A) or rose Bengal (B). Control cultures (closed circles) or cultures enriched with zeaxanthin and α -tocopherol (open squares) were incubated with a range of concentrations of MC-540 or RB and exposed to green light followed by the MTT assay as an indicator of toxicity. Data are the percent of control cultures (irradiated without photosensitizers) expressed as means of triplicate cultures; error bars indicate SD.

Figure 2.

Accumulation of lipid hydroperoxides 7 α / β -OOH (open triangles) and 5 α -OOH (black squares) in ARPE-19 cells undergoing photosensitized oxidation. Cells were incubated with a range of concentrations of MC-540 (A) or RB (B) and exposed to green light. After irradiation, lipids were extracted from 5×10^6 of cells and subjected to HPLC-EC (Hg) analysis. Values are means of triplicate cultures; error bars indicate SD.

Figure 3.

The effect of antioxidants on the photosensitized generation of cholesterol hydroperoxide 5α-OOH (A, B) and 7α/β-OOH (C, D) in ARPE-19 cells without photosensitizers (controls) or in cells pre-loaded with (A,C) MC-540 or (B, D) RB. Cultures without antioxidants (black bars) or enriched with zeaxanthin and α-tocopherol (open bars) were incubated with a range of concentrations of MC-540 or RB and exposed to green light. After irradiation, lipids were extracted from 5×10^6 cells and subjected to HPLC-EC (Hg) analysis. Values are means of triplicate cultures; error bars indicate SD. Data were corrected for any variation in the amount of extracted lipids using cholesterol as an internal standard. Asterisks indicate significant differences between non-supplemented and antioxidant-treated cultures (*t*-test, $P < 0.05$).

Figure 4.

The effect of antioxidants on the specific phagocytic activity of ARPE-19 cells subjected to photoinduced oxidative stress with MC-540 (A) or RB (B). Cultures without antioxidants (black bars) or enriched with zeaxanthin and α -tocopherol (open bars) were incubated with a range of concentrations of MC-540 or RB and exposed to green light. POS-FITC were delivered to cultures and uptake was quantified by flow cytometry. Data were normalized to control cultures lacking photosensitizers. Values are means of six replicate cultures; error bars indicate SD. Asterisks indicate significant differences between non-supplemented and antioxidant-treated cultures (t -test, $P < 0.05$).

Figure 5.

Western blot analysis for (A) the αv integrin subunit, (B) the $\beta 5$ integrin subunit, (C) MerTK, or (D) actin in antioxidant supplemented (open bars) or non-supplemented ARPE-19 cultures (black bars). Data are the mean band densities, given in arbitrary units (AU), from extracts of triplicate culture wells from each group in a representative experiment; error bars indicate SD ($n=4$). Outcomes do not differ significantly for any protein (t -test analyses).

Figure 6.

Western blot analysis for MerTK protein in ARPE-19 cultures without antioxidants or supplemented with zeaxanthin/ α -tocopherol and subjected to photodynamic stress mediated by (A) 8 μ M MC-540 or (B) 600 nM RB followed by light irradiation. Blots from triplicate cultures for each group are shown. Graphs show densitometric analysis of the bands for non-supplemented (black bars) or antioxidant supplemented cultures (open bars). Band densities in the photosensitizer-treated groups are expressed as a percent of their respective controls; error bars indicate SD. MerTK signals without versus with antioxidants differ significantly for both photosensitizers (t -test, $P < 0.05$).

Figure 7.

Western blot analysis for (A,B) αv integrin and (C,D) β5 integrin subunits in ARPE-19 cultures without antioxidants or supplemented with zeaxanthin/α-tocopherol and subjected to photodynamic stress mediated by (A,C) 8 μM MC-540 or (B,D) 600 nM RB followed by light irradiation. Blots from triplicate cultures for each group are shown. Graphs show densitometric analysis of the bands for non-supplemented (black bars) or antioxidant supplemented cultures (open bars) with the band densities in the photosensitizer-treated groups expressed as a percent of their respective controls. Error bars indicate SD. αv integrin and β5 integrin signals without versus with antioxidants differ significantly for both photosensitizers (*t*-test, *P*<0.05).

Figure 8.

Western blot analysis for actin in ARPE-19 cultures without antioxidants or supplemented with zeaxanthin/ α -tocopherol and subjected to photodynamic stress mediated by 8 μ M MC-540 followed by green light irradiation. Blots from triplicate cultures for each group are shown. Graphs show densitometric analysis of the bands for non-supplemented (black bars) or antioxidant supplemented cultures (open bars). Band densities in the photosensitizer-treated groups are expressed as a percent of their respective controls; error bars indicate SD. Actin signals without versus with antioxidants do not differ significantly (t -test analysis, $P < 0.05$).