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Retinal carotenoids can attenuate formation of A2E in the retinal pigment epithelium

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ABSTRACT

A2E, an important constituent of lipofuscin in human retinal pigment epithelium (RPE), is thought to mediate light-induced oxidative damage associated with aging and other ocular disorders. Ocular carotenoids in overlying retinal tissues were measured by HPLC and mass spectrometry and were correlated with levels of RPE A2E. We observed a statistically significant increase in total A2E levels in human RPE/choroid with age, and A2E levels in macular regions were approximately 1/3 lower than in peripheral retinal regions of the same size. There was a statistically significant inverse correlation between peripheral retina carotenoids and peripheral RPE/choroid A2E. Prospective carotenoid supplementation studies in Japanese quail demonstrated nearly complete inhibition of A2E formation and oxidation. These findings support current recommendations to increase dietary intake of xanthophyll carotenoids in individuals at risk for macular degeneration and highlight a new potential mechanism for their protective effects—inhibition of A2E formation and oxidation in the eye.

Introduction

Age-related macular degeneration (AMD) is the major cause of irreversible blindness in developed countries, yet its molecular pathophysiology remains inadequately understood [1]. Cellular damage due to high levels of oxidative stress appears to be one of the main pathological explanations for age-related ocular diseases including AMD [2], and cellular accumulation of lipofuscin, a complex mixture of highly fluorescent retinoid and phospholipid metabolites, is considered to be a primary pathogenic biomarker of aging in the retinal pigment epithelium (RPE) [3].

A2E is the one of the important constituents of lipofuscin in the RPE. Chemically, it is a combination of two all-*trans*-retinal molecules and one ethanolamine molecule [4]. Levels of A2E and other lipofuscin components rise with age, with light exposure, and with development of AMD, and early onset macular disorders such as Stargardt and Best diseases are notable for unusually high levels of A2E in humans and in animal models [5–9]. Studies have demonstrated that lipofuscin components such as A2E and its *cis* isomers can act as blue-light-mediated photosensitizers for the generation of reactive oxygen species that could cause damage and cell death in the macula, potentially leading to loss of central vision [10,11].

On the other hand, the dietary xanthophyll carotenoids lutein and zeaxanthin are concentrated at very high levels in the human macula and to a lesser extent in the peripheral retina where they are believed to limit retinal oxidative damage by absorbing incoming blue light and/or by quenching reactive oxygen intermediates [12,13]. *In vitro* studies have suggested that the ocular carotenoids may alleviate A2E-mediated oxidative damage either by direct quenching or by screening phototoxic blue light [14], but *in vivo* evidence is notably lacking, in part due to the difficulty in obtaining human ocular tissues and the rarity of non-primate small animal models that accumulate significant levels of both A2E and ocular carotenoids. Here we report the relationship of A2E and carotenoids in the macula and peripheral retina of a large collection of human eyes, and we study the inhibition of A2E formation by dietary carotenoids in the Japanese quail *Coturnix japonica*, a bird that has substantial ocular levels of both A2E and carotenoids.

Materials and methods

Chemicals

Organic solvents were HPLC grade from Fisher Scientific (Hampton, NH). Standards of A2E and iso-A2E were prepared and column purified in the laboratory of Dr. Heidi R. Volmer-Snarr, Department

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of Chemistry and Biochemistry, Brigham Young University, Provo, Utah. They were dissolved in methanol (MeOH) at a concentration of 1 μg/ml, stored at −70 °C, and brought to room temperature before use. The concentration of the stock solution of A2E was confirmed spectroscopically [E (1 M) at 439 nm = 36900] using a published extinction coefficient [4]. Similar standard stock solutions of lutein (Kemin Health, Des Moines, IA) and zeaxanthin (DSM, Kaiseraugst, Switzerland) were prepared, and concentrations were confirmed using published extinction coefficients [15].

Tissue procurement and processing

Human donor human eyes were obtained from the Utah Lions Eye Bank within 24 h after death after corneas had been harvested for transplantation. None of the donors had a known history of eye disease. Tissue procurement and distribution complied with the tenets of the Declaration of Helsinki. The time between donor death and enucleation was not more than 4 h. Dissections were carried out 6–24 h after donor death in a dim light environment. These data exclude outliers with unusually high levels of ocular carotenoids who had been consuming high-dose lutein supplements regularly prior to death because of potential confounding effects on data interpretation since exact dosage and duration of supplementation were unknown [16]. All eyecups were visually inspected with a handheld magnifier to exclude the presence of obvious ocular pathology such as intermediate or large drusen, hemorrhages, or scars. After carefully removing adherent vitreous, macular and mid-peripheral retinal tissues were excised with an 8-mm circular trephine. The underlying RPE/choroid layer was then carefully excised using the same trephine. Diverse non-human mammalian eyes were obtained from neighboring laboratories or local slaughterhouses. Since non-primate animal eyes do not have a macula, the entire retina and RPE/choroid were isolated and processed. Wet weights were recorded for all collected tissues after blotting excess moisture. Total protein levels and protein separation patterns determined by Bradford assay and by one-dimensional SDS gel electrophoresis, respectively, were similar in both macular and retinal RPE/choroid punches from humans (~250 μg/8-mm of tissue).

Extraction of A2E from RPE/choroid

A2E and its isomers were extracted and isolated from RPE/choroid using a previously described method [8]. RPE samples were homogenized in 1:1 $CHCl_3$/MeOH (2 ml) and 0.01 M phosphate-buffered saline (PBS) (1 ml). The homogenizer was washed with 1:1 $CHCl_3$/MeOH (2 ml), 0.01 M PBS (1 ml), and then $CHCl_3$ (2 ml) and CH_2Cl_2 (2 ml) were added to remove any remaining material. All solutions were combined, and the organic layer was extracted from the aqueous layer. The combined organic extracts were evaporated to dryness under vacuum at room temperature. The residue was dissolved in MeOH for HPLC. The vials were centrifuged at approximately 2000*g* to remove the minor amounts of insoluble solid particles prior to analysis.

Extraction of carotenoids from retina

Tissues were homogenized and extracted three times with tetrahydrofuran containing 0.01% (w/v) butylated hydroxytoluene by sonication at 5–10 °C for 30 min each time. The combined organic extracts were evaporated to dryness under vacuum at room temperature. The dried residue was redissolved in one ml of HPLC mobile phase and centrifuged at approximately 2000*g* for 10 min to remove the minor amounts of insoluble solid particles prior to analysis. The majority of carotenoids in bird retinas are esterified [17], so after the initial extraction, the dried carotenoid residue was redissolved in hexane and subjected to saponification in 1.8% (w/v) methanolic potassium hydroxide (KOH) for 2 h at room temperature. After saponification, the samples were washed with water until the samples achieved neutral pH. The vials were centrifuged at approximately 2000*g* to remove the minor amounts of insoluble solid particles. The solution was evaporated to dryness on a rotary evaporator under reduced pressure at room temperature and reconstituted in the appropriate HPLC solvents.

HPLC conditions

HPLC analysis was performed on a Thermo Separations (San Jose, CA) HPLC system with binary gradient pumps, a refrigerated autosampler, a UV6000 photodiode-array detector (PDA), and an MSQ single quadrupole mass spectrometer. Peak identities were confirmed by PDA and mass spectra and by co-elution with authentic standards as necessary. Calibration was by external standardization curves with authentic standards. We do not routinely use internal standards because they may interfere with low-level analytes in small biological samples [18]. Typical reproducibility with external standardization in our laboratory is ±5%.

A2E HPLC analysis

The dried A2E samples were re-dissolved in 100 μl of MeOH. A gradient of 84–100% acetonitrile (A) with 0.05% trifluoroacetic acid in H_2O (B) over 35 min was used to separate A2E at a flow rate of 1.0 ml min^{-1} on a reverse-phase C18 column (4.6 × 250 mm, Phenomenex, Atlanta, GA). The column was maintained at room temperature, and the HPLC PDA detector was operated at 440 nm.

Carotenoid HPLC analysis

The dried extracts were re-dissolved in 100 μl of HPLC mobile phase [hexane: dichloromethane: methanol: *N*,*N*′-di-isopropylethylamine (80:19.2:0.7:0.1 v v^{-1})]. HPLC separation was carried out at a flow rate of 1.0 ml min^{-1} on a cyano column (Microsorb 25 cm length × 4.6 mm id, particle size 5 μm, Rainin Instrument Co., Woburn, MA). The column was maintained at room temperature, and the HPLC PDA detector was operated at 450 nm.

Mass spectrometry (MS) equipment and conditions

MS analysis was performed using a Thermo Separations (San Jose, CA) MSQ single quadrupole mass spectrometer, equipped with an electron spray ionization (ESI) source and an atmospheric pressure chemical ionization (APCI) source. A2E and carotenoids were ionized in positive ion ESI and APCI modes, respectively. To avoid overloading of eluted solvent molecules in the mass spectrometer and to optimize ionization conditions, 50% (v/v) of the eluate was directed to waste with the help of a diverter valve after the PDA detector. The delay time from PDA to MS was 0.13 min. The protonated precursor molecular ions were initially acquired in full-scan mode from 300–1000 Da with 0.2 step size, revealing the molecular masses of the components. Selected ion monitoring (SIM) was performed using dwell time of 200 ms for each channel. In SIM mode, the *m*/*z* channels 592 ± 3, 608 ± 1.5, 624 ± 1.5, and 640 ± 1.5 were used for A2E and its oxidative products. Typical detection conditions for A2E were: RF lens bias voltage 0.1 V, cone voltage 80 V and heater temperature 550 °C. The ion source and tuning lens parameters were optimized automatically by infusing A2E samples via the injector. For carotenoids, the *m*/*z* channels 551 ± 0.7 and 569 ± 0.7 were used for lutein, and 569 ± 0.8 for zeaxanthin. Typical detection conditions were: corona discharge current 5 μA, cone voltage 80 V, and probe temperature 500 °C.