Research Article

Journal of MASS SPECTROMETRY

Received: 6 July 2010 Accepted: 1 November 2010 Published online in Wiley Online Library: 23 December 2010

(wileyonlinelibrary.com) DOI 10.1002/jms.1865

Compositional studies of human RPE lipofuscin: mechanisms of molecular modifications

L. S. Murdaugh,[a] S. Mandal,[a] A. E. Dill,[a†] J. Dillon,[a,b] J. D. Simon[c] and E. R. Gaillard[a,b*]

The accumulation of lipofuscin has previously been implicated in several retinal diseases including Best's macular dystrophy, Stargardt's disease and age-related macular degeneration (AMD). Previously one of the major fluorophores of lipofuscin was identified as a bis-retinoid pyridinium salt called A2E, which is known to photochemically cause damage. In addition to A2E, there are numerous components in RPE lipofuscin that are unidentified. These compounds were determined to be structurally related to A2E by their fragmentation pattern with losses of 106, 190, 174 and/or 150 amu from the parent ion and the formation of fragments of *ca* 592 amu. The vast majority consists of relatively hydrophobic components corresponding to derivatized A2E with molecular weights in discrete groups of 800–900, 970–1080 and >1200 *m/z* regions. In order to determine the mechanism of these modifications, A2E was chemically modified by; (1) the formation of specific esters, (2) reaction with specific aldehydes and (3) spontaneous auto-oxidation. The contribution of ester formation to the naturally occurring components of lipofuscin was discounted since their fragmentation patterns were different to those found *in vivo*. Alternatively, reactions with specific aldehydes result in nearly identical products as those found *in vivo*. Artificial aging of RPE lipofuscin gives a complex mixture of structurally related components. This results from the auto- and/or photooxidation of A2E to form aldehydes, which then back react with A2E giving a series of higher molecular weight products. The majority of these modifications result in compounds that are much more hydrophobic than A2E. These higher molecular weight materials have increased values of log *P* compared to A2E. This increase in hydrophobicity most likely aids in the sequestering of A2E into granules with the concomitant diminution of its reactivity. Therefore, these processes may serve as protective mechanisms for the RPE. **Copyright © 2010 John Wiley & Sons, Ltd.**

Supporting information may be found in the online version of this article.

Keywords: lipofuscin; A2E; blue light damage; oxidative stress; age-related macular degeneration

Introduction

As organisms age, many metabolically active post-mitotic cells accumulate autofluorescent lysosomal storage bodies known as lipofuscin. Lipofuscin is a brown-yellow, electron-dense, age-related pigment that is composed of a complex heterogeneous mixture of lipid–protein aggregates that form clusters of granules in the RPE. The accumulation of lipofuscin has previously been implicated in several retinal diseases including Best's macular dystrophy, Stargardt's disease and age-related macular degeneration (AMD).[1–8] Within the human eye, the granules are believed to form from the indigestible material of phagocytized photoreceptor outer segments[9,10] and may account for up to 19% of the cytoplasmic volume by the age of 80.[3,11,12] Lipofuscin has also been shown to generate a series of reactive oxygen species (ROS), which include singlet oxygen, hydrogen peroxide and superoxide anions.[4,11,13–15] Considered photochemically toxic, lipofuscin was found to decrease phagocytic capacity.[16] Previous studies have shown that both the isolated granules and the organic soluble extract of lipofuscin are photoreactive.[4,14,17] Additional studies indicate that light exposure, the accumulation of these photoreactive granules, photoreceptor cell death and the onset of AMD are all related.[2,18,19]

One of the major fluorophores of lipofuscin, A2E, was first isolated from lipofuscin granules by Eldred.[20] Structurally, A2E is a pyridinium bis-retinoid, which is synthesized using 2 mol of all-*trans*-retinal (RAL) and 1 mol of ethanolamine.[21–23] The photooxidation of A2E results in the generation of ROS, such as peroxide and superoxide radicals.[24,25] Ben-Shabat *et al*.[26] were the first to propose the photooxidation of A2E results in higher molecular weight compounds that differ by 16 amu resulting in epoxide formation along the polyene chain. However, the survival of these epoxides in the acidic environment of lysosomes could not be explained. Dillon *et al*.[27] proposed that the epoxides of A2E would undergo rearrangement to form furanoid oxide structures,

* *Correspondence to: E. R. Gaillard, Department of Chemistry and Biochemistry, Northern Illinois University, DeKalb, IL 60115, USA. E-mail: gaillard@niu.edu*

† *Current address: A. E. Dill, GreatPoint Energy, 2225 Harrison St., Chicago, IL 60612, USA.*

a *Department of Chemistry and Biochemistry, Northern Illinois University, DeKalb, IL, USA*

b *Department of Ophthalmology, Columbia University, New York, NY, USA*

c *Department of Chemistry, Duke University, Durham, NC, USA*

Copyright © 2010 John Wiley & Sons, Ltd.

Journal of MASS SPECTROMETRY

which are relatively stable in the acidic lysosomal environment. Furthermore, oxidative cleavage of side chains results in the formation of highly reactive aldehydes and ketones, which could readily react with cell constituents and cause irreversible damage.[28] This explanation is based on the structural similarities between carotenoids and A2E. In addition, the amphiphilic structure of A2E is responsible for detergent-like action in membrane disruption. The quaternary amine structure also aids in the inhibition of lysosomal function by complexing to specific lysosomal enzymes.[21]

Besides A2E, there are numerous components in RPE lipofuscin that are structurally related to A2E as determined by their fragmentation pattern with losses of 106, 190, 174 and/or 150 amu from the parent ion and the formation of fragments of *ca* 592 amu. The vast majority consists of relatively hydrophobic components corresponding to derivatized A2E with molecular weights in discrete groups of 800–900, 970–1080 and >1200 *m/z* regions. These modified components increase the hydrophobicity of A2E and may explain the formation of lipofuscin granules in the RPE. The present study is part of a continuing effort to identify the molecular modifications to the structure of A2E[27,28] and their mechanism of formation.

Materials and Methods

Chemicals

The chemicals used for the experiments were of the highest purity that are commercially available. All solvents used were HPLC grade and were purchased from Thermo Fisher Scientific (Waltham, MA, USA). RAL was obtained from A.G. Scientific (San Diego, CA, USA). Ethanolamine was obtained from ACROS Organics (NJ, USA). Water was purified using a Millipore Milli-Q Plus PurePak 2 water purification system.

Synthesis of A2E

A2E was prepared from RAL and ethanolamine in acetic acid and ethanol as previously described by Parish *et al*.[23] The mixture was stirred in the dark for 3 days at room temperature. After excess solvent was removed, the A2E was separated from the initial reaction mixture using 1050 Ti HPLC (Hewlett Packard, France) and a phenomenex 4-μm C18 RP column. Using an isocratic gradient of MeOH/H_2O (90 : 10) and a flow rate of 1.0 ml/min, the retention time of A2E was approximately 28 min monitored with a photodiode array detector at 430 nm. The concentration of the purified A2E was determined by measuring the absorbance at 439 nm using an Ocean Optics spectrometer, given an extinction coefficient of 36 900 l/mol cm.[23] After collection, the pure A2E fraction was dried under argon and stored at −70 °C until further analysis.

Synthesis of A2E esters

HPLC purified A2E was used for this reaction. A2E was dissolved in chloroform and transferred to a three-neck flask. The flask was continuously purged with argon gas on one side and fitted to a drying tube on the other side to absorb the hydrochloric acid from the reaction. A2E was reacted with acetyl chloride, hexanoyl chloride and cinnamoyl chloride separately. The reactions were quenched with sodium hydroxide and water and dried under argon. Analysis of the three reaction mixtures was performed using ESI/MS by re-dissolving in acetonitrile.

Reaction of A2E with cinnamaldehyde and benzaldehyde

A2E collected from the HPLC was dried under argon. A2E (30 μmol/l) was then resuspended in 4 ml of acetonitrile and then treated with cinnamaldehyde (15 μM) or benzaldehyde (15 μM). The reaction was run for approximately 12 h, an aliquot was then diluted with acetonitrile and analyzed with ESI-MS with the same instrument settings as below.

Lipofuscin

Human RPE lipofuscin granules were extracted and isolated from donor globes (Midwest Eye Banks and Transplantation Centers) as previously described by Feeney-Burns and Eldred.[10] The organic soluble portion was obtained from the samples by Folch extraction using a 1 : 1 : 1 ratio of $CHCl_3$/CH_3OH/H_2O. The organic soluble portion was then dried under argon, resuspended in 1 ml MeOH and analyzed using ESI-MS/MS with a PDA detector.

Artificially aged (auto-oxidized) A2E

After synthesis and purification, a 2-ml aliquot of 15 μM A2E was transferred to a 4 °C refrigerator in the dark. An aliquot (20 μl) was removed from the sample at times: 0, 30 and 60 days and analyzed on ESI-MS/MS with PDA detector.

Reaction of A2E with all-trans-retinal (RAL)

After synthesis and purification, a 5-ml aliquot of 50 μM A2E was mixed with 100 μM RAL and a catalytic amount of acetic acid. The mixture was then bubbled with argon and irradiated for 1 h using a Philips special blue light (Oriel, Stratford, CT, USA, model number 6292) in a glass irradiating chamber fitted with a quarter inch sheet of plexiglass cover to block the small amount of UV output from the lamp. An aliquot was then removed and diluted with methanol and analyzed on ESI-MS/MS with PDA detector.

LC–MS analysis

All samples were analyzed on a Thermo Finnigan LCQ Advantage. The mass spectrometer was set to positive ion mode with a capillary temperature of 200 °C, source voltage of 4.0 kV, capillary voltage of 42 V and a tube lens offset of 50 V. The mass-to-charge ratios were collected from 200 to 2000 and normalized collision energy of 35% was used for the MS/MS data. The lipofuscin and purified A2E samples were separated using Surveyor LC system with a Synergi Max-RP C12 column. The flow rate was set to 0.2 ml/min with a mobile phase of MeOH balanced with H_2O (both containing 0.1% formic acid) using a gradient of 80% MeOH for 30 min and 80–100% MeOH for 90 min.

Results

Besides A2E, there are numerous components in RPE lipofuscin that are structurally related to A2E. This is determined by their fragmentation pattern with losses of 106, 190, 174 and/or 150 amu from the parent ion and the formation of fragments of *ca* 592 amu. The vast majority consists of relatively hydrophobic components corresponding to derivatized A2E with molecular weights in discrete groups of 800–900, 970–1080 and >1200 *m/z* regions. Since the RPE stores retinal as a fatty acid ester, our first hypothesis was that the hydrophobic components of A2E were a series of fatty acid derivatives.

 Copyright © 2010 John Wiley & Sons, Ltd.

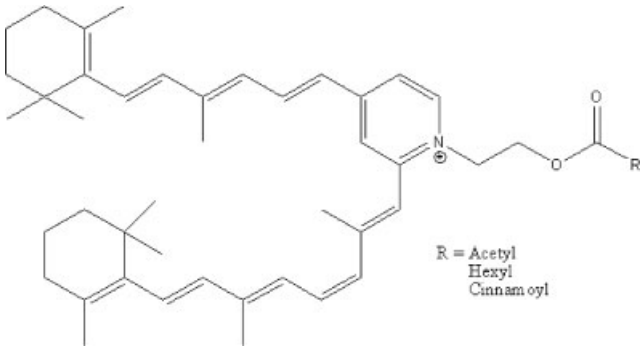

Figure 1. Product from esterification reaction with A2E and alkyl chloride. R equals acetyl, hexanoyl or cinnamoyl (C_9H_7ClO).

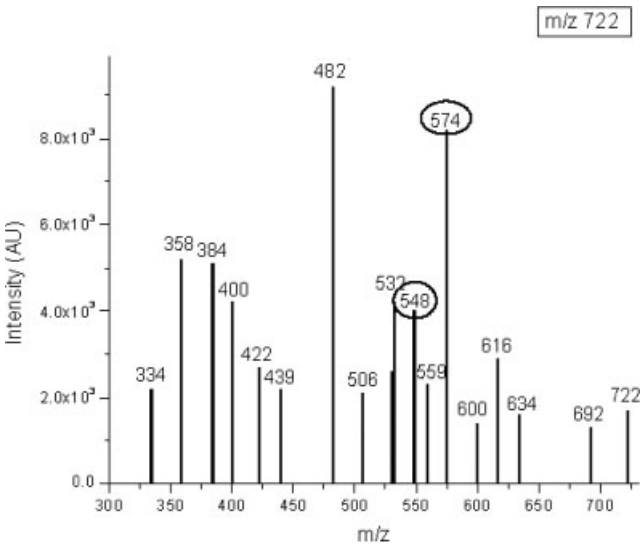

Figure 2. The MS/MS of cinnamoyl ester (*m/z* 722).

A2E was first treated with acetyl, hexanoyl or cinnamoyl chloride to synthesize the esters as indicated in Fig. 1, whereas Fig. 2 displays the MS/MS obtained from the cinnamoyl ester. All esters showed the characteristic losses of 106 and 190[29] but also gave spectra with major fragments of *m/z* 548 and/or 574. Further fragmentation of this major peak (*m/z* 548) yielded fragments, with *m/z* 358, 410 and 374 (Supporting Information Fig. 1). The two major daughter fragments of the esters can readily be explained by the rearrangement depicted in Fig. 3 resulting from de-esterification with the loss of ethanol to form *m/z* 548 (a) or a McLafferty rearrangement resulting in *m/z* 574 (b). In addition, it is possible to form *m/z* 548 by the loss of 174 from the parent *m/z* 722 for the cinnamoyl ester. These fragments are not found in the lipofuscin components.

A second hypothesis that may account for the hydrophobic mixture in lipofuscin involved reactions with A2E-derived aldehydes.[28] It was proposed that once formed these aldehydes could then react with other A2E molecules forming higher molecular weight species. To investigate this hypothesis, synthesized A2E was allowed to incubate at 4 °C for 60 days. This artificially aged A2E sample led to a complex mixture, which included many of the compounds found *in vivo* and appeared as clusters in three *m/z* regions (centered at 850, 1100 and 1400). Figure 4 displays some of the compounds found in the artificially aged A2E sample, which are similar to the lipofuscin mixture. The MS/MS of one of the major peaks with *m/z* 858 is displayed as the inset in Fig. 4 with characteristic losses of 106, 150, 174 and 190 identified.[30] To further investigate the formation of the higher molecular weight compounds formed from the reaction of A2E with aldehydes, A2E was reacted with specific aldehydes such as cinnamaldehyde, benzaldehyde and RAL for approximately 12 h in the dark or A2E was photolyzed for 3 h with a subsequent addition of the aldehyde. The resulting full mass spectra from reaction with cinnamaldehyde showed completely oxidized A2E and peaks with the oxidized A2E and attached aldehydes (Fig. 5(a) and (b)). The benzaldehyde reaction led to similar products (Supporting Information Fig. 2). Similar to the human lipofuscin data, the reactions with A2E appear as a series of discrete groups.[30] For both the cinnamaldehyde and benzaldehyde reactions, Group I is A2E and its oxidation products, Group II is Group I plus the addition of one aldehyde and Group III is the addition of a second aldehyde.

The fragmentation pattern of one of the higher molecular weight compounds in the A2E–cinnamaldehyde (*m/z* 790) condensation is displayed in Fig. 5(b). The fragmentation of major peaks showed similar patterns as seen in the lipofuscin components with the loss of 150, but did not have the peaks corresponding to losses of 190 and 174 fragments. The loss of 148 in the A2E–cinnamaldehyde spectrum could be attributed to cinnamic acid. The loss of 106 also signifies that the side chains of A2E are intact and the modifications are occurring at the ends of the polyene chain. The loss of xylene is commonly observed in polyene compounds that have more than four conjugated bonds.[31,32]

The photolysis of A2E and RAL was also performed. The full mass spectrum displayed in Fig. 6 indicates the formation of three main products with *m/z* 858, 920 and 1188 after 1 h of irradiation. The fragmentation pattern of *m/z* 858 displayed in Fig. 7 shows the same characteristic losses of 106, 150, 174 and 190 and the parent ion with *m/z* 858 as seen in human retinal lipofuscin (Fig. 8). Fragmentation of compounds with *m/z* 1188 and 920 both contain a major daughter ion with *m/z* 858 (Supporting Information Figs 4 and 5). This reaction was also performed without irradiation; however, the formation of products with *m/z* 920 and 1188 was much slower, appearing after 18 h. The peak with *m/z* 920 was also found in lipofuscin but with a lower abundance compared with the artificially aged A2E.

Discussion

Hydrophobic substances, like lipids, have the ability to bind to cellular membranes and hydrophobic proteins, which disrupt the normal cellular function. Hence, these hydrophobic substances are stored in small pockets known as lipid droplets or adiposomes. In the eye, the retinyl ester storage particles in the RPE are called retinosomes. These storage particles are inert and prevent any interaction of hydrophobic lipids with cellular components.[33]

In the RPE, RAL, an intermediate of the visual cycle is converted to all-*trans* retinyl ester by lecithin retinal acyl transferase (L-RAT).[34–37] The retinyl ester then self-aggregates into a retinosome,[33] which later reacts with visual enzymes to produce 11-*cis*-retinal. Similarly, A2E that accumulates in the RPE lysosomes may also be subjected to esterification or modification with the reactive aldehydes that result from oxidation of A2E.[27] Furthermore, the accumulation of A2E is indirectly associated with decreased efflux of cholesterol from the RPE cells.[38] Since

Copyright © 2010 John Wiley & Sons, Ltd.

Journal of MASS SPECTROMETRY

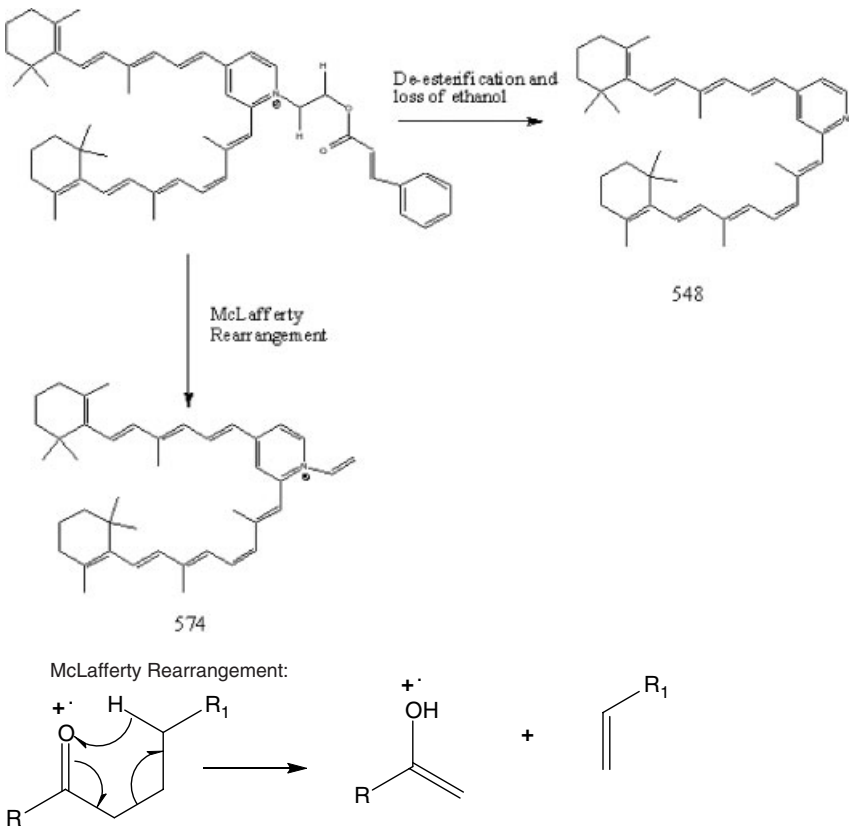

Figure 3. Scheme for the formation of the two major fragments (*m/z* 574 and 548) observed in the MS/MS spectrum of esterified A2E. The *m/z* 548 species is proposed to be formed through de-esterification and the loss of ethanol. The *m/z* 574 species is proposed to form through McLafferty rearrangement (see inset).

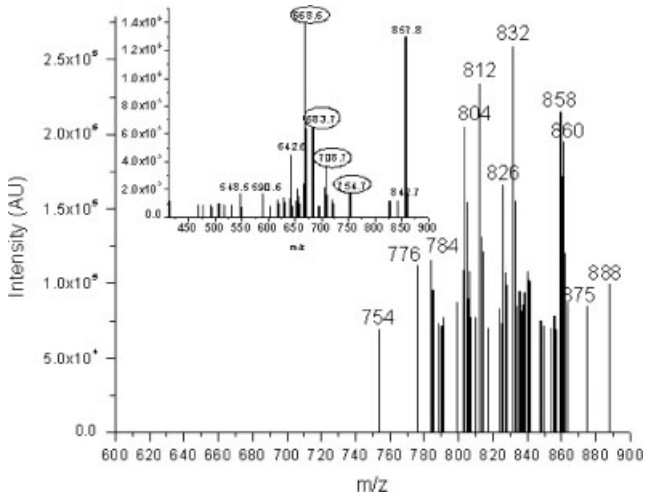

Figure 4. The mass spectrum of artificially aged A2E at retention time 89.18 min (inset MS/MS 858 from the companion paper[30]).

the phagocytic activity of RPE is reduced considerably with the accumulation of lipofuscin, the major transcription factors such as A2-P and PPARγ,[39] which maintains the acid lipase activity and transcription of major cholesterol transporters, are less expressed leading to decreased cholesterol efflux and increased cholesteryl ester formation.[38] Based on the activity of the acid lipase and RPE lysosomal enzymes, A2E could exist as both an A2E ester and free A2E. In addition, cholesterol accumulation can be further accelerated by age-dependent thickening of Bruch's membrane (BM). BM is located between the choriocapillaris and the RPE and is found to accumulate extracellular matrix materials that can interact with lipoproteins.[40]

Esterification of A2E was performed with acetyl chloride and hexanoyl chloride. Both the reactions were used as model systems to represent the possible short and long chain fatty acids that exist in the lipofuscin extract that could derivatize A2E to form esters. The fragmentation pattern for both the esters had patterns that could not be found in the human lipofuscin extract. However, the major fragment with *m/z* 548 was identified in the full mass spectrum of human lipofuscin extract and had similar fragments to that of the fragment with similar *m/z* from A2E ester. The structural make up of both the compounds could be similar. Figure 3 illustrates a possible structure for the species with *m/z* 548, which is A2E after the loss of the ethanol group. Supporting information (supplemental figure 1) displays the MS/MS data for the compound with *m/z* 548, which includes a major fragment with *m/z* 358. Since both the esterification products displayed the same fragmentation pattern, A2E was treated with the aromatic cinnamoyl chloride, which has structural similarity to A2E. The fragmentation pattern of the A2E cinnamoyl chloride ester differed from the acetyl and hexanoyl esters, the major peak had *m/z* 574, which was attributed to McLafferty rearrangement illustrated in Fig. 3. This fragmentation was not observed in human RPE samples indicating that A2E is probably not stored as esters.

Oxidation of A2E results in the formation of highly reactive aldehydes that result from A2E side chain cleavage. The higher molecular weight compounds are not stored as esters but may

 Copyright © 2010 John Wiley & Sons, Ltd.

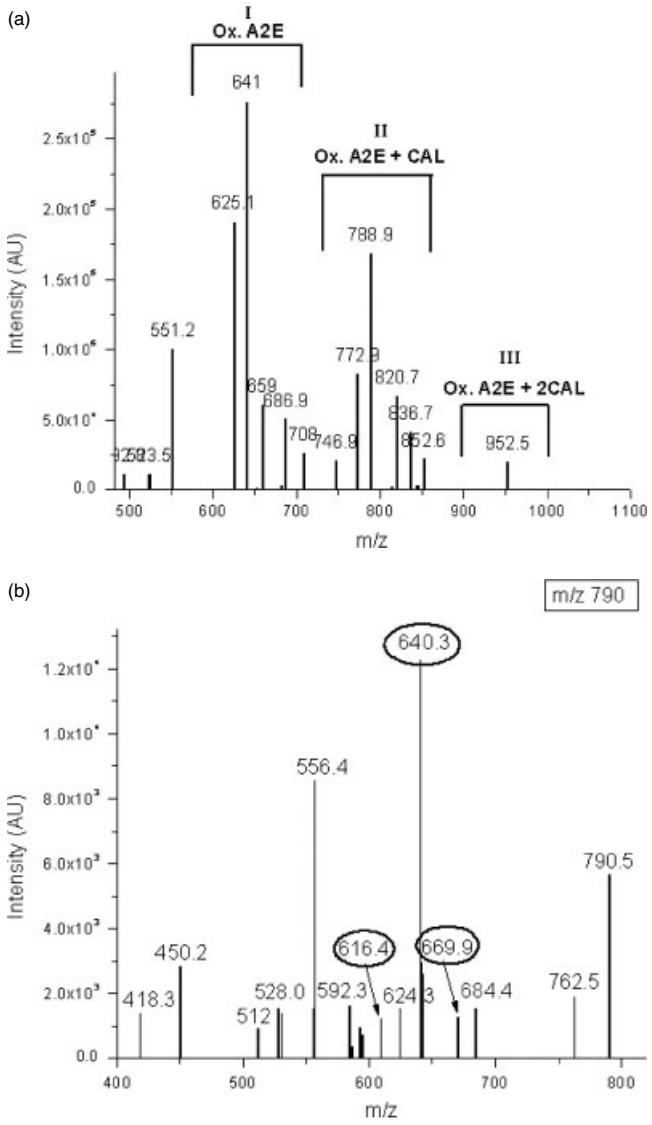

Figure 5. (a) The full mass spectrum of the reaction between A2E and cinnamaldehyde (CAL) and (b) the MS/MS of *m/z* 790 from the A2E–cinnamaldehyde reaction mixture.

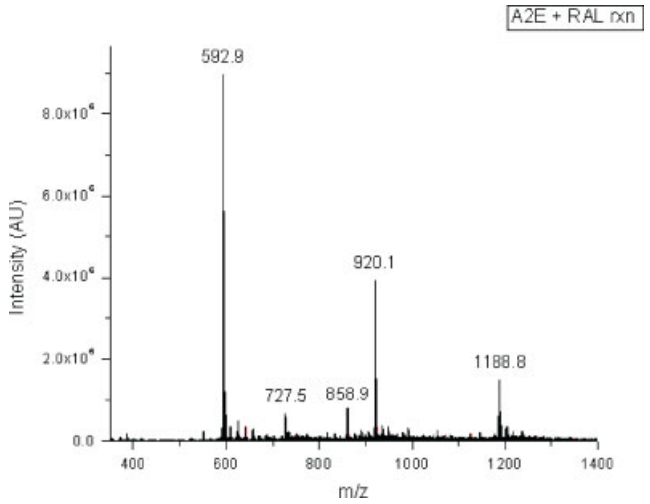

Figure 6. The mass spectrum of A2E photolyzed in the presence of all-*trans*-retinal.

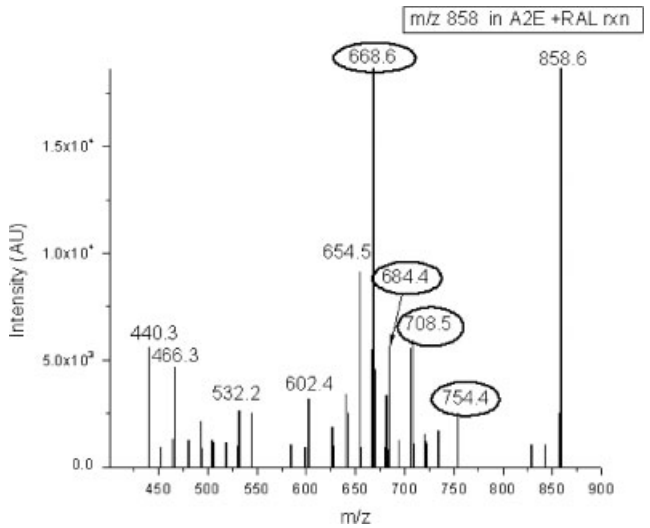

Figure 7. The MS/MS of *m/z* 858 from the A2E–all-*trans*-retinal reaction. Peaks corresponding to *m/z* 858 with the loss of 106 (*m/z* 754), 150 (*m/z* 708), 174 (*m/z* 684) and 190 (*m/z* 668) are identified.

be a result of the attachment of aldehydes to oxidized A2E structures. When A2E was allowed to incubate for 60 days, several compounds are formed that are also observed in human lipofuscin. The auto-oxidation of A2E in the presence of cinnamaldehyde and benzaldehyde yielded a series of compounds including oxidation and addition products. The fragmentation patterns and characteristic losses of these products were similar to those found in oxidized A2E. The photolysis of A2E in the presence of retinal also generated compounds that had the same characteristic fragmentation patterns with losses of 106, 150, 174 and 190 found in human retinal lipofuscin.[29]

In addition, Simon *et al*. recently reported that lipofuscin is an aggregated material consisting of a variety of different compounds including blue-absorbing chromophores and yellow-emitting fluorophores. However, in contrast to previous literature reports, A2E is not the dominant blue-absorbing chromophore or yellow-emitting fluorophore in lipofuscin. Excited state A2E becomes deactivated by energy transfer.[41] This absence of fluorescence suggests that A2E may self-quench as it aggregates and forms higher molecular weight products. This supports our result indicating that the majority of components in the hydrophobic portion of RPE lipofuscin granules consist of derivatized A2E. We hypothesized that auto- and/or photooxidation of A2E forms aldehydes which then further react with A2E to yield a series of higher molecular weight compounds that are much more hydrophobic than A2E. These compounds have increasing values of log *P* (hydrophobicity factor) which induces the sequestering of these derivatives into granules with a concomitant diminution in reactivity. These reactions also trap resultant aldehydes which would oxidize cellular components with concomitant cellular damage.

Supporting information

Supporting information may be found in the online version of this article.

Copyright © 2010 John Wiley & Sons, Ltd.

Journal of MASS SPECTROMETRY

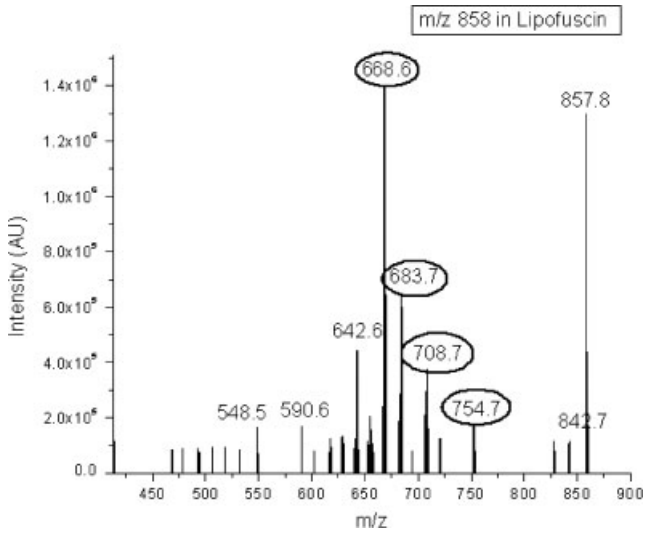

Figure 8. The MS/MS of *m/z* 858 in lipofuscin. Peaks corresponding to *m/z* 858 with the loss of 106 (*m/z* 752), 150 (*m/z* 708), 174 (*m/z* 684) and 190 (*m/z* 668) are identified.

References

[1] F. C. Delori, D. G. Goger, C. K. Dorey. Age-related accumulation and spatial distribution of lipofuscin in RPE of normal subjects. *Invest. Ophthalmol. Vis. Sci.* **2001**, *42*, 1855.

[2] C. K. Dorey, G. Wu, D. Ebenstein, A. Garsd, J. J. Weiter. Cell loss in the aging retina. Relationship to lipofuscin accumulation and macular degeneration. *Invest. Ophthalmol. Vis. Sci.* **1989**, *30*, 1691.

[3] L. Feeney-Burns, E. S. Hilderbrand, S. Eldridge. Aging human RPE: morphometric analysis of macular, equatorial, and peripheral cells. *Invest. Ophthalmol. Vis. Sci.* **1984**, *25*, 195.

[4] E. R. Gaillard, S. J. Atherton, G. Eldred, J. Dillon. Photophysical studies on human retinal lipofuscin. *Photochem. Photobiol.* **1995**, *61*, 448.

[5] F. G. Holz, F. Schutt, J. Kopitz, G. E. Eldred, F. E. Kruse, H. E. Volcker, M. Cantz. *Investigative ophthalmology & visual science* **1999**, *40*, 737.

[6] P. F. Lopez, I. H. Maumenee, Z. de la Cruz, W. R. Green. Autosomal-dominant fundus flavimaculatus. Clinicopathologic correlation. *Ophthalmology* **1990**, *97*, 798.

[7] M. F. Rabb, M. O. Tso, G. A. Fishman. Cone-rod dystrophy. A clinical and histopathologic report. *Ophthalmology* **1986**, *93*, 1443.

[8] T. A. Weingeist, J. L. Kobrin, R. C. Watzke. Histopathology of Best's macular dystrophy. *Arch. Ophthalmol.* **1982**, *100*, 1108.

[9] M. Boulton, N. M. McKechnie, J. Breda, M. Bayly, J. Marshall. The formation of autofluorescent granules in cultured human RPE. *Invest. Ophthalmol. Vis. Sci.* **1989**, *30*, 82.

[10] L. Feeney-Burns, G. E. Eldred. The fate of the phagosome: conversion to 'age pigment' and impact in human retinal pigment epithelium. *Trans. Ophthalmol. Soc. UK* **1983**, *103*(Pt 4), 416.

[11] S. Davies, M. H. Elliott, E. Floor, T. G. Truscott, M. Zareba, T. Sarna, F. A. Shamsi, M. E. Boulton. *Free radical biology & medicine* **2001**, *31*, 256.

[12] J. J. Weiter, F. C. Delori, G. L. Wing, K. A. Fitch. Retinal pigment epithelial lipofuscin and melanin and choroidal melanin in human eyes. *Invest. Ophthalmol. Vis. Sci.* **1986**, *27*, 145.

[13] L. B. Avalle, J. Dillon, E. R. Gaillard. Action spectrum for singlet oxygen production by human retinal lipofuscin. *Photochem. Photobiol.* **2005**, *81*, 1347.

[14] M. Rozanowska, J. Wessels, M. Boulton, J. M. Burke, M. A. Rodgers, T. G. Truscott, T. Sarna. *Free radical biology & medicine* **1998**, *24*, 1107.

[15] J. Wassell, S. Davies, W. Bardsley, M. Boulton. The photoreactivity of the retinal age pigment lipofuscin. *J. Biol. Chem.* **1999**, *274*, 23828.

[16] S. Sundelin, U. Wihlmark, S. E. Nilsson, U. T. Brunk. Lipofuscin accumulation in cultured retinal pigment epithelial cells reduces their phagocytic capacity. *Curr. Eye Res.* **1998**, *17*, 851.

[17] B. S. Winkler, M. E. Boulton, J. D. Gottsch, P. Sternberg. Oxidative damage and age-related macular degeneration. *Mol. Vis.* **1999**, *5*, 32.

[18] F. G. Holz, C. Bellman, S. Staudt, F. Schutt, H. E. Volcker. Fundus autofluorescence and development of geographic atrophy in age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.* **2001**, *42*, 1051.

[19] H. R. Taylor, B. Munoz, S. West, N. M. Bressler, S. B. Bressler, F. S. Rosenthal. Visible light and risk of age-related macular degeneration. *Trans. Am. Ophthalmol. Soc.* **1990**, *88*, 163; discussion 173.

[20] G. E. Eldred. Age pigment structure. *Nature* **1993**, *364*, 396.

[21] G. E. Eldred, M. L. Katz. Fluorophores of the human retinal pigment epithelium: separation and spectral characterization. *Exp. Eye Res* **1988**, *47*, 71.

[22] G. E. Eldred, M. R. Lasky. Retinal age pigments generated by self-assembling lysosomotropic detergents. *Nature* **1993**, *361*, 724.

[23] C. A. Parish, M. Hashimoto, K. Nakanishi, J. Dillon, J. Sparrow. Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 14609.

[24] L. Ragauskaite, R. C. Heckathorn, E. R. Gaillard. Environmental effects on the photochemistry of A2-E, a component of human retinal lipofuscin. *Photochem. Photobiol.* **2001**, *74*, 483.

[25] K. Reszka, G. E. Eldred, R. H. Wang, C. Chignell, J. Dillon. The photochemistry of human retinal lipofuscin as studied by EPR. *Photochem. Photobiol.* **1995**, *62*, 1005.

[26] S. Ben-Shabat, Y. Itagaki, S. Jockusch, J. R. Sparrow, N. J. Turro, K. Nakanishi. Formation of a nonaoxirane from A2E, a lipofuscin fluorophore related to macular degeneration, and evidence of singlet oxygen involvement. *Angew. Chem. (Int. Ed.)* **2002**, *41*, 814.

[27] J. Dillon, Z. Wang, L. B. Avalle, E. R. Gaillard. The photochemical oxidation of A2E results in the formation of a 5,8,5′,8′-bis-furanoid oxide. *Exp. Eye Res.* **2004**, *79*, 537.

[28] Z. Wang, L. M. Keller, J. Dillon, E. R. Gaillard. Oxidation of A2E results in the formation of highly reactive aldehydes and ketones. *Photochem. Photobiol.* **2006**, *82*, 1251.

[29] E. R. Gaillard, L. B. Avalle, L. M. Keller, Z. Wang, K. J. Reszka, J. P. Dillon. A mechanistic study of the photooxidation of A2E, a component of human retinal lipofuscin. *Exp. Eye Res.* **2004**, *79*, 313.

[30] L. S. Murdaugh, L. B. Avalle, S. Mandal, A. E. Dill, J. Dillon, J. D. Simon, E. R. Gaillard. *J. Mass Spectrom.* **2010**, *45*, 1139.

[31] C. R. Enzell, R. A. Appleton, I. Wahlberg. Terpenes and terpenoids. In *Biochemical Applications of Mass Spectrometry*, G. R. Waller (Ed.). Wiley: New York, **1972**, 375.

[32] G. Waller. *Biochemical Applications of Mass Spectrometry*. Wiley-Interscience: New York, **1972**, 377.

[33] Y. Imanishi, V. Gerke, K. Palczewski. Retinosomes: new insights into intracellular managing of hydrophobic substances in lipid bodies. *J. Cell Biol.* **2004**, *166*, 447.

[34] R. J. Barry, F. J. Canada, R. R. Rando. Solubilization and partial purification of retinyl ester synthetase and retinoid isomerase from bovine ocular pigment epithelium. *J. Biol. Chem.* **1989**, *264*, 9231.

[35] P. N. MacDonald, D. E. Ong. Evidence for a lecithin-retinol acyltransferase activity in the rat small intestine. *J. Biol. Chem.* **1988**, *263*, 12478.

[36] R. R. Rando. The biochemistry of the visual cycle. *Chem. Rev.* **2001**, *101*, 1881.

[37] J. C. Saari, D. L. Bredberg. Lecithin : retinol acyltransferase in retinal pigment epithelial microsomes. *J. Biol. Chem.* **1989**, *264*, 8636.

[38] A. Lakkaraju, S. C. Finnemann, E. Rodriguez-Boulan. The lipofuscin fluorophore A2E perturbs cholesterol metabolism in retinal pigment epithelial cells. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 11026.

[39] A. V. Ershov, N. Parkins, W. J. Lukiw, N. G. Bazan. Modulation of early response gene expression by prostaglandins in cultured rat retinal pigment epithelium cells. *Curr. Eye Res.* **2000**, *21*, 968.

[40] C. A. Curcio, C. L. Millican, T. Bailey, H. S. Kruth. Accumulation of cholesterol with age in human Bruch's membrane. *Invest. Ophthalmol. Vis. Sci.* **2001**, *42*, 265.

[41] N. M. Haralampus-Grynaviski, L. E. Lamb, C. M. Clancy, C. Skumatz, J. M. Burke, T. Sarna, J. D. Simon. *Proceedings of the National Academy of Sciences of the United States of America* **2003**, *100*, 3179.

 Copyright © 2010 John Wiley & Sons, Ltd.