Spatial Localization of A2E in the Retinal Pigment Epithelium

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PURPOSE. Lipofuscin, a fluorescent lysosomal pigment made of lipophilic molecules, is associated with age-related pathophysiological processes in the retinal pigment epithelium (RPE). The best-characterized components of lipofuscin are A2E and its oxides, but a direct spatial correlation with lipofuscin has not previously been possible.

METHODS. Lipofuscin fluorescence was mapped across the RPE of *Abca4*^{-/-} and *Sv129* (background strain control) mice. In the same tissues, they determined the spatial distribution of A2E and its oxides by using the high molecular specificity of matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS). The fluorescence and tandem mass spectra taken directly from the tissue were compared with those of synthetic A2E standard.

RESULTS. In 2-month-old mice, A2E was found in the center of the retinal pigment epithelial tissue; with age, A2E increased across the tissue. With high levels of A2E, there was a marked correlation between A2E and lipofuscin, but with low levels this correlation diminished. The distributions of the oxidized forms of A2E were also determined. The amount of oxidation on A2E remained constant over 6 months, implying that A2E does not become increasingly oxidized with age in this time frame.

CONCLUSIONS. This report is the first description of the spatial imaging of a specific retinoid from fresh tissue and the first description of a direct correlation of A2E with lipofuscin. The molecule-specific imaging of lipofuscin components from the RPE suggests wide applicability to other small molecules and pharmaceuticals for the molecular characterization and treatment of age-related macular degeneration. (*Invest Ophthalmol Vis Sci.* 2011;52:3926–3933) DOI:10.1167/iovs.10-7020

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One of the hallmarks of aging in the eye is the accumulation of autofluorescent degradation material in the lysosomal storage bodies of the retinal pigment epithelium (RPE), termed lipofuscin. Lipofuscin is a complex mixture of molecules¹ principally defined by fluorescence.² In the human RPE, it is detected as early as 1 year of age.³ Through oxidative stress generated by interaction with blue light, lipofuscin has been implicated in progressive retinal pigment epithelial cytotoxicity⁴ and the eventual development of age-related macular degeneration (AMD).⁵ However, lipofuscin is not confined to the RPE or the eye because it is present in most major organs, including the brain and heart.⁶ In human postmitotic cells, lipofuscin can first be detected during infancy (see Ref. 7 for review). The exact composition of lipofuscin is not known in any tissue, but it is expected to vary from tissue to tissue. Therefore, although lipofuscin-induced pathogenesis is implicated in multiple nonvisual diseases,^{8–11} the pathophysiological effects of lipofuscin are also anticipated to differ throughout the body.

The composition of lipofuscin is best characterized in the RPE,¹² where its accumulation is thought to depend on the availability of all-*trans* retinal, a potentially highly toxic allelic aldehyde, produced in the photoreceptor outer segments on light exposure.¹³ When the production of all-*trans* retinal (such as in RPE65 deficiency)¹⁴ is suppressed, the level of fluorescence associated with lipofuscin is decreased. Normally, the majority of all-*trans* retinal is reduced to all-*trans* retinol in the photoreceptors, which is then transported to the RPE to reform 11-*cis* retinal, the chromophore of vertebrate visual pigments. Excess all-*trans* retinal can form bis-retinoids, which, as the photoreceptor outer segments are being phagocytized by the retinal pigment epithelial cells on a daily basis, accumulate in the RPE, where they are only partially degraded.¹⁵ To date, 21 bis-retinoids have been identified and are thought to be the components of the RPE lipofuscin responsible for age-related cell damage.¹⁶

The best-studied bis-retinoid and the first component of lipofuscin to be identified is A2E (Fig. 1).^{16–18} A direct correlation of A2E levels with lipofuscin has not been possible because the spatial localization of A2E presents a difficult challenge. Immunolocalization techniques are not applicable, and fluorescence methods are not specific enough because several chemically similar bis-retinoid compounds fluoresce in the same region. Moreover, levels of A2E are normally measured from organic extracts of lipofuscin from tissue, followed by chromatographic analysis by high-performance liquid chromatography (HPLC). Still, the individual components identified with these extraction methods have not been found to adequately correlate with total lipofuscin fluorescence.⁷ Especially at lower levels of A2E, other compounds can dominate the same chromatographic region.¹⁹ Recently, the Bernstein group,²⁰ using 8-mm punches from human RPE and subsequent extraction of A2E, demonstrated that its concentration is highest in

FIGURE 1. The chemical structure of A2E. Backbone fragmentation and the molecular weight of the resultant fragment ions are indicated. The highlight of m/z 418 shows that it is characteristically the most intense ion in the fragment spectrum (when compared with Figs. 2E and 2F).

the periphery of the preparation, contrary to what was expected from lipofuscin fluorescence.²¹

To achieve high resolution (150 μm) and molecule-specific spatial information on A2E and its oxides across the native RPE, we adapted and used matrix-assisted laser desorption-ionization mass spectrometric imaging (MALDI-IMS).²² In MALDI imaging, the entire surface of a tissue (whole mount or section) is coated with an organic acid matrix (benzoic or cinnamic acid derivatives), and then mass spectra are collected across the surface at defined step sizes, resulting in a multidimensional data set (X- and Y-dimensions, m/z and ion intensity). The MALDI images are essentially cross-sections of this data set for selected individual ions in the mass spectra, reflecting the intensity distributions of molecular ions across the observed surface. The positively charged pyridinium ion in A2E produces strong signals allowing for sensitive detection.²³ Additionally, the MALDI-IMS technology allows molecule-specific imaging of many compounds in a single experiment.

In this article, we report the spatial localization of A2E and its oxidized derivatives in the RPE of wild-type and *Abca4*^{-/-} mice. The latter is a model for Stargardt disease, in which the ABCA4 transport-protein is deficient and retinoid processing is compromised, resulting in high levels of both A2E and lipofuscin.²⁴ The results for this model are evaluated in comparison to their background strain, the *Sv129* mouse. This molecular imaging methodology is the first technique to allow mapping of the localization of lipofuscin components in native tissue with high spatial resolution. There are no other known methods for the spatial localization of these relatively low molecular weight compounds. Moreover, because the method requires minimal sample manipulations, we were also able for the first time to spatially correlate A2E levels with the distribution of lipofuscin fluorescence within the same tissue.

METHODS

Animals

Abca4^{-/-} mice were bred from pairs generously provided by Gabriel Travis (University of California at Los Angeles); *Sv129* mice were obtained from Harlan Laboratories (Indianapolis, IN). Mice were maintained and bred in the Medical University of South Carolina core animal facilities under 12-hour light/12-hour dark cyclic light conditions. *Sv129* (2 and 6 months old, $n = 4$ per age group) and *Abca4*^{-/-} (2 and

6 months old, $n = 4$ per age group) mice were dark adapted overnight and killed under dim red light. The eyes were enucleated; then, with the use of an infrared camera, they were hemisected and, immersed in mammalian Ringer's,²⁵ and the anterior segment and the neural retina were carefully removed with forceps. The eyecups were flattened by small incisions, mounted with their scleral-side down on square indium tin oxide-coated conductive glass slides (Delta Technologies Ltd, Stillwater, MN), and desiccated. Care was taken to retain the original orientation and stereo position of the eyes. All animal procedures were designed and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Medical University of South Carolina Animal Care and Use Committee.

Synthesis of A2E

A2E was synthesized as previously described from ethanolamine (Sigma, St. Louis, MO) and *all-trans* retinal (Sigma).²⁶ The product was characterized by absorption spectra (Breeze 2 with a 2998 photodiode array detector; Waters, Milford, MA), nuclear magnetic resonance (Avance II with a 600 MHz Ultrashield magnet; Bruker, Billerica, MA), and mass spectrometric (LTQ-XL; Thermo Fisher, Waltham, MA) analyses. The obtained spectra and peak shifts were in good agreement with those published in the literature.²⁶ The material was aliquotted (3 mM) in benzene (Sigma) and stored at -80°C under argon until used. All samples were stored in light-tight containers, and all subsequent sample and tissue preparation procedures were performed under dim red light. The expected molecular weight value for A2E is $m/z = 592$; for singly oxidized A2E it is $m/z = 608$; and for doubly oxidized A2E it is $m/z = 624$.

Fluorescence Measurements

The slides with the tissue were placed on the stage of a laser scanning confocal microscope (SP2 RS; Leica, Wetzlar, Germany). Lipofuscin fluorescence was imaged with a 10 $\times$ (NA = 0.3) or a 20 $\times$ (NA = 0.5) objective using 488 nm excitation (argon laser) and collecting emission from 565 to 725 nm, with the pinhole fully open (i.e., nonconfocal mode). Fluorescence images of overlapping fields were collected and assembled to provide a single image of the entire eyecup. To facilitate comparisons, the intensities in these images were normalized to total fluorescence. Fluorescence emission spectra of lipofuscin and synthetic A2E were measured in the same microscope with the same settings. The emission spectrum of synthetic A2E was measured by adding it to purified rod outer segment membranes (rhodopsin concentration of 40 μM) at a concentration of 100 μM ; 30 μL solution was placed on a glass histology slide and covered with a coverslip. To obtain fluorescence maps over entire eyecups, fluorescence images of lipofuscin were first collected as individual fields that were then joined by overlapping areas into a whole individual eyecup. Then the intensities of the pixels of the eyecup field were normalized to the total eyecup fluorescence.

MALDI Imaging of Retinal Pigment Epithelial Tissue

The slides with the tissue were prepared for MALDI imaging by automated matrix deposition (Portrait 630; Labcyte, Sunnyvale, CA). Fifty iterations (one 170-pL droplet per iteration of 10 mg/mL 2',5'-dihydroxyacetophenone in 70:30 ethanol/water [vol/vol]) were applied across each tissue with a center-to-center droplet distance of 300 μm , and shifts in x or y , or both, were applied so that the final center-to-center spot distance was 150 μm . This procedure ensured no delocalization of analytes to adjacent matrix spots. MALDI data were collected in the positive ion mode at +25 kV accelerating potential on a TOF mass spectrometer (ultrafleXtreme; Bruker) operating in reflector mode with the laser repetition rate set to 500 Hz. Before data collection, a linear external calibration was applied using leucine enkephalin (m/z 556.28; Bruker) and angiotensin II (m/z 1046.54; Bruker). Imaging data sets were acquired over paired (left and right eyes) mouse

tissues in the m/z 420 to 1400 range, with a raster step size of $150\ \mu\text{m}$ and 500 laser shots per spectrum. After data acquisition, MALDI images were reconstructed using acquisition and evaluation software (Flex-Imaging 2.0; Bruker). Each m/z signal was plotted $\pm 0.5\ m/z$ units. For display purposes, in each individual sample, the data were normalized to total ion current and interpolated (which applies a linear pixel intensity change between adjacent sampling locations for the plotted m/z value), and pixel intensities for all data sets were plotted on the same relative intensity scale (0–80: pink, ~ 70 ; red, ~ 50 ; green, ~ 30 ; light blue, ~ 20 ; dark blue, ~ 10) to facilitate signal intensity comparison.

Identification of A2E by Tandem Mass Spectrometry

Tandem mass spectrometry was performed directly from samples that had previously undergone MALDI imaging analysis. Therefore, tandem

mass spectra of m/z 592, m/z 608, and m/z 624 were collected using an ion trap instrument (MALDI Duo LTQ; Thermo Fisher). The precursor ion was isolated with a 2-amu-wide window centered on the precursor m/z value and subjected to collision-induced dissociation, with collision energy set to 75%. The acquired spectra were compared with synthetic A2E prepared by the dried droplet method with matrix and solvent conditions identical to those used for tissue analysis.

Correlation of A2E and Lipofuscin

To calculate the correlation of lipofuscin fluorescence and A2E distribution, both images were transformed to 8-bit absolute intensities using the ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). Then the intensity interpolation was removed from the MALDI images and was replaced by squares (12–14 pixels each, depending on the individual images) that matched the intensity of the

FIGURE 2. Lipofuscin and A2E in mouse retinal pigment epithelial tissues. Summary mass spectra from retinal pigment epithelial tissues of 6-month-old *Sv129* (A) and *Abca4*^{-/-} mice (B). Consistent with the molecular weight of A2E, m/z 592 was a major signal in the spectra, which were normalized to a common signal at m/z 783. Insets: fluorescence micrographs from the RPE of the same animals ($\lambda_{\text{exc}} = 488\ \text{nm}$, 20 $\times$ objective) shown at the same intensity scaling. Scale bar, 50 μm . Fluorescence emission spectra of 100 μM synthetic A2E mixed into bovine rod outer segments containing 40 μM rhodopsin (C) and of lipofuscin from 6-month-old *Sv129* retinal pigment epithelial tissue (D). The emission spectra (488 nm excitation, 20 $\times$ objective) represent an average of 27 individual areas with lipofuscin accumulation. Tandem mass spectra recorded from a sample of synthetic A2E (E) and directly from matrix-coated 6-month-old *Abca4*^{-/-} mouse retinal pigment epithelial tissue (F). The selected precursor ion for the MS/MS experiment was m/z 592.

FIGURE 3. Lipofuscin fluorescence and MALDI images of A2E in mouse RPE. RPE tissue images from 2-month-old *Sv129* (A) and *Abca4*^{−/−} (B) mice and 6-month-old *Sv129* (C) and *Abca4*^{−/−} (D) mice. Per panel, *left*: fluorescence intensity images; *right*: MALDI images of A2E in the same tissue. Fluorescence images were acquired as micrographs ($\lambda_{\text{exc}} = 488 \text{ nm}$, 10 $\times$ objective) of individual fields and joined by overlapping areas. Images are shown at the same intensity scaling. The MALDI images were acquired after the tissue was spotted with MALDI matrix at 150 μm resolution. The pixel intensity is proportional to A2E quantity, with the scale normalized to total ion current. All images are oriented as follows: dorsal (*top*); ventral (*bottom*); nasal (*left*, A, B; *right*, C, D); temporal (*right*, A, B; *left*, C, D). *Left*: 2-month-old animals are represented on the *left* and 6-month-old animals are represented on the *right*. Scale bar, 1 mm.

collected data. Thereafter, the fluorescent images were remapped to the resolution of the processed MALDI images by pixel averaging, and the image intensity of these images was rescaled so that the average and mean intensity matched those of the corresponding MALDI images. The similarity between the resultant fluorescent and A2E images was quantitated by the Pearson correlation function. To further visualize the correlation, the images used for the correlation calculations were blurred with a 9-pixel filter, and a false-color intensity scale was applied (see Figs. 4 and 6).

Quantitation of A2E and Its Oxides

The height of the first peak within an isotopic envelope was used to obtain data for relative quantitation and comparison between different

tissue samples. Total A2E was determined as A2E and all its oxides together and was normalized in each sample to a signal at m/z 783, which was common in all samples ($N = 32$) with similar intensities (12 ± 0.6). Values were calculated as the mean $\pm$ SE. The number of oxygen per A2E molecule was calculated by the following formula: $\Sigma iA_i/\Sigma A_i$, where A_i is the area under the curve for the i^{th} oxidation level.

RESULTS

Detection of A2E in the Unperturbed Mouse RPE

To determine whether A2E could be detected in retinal pigment epithelial tissue, mass spectra were collected in the m/z

FIGURE 4. Visual correlation of lipofuscin and A2E in mouse RPE. Images from 2-month-old *Sv129* (A), 2-month-old *Abca4*^{−/−} (B), 6-month-old *Sv129* (C), and 6-month-old *Abca4*^{−/−} (D) mice. The figures were generated from the data shown in Figure 3 by removing the intensity interpolation from the MALDI images and remapping the fluorescence images to achieve the same resolution by pixel averaging. After intensity matching, the images were blurred with a 9-pixel filter. In each panel, the *left image* represents lipofuscin fluorescence intensity and the *right image* represents the MALDI image of A2E in the same tissue. Scale bar, 1 mm.

TABLE 1. Correlation between A2E Distribution and Lipofuscin Fluorescence

Mouse RPE Tissue and Age	Pearson Correlation Coefficient*
<i>Sv129</i> at 2 months	0.398
<i>Abca4</i> ^{-/-} at 2 months	0.572
<i>Sv129</i> at 6 months	0.659
<i>Abca4</i> ^{-/-} at 6 months	0.824

* Calculated between MALDI images of A2E and images of lipofuscin fluorescence (seen in each panel of Fig. 3), which were remapped to the same resolution by pixel averaging.

420 to 1400 range from eyecups prepared for MALDI imaging. In the 6-month-old *Sv129* eyecup (Fig. 2A), m/z 592 was one of several abundant signals; however, in the age-matched *Abca4*^{-/-} tissue (Fig. 2B), m/z 592 was the most abundant signal. For a direct comparison between samples, the ion intensities were normalized to the intensity of a common signal at m/z 783. These results confirmed that a signal expected from the molecular ion of A2E (predicted m/z 592.45) was produced directly from the tissue. Moreover, the higher accumulation in *Abca4*^{-/-} mice compared to their *wt* counterparts was also consistent with the known high accumulation of A2E in these animals.²⁴

Characteristic lipofuscin fluorescence emanated from lysosomal granules within the retinal pigment epithelial cells. The insets in Figures 2A and 2B show micrographs ($\lambda_{exc}=488$ nm) from the RPE of 6-month-old *Sv129* and *Abca4*^{-/-} animals. As expected, fluorescence was more intense and dense in the *Abca4*^{-/-} mice than in *Sv129* mice. This fluorescence is consistent with the presence of A2E, as shown by the similarity of the emission spectrum collected from an eyecup to that of synthetic A2E. To simulate a native fluorophore environment, synthetic A2E (100 μ M) was suspended in a bovine rod outer segment preparation. A2E has an emission maximum of 600 nm with 488 nm excitation in this environment (Fig. 2C), in agreement with previous reports.²⁷ The fluorescent spectrum of lipofuscin from an eyecup of a 6-month-old *Abca4*^{-/-} mouse is shown in Figure 2D.

Although the m/z 592 signals in the mass spectra were consistent with the presence of A2E, we used the technique of tandem (MS/MS) mass spectrometry to confirm the peak identity. MS/MS spectra were recorded from a MALDI spot of synthetic A2E, which had only a single signal at m/z = 592 (Fig. 2E). An MS/MS spectrum of m/z 592 was also collected directly from the matrix-coated retinal pigment epithelial tissue of a 6-month-old *Abca4*^{-/-} mouse (Fig. 2F). The two MS/MS spectra had the same fragmentation ion patterns, confirming that the ion at m/z 592 from the tissue samples was indeed A2E.

Spatial Distribution of A2E in the Mouse RPE

MALDI imaging requires minimal sample manipulations; therefore, it was possible to directly compare the distribution of lipofuscin fluorescence with that of A2E in the same tissue. In the dual imaging experiments, first autofluorescence images of lipofuscin were collected as individual fields and joined by overlapping areas to map the entire mouse retinal pigment epithelial tissue. Thereafter, the same tissue was spot-coated with MALDI matrix, and mass spectra were collected from the entire surface. MALDI images of A2E were generated by plotting the relative intensities of the m/z 592 signal from individual mass spectra over the entire surface normalized to the total ion current. The intensities are proportional to the quantities of lipofuscin and A2E, respectively.

Results of the comparison of lipofuscin autofluorescence with tissue images of A2E are summarized in Figure 3. For this comparison, images from 2-month-old (Figs. 3A, 3B) and 6-month-old (Figs. 3C, 3D) *Sv129* and *Abca4*^{-/-} mice were used. In each panel, the image on the left represents lipofuscin fluorescence, and the image on the right represents the MALDI image of A2E in the same tissue. As anticipated, A2E accumulates with age in both strains, but in the *Abca4*^{-/-} mouse, the abundance of A2E is much higher than in the wild-type controls. In both the transgenic and the *wt* mouse, the abundance of A2E is concentric, highest in the center and diminishing toward the periphery. Overall, there appeared to be a remarkable correlation between the MALDI images of A2E and lipofuscin fluorescence (Fig. 4).

Table 1 summarizes the correlation data between the fluorescence and MALDI images of A2E. All correlations of fluorescence and A2E in the same tissues were elevated ($r > 0.4$) compared with cross-correlations between different tissues ($r < 0.2$). The highest correlations were obtained when the levels of A2E were also the highest (in *Abca4*^{-/-} animals and increasing with age).

Spatial Distribution of Oxidized A2E Products in the Mouse RPE

A major advantage of the MALDI imaging technology is the potential to simultaneously image any molecule having a mass within the targeted range (m/z 420 - 1400, in our case). In the MALDI images of oxidized A2E, we have detected up to three oxidations on A2E (confirmed by MS/MS). Figure 5 shows images of singly (m/z 608; Fig. 5A), doubly (m/z 624; Fig. 5B), and triply (m/z 640; Fig. 5C) oxidized A2E in the RPE of a 6-month-old *Abca4*^{-/-} mouse. These images were simultaneously acquired from the same tissue as in Figure 3D.

Although the signal intensities decrease with the increasing oxidation levels, the spatial distribution of the three oxidized products closely follows the distribution of A2E and lipofuscin fluorescence (Fig. 6). The correlation between A2E, oxidized

FIGURE 5. MALDI images of oxidized A2E in 6-month-old *Abca4*^{-/-} mouse RPE. Singly oxidized A2E (m/z 608; A), doubly oxidized A2E (m/z 624; B), and triply oxidized A2E (m/z 640; C). Images were extracted from the same data set used to generate the A2E image seen in Figure 3D. Oxidation states higher than triply oxidized A2E were not detected. Scale bar, 1 mm.

FIGURE 6. Visual correlation of A2E oxides in 6-month-old *Abca4*^{−/−} mouse RPE. Singly oxidized A2E (A), doubly oxidized A2E (B), and triply oxidized A2E (C). The figure represents MALDI images of A2E oxides and was generated from the data shown in Figure 5 by removing the intensity interpolation from the MALDI images. The resultant images were blurred with a 9-pixel filter. Scale bar, 1 mm.

A2E, and lipofuscin fluorescence was relatively high ($r > 0.4$; see Table 2), with the highest correlation observed for the single oxidation state.

Quantitation of A2E from Unperturbed Mouse RPE

The primary information displayed in our MALDI images is qualitative; however, quantitative changes in relative abundance can also be interpreted with a well-chosen internal standard. To accomplish this, summary MALDI spectra were generated, representing an average of all the individual MALDI spectra in the respective imaging data set. We have used the summary MALDI spectra from 2- and 6-month-old *Abca4*^{−/−} and *Sv129* mice to determine changes in levels of A2E and A2E oxidation in mouse retinal pigment epithelial tissues.

Figure 7 shows A2E and its oxides in a mass spectrum from 6-month-old *Abca4*^{−/−} retinal pigment epithelial tissue. As can be seen, the mass resolution was high enough to detect the isotopic distribution of the molecules (A2E and oxidized A2E are gray shaded). The results of the relative quantitation between the two different mouse strains and ages are summarized in the inset. The left panel of the inset shows that total A2E significantly increased with age (from 0.99 ± 0.28 to 3.02 ± 0.51 in *Abca4*^{−/−}; and from 0.11 ± 0.04 to 1.19 ± 0.27 in *Sv129* mice; the values represent arbitrary units, $n = 8$ in each condition, $P < 0.05$). At both ages, A2E levels were significantly higher in the *Abca4*^{−/−} mice compared with the *Sv129* animals ($P < 0.05$). The right panel of the inset shows that the amount of oxygen per A2E molecule did not change significantly with age and that it was independent of the mouse strain. On average ($n = 32$), there was 0.17 ± 0.03 oxygen per A2E molecule. Thus, although total levels of oxidized A2E do increase with age, the relative levels of oxidized to unoxidized A2E remain constant.

DISCUSSION

The accumulation of lipofuscin is believed to be a contributing factor in AMD development. However, the identification of any

pathogenic components has proven to be an elusive target because of the lack of suitable methodologies. Lipofuscin, defined on the basis of its fluorescence, can be detected and imaged at very high sensitivity and spatial resolution by fluorescence microscopy. On the other hand, the analysis of the lipofuscin components typically requires tissue homogenization, lipid extraction, HPLC, and subsequent analysis by spectroscopy or mass spectrometry. These analytical methods are able to detect molecular composition at high sensitivity; however, they preclude spatial localization.

Studies on the compositional analysis of lipofuscin have identified A2E as a major component in the RPE.^{18,28} We have developed a new, highly sensitive mass spectrometry-based method for the quantitation of A2E in retinal pigment epithelial extracts and compared the levels of A2E obtained by this method to traditional HPLC and absorbance (430 nm) detection.¹⁹ Although there was a reasonable correlation between the two approaches, the HPLC method tends to overestimate the amount of A2E, particularly at low levels of A2E, perhaps because other compounds can coelute with A2E. These observations are in agreement with the idea that optical methods are not specific enough to identify the components of lipofuscin and that it is beneficial to be able to detect molecules using the high specificity of mass spectrometry. On the other hand, without the ability to spatially identify lipofuscin components, it is difficult to attribute lipofuscin fluorescence and toxicity to

FIGURE 7. A2E and A2E oxidation levels in mouse retinal pigment epithelial tissues. Mass spectrum from a 6-month-old *Abca4*^{−/−} retinal pigment epithelial tissue (from Fig. 2B between 590 and 650 m/z). Isotopic envelopes for A2E and oxidized A2E are labeled and shown shaded. Inset: total A2E increases with age in both mouse strains (left). However, the amount of oxygen per A2E molecule was stable, and the oxidation profiles of the two strains were not significantly different (right). $n = 8$ in each condition. * $P < 0.05$.

TABLE 2. Pearson Correlations between A2E, Its Oxides, and Lipofuscin Fluorescence in 6-Month-Old *Abca4*^{−/−} Mice

A2E Species	Fluorescence*	A2E	A2E-ox	A2E-2ox
A2E	0.837	1.000	—	—
A2E-ox	0.771	0.922	1.000	—
A2E-2ox	0.740	0.879	0.927	1.000
A2E-3ox	0.434	0.479	0.508	0.640

*The image of lipofuscin fluorescence (seen in Fig. 3D) was remapped to the same resolution as the MALDI images of A2E (seen in Fig. 3D) and its oxides (seen in panels of Fig. 5) by pixel averaging.

the accumulation of any of its individual components. Therefore, for A2E, *bis*-retinoids, and other fluorescent small molecules that constitute lipofuscin, it is highly advantageous to have an analytical method at hand that can provide molecule-specific data directly comparable to fluorescence maps.

The usefulness of MALDI imaging has been demonstrated before for the spatial localization of proteins and peptides,^{22,29} lipids,³⁰ and small molecule pharmaceuticals.³¹ Within the eye, MALDI imaging has been used for lens proteins^{32,33} and, more recently, retina lipids.³⁴ However, MALDI imaging is also an appropriate analytical tool with which to address the question of the spatial distribution of lipofuscin components. Moreover, one of the great advantages of MALDI imaging is that many compounds can be imaged in a single experiment. Thus, the methodology is ideal for the simultaneous analysis of multiple lipofuscin components and has an enormous potential for addressing both disease processes and the underlying mechanistic questions of lipofuscin-related pathologies in the RPE.

With MALDI imaging we have successfully detected, identified, and imaged A2E in mouse tissue at 150- μ m resolution with spot matrix deposition. Although the technique has limitations in terms of resolution (25 μ m with spray matrix deposition), spot-coating resulted in less sample-to-sample variation, which increases confidence in signal comparison between the different experimental groups. Given that our experiments used native tissue and required minimal sample manipulations, we were also able to directly compare the spatial distribution of A2E and lipofuscin in the same tissue. This ability allows comparison of our results to data from the literature because traditional lipofuscin analysis was based on fluorescence. Overall, there was a marked agreement between the MALDI images of A2E and lipofuscin fluorescence. The correlation deviated from being perfect because the fluorescence data included any molecule fluorescing in the range being measured (including flavoproteins, connective tissue, and other *bis*-retinoids). However, the MALDI imaging and fluorescent data are in good agreement where high levels of A2E (e.g., 6-month-old *Abca4*^{-/-} mice) are present. In such cases, fluorescence is a reasonable method for assessing the spatial distribution of A2E.

The distribution profile of A2E in the mouse retina was not entirely uniform. In the young *wt* retina, A2E was localized mainly in the center of the preparation. However, with age, the distribution becomes more uniform across the entire retinal pigment epithelial tissue. The technology presented in this article will be invaluable for spatially analyzing lipofuscin composition in the human RPE, which has a different histologic organization.

A2E is known to be readily oxidized, and this oxidation is proposed to be involved in disease pathogenesis.³⁵ In vitro, up to nine oxidations have been detected to be added to A2E.³⁶ However, we could readily detect only the first two oxidation states in most tissues, and no more than three oxidations in samples with high levels of A2E. The distribution of oxidized A2E followed closely that of A2E. Oxidation has been reported as 7,8-epoxide and 5,8-furanoid on A2E.³⁷⁻³⁹ Recently, we have also found oxidation at the 9,10 position, most likely as the epoxide.¹⁹ The collected MS/MS spectra on oxidized A2E in our experiments confirm these oxidation sites. To quantitate changes in A2E and A2E oxidations, we have used summary MALDI spectra. These spectra are descriptive of the observed tissue as a whole and are, therefore, comparable to the ones obtained from tissue extraction experiments. Although both A2E and oxidized A2E increased significantly with age, and this was significantly higher in the *Abca4*^{-/-} animals, the relative amount of oxidation was stable (0.17 ± 0.03 oxygen per A2E). These data show that the presence of oxidized A2E depends on total A2E levels, indicating that elevated levels of total A2E

must accumulate before levels of oxidized A2E become significant.

In conclusion, we have developed a new MALDI imaging technique to achieve multiplex and molecule-specific imaging of A2E and its oxides. The MALDI-imaging data acquired directly from the tissue were comparable to fluorescence maps and were used to correlate the localization of *bis*-retinoids with lipofuscin fluorescence. A major strength of the MALDI imaging technique is that many compounds can be imaged from a single experiment. In this report, we have focused on free A2E in the RPE. However, the method is readily applicable to imaging numerous other molecular species (such as lipid-linked *bis*-retinoids); thus, these techniques have an enormous potential for vision biology studies for addressing age- and lipofuscin-related abnormalities in the RPE such as AMD.

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