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Molecule-Specific Imaging and Quantitation of A2E in the RPE

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11.1 Introduction

Lipofuscin accumulates with age in the lysosomal storage bodies of the RPE and has been implicated in progressive RPE toxicity, which can eventually lead to retinal degeneration (Winkler et al. 1999). Chemically, lipofuscin is a complex mixture of partially degraded molecules characterized by orange fluorescence (Delori et al. 1995). The *bis*-retinoid A2E was identified from organic extracts of lipofuscin (Sakai et al. 1996). In vivo, *bis*-retinoid precursors form in the photoreceptor outer segments when the all-*trans* retinal to retinol conversion is delayed. A2E is generated from these precursors in the RPE after outer segment phagocytosis. The *bis*-retinoids have received considerable attention, as A2E (and especially its oxides) has been shown to produce RPE damage (Eldred and Lasky 1993).

However, a direct correlation of A2E with lipofuscin has not been possible because fluorescence is not specific enough, and immunolocalization techniques are not applicable for *bis*-retinoids. To understand the spatial localization of lipofuscin components, we utilized matrix-assisted laser desorption/ionization (MALDI) imaging (Caprioli et al. 1997). In this technique, mass information is collected systematically over the surface of a matrix-coated tissue section, and the resulting images represent intensity distributions of molecular signals across the observed surface. With this technique, we analyzed *Abca4*^{-/-} (a model for Stargardt disease with high levels of both A2E and lipofuscin), *Rpe65*^{-/-} (a model for blocked visual cycle, where A2E is not formed), and *wt* mice. The collected MALDI imaging data were utilized to identify *bis*-retinoid components of lipofuscin and to

determine their spatial distributions. To further understand the accumulation of A2E, we employed an LC-MS/MS method for the highly sensitive (femtomoles) absolute quantitation of *bis*-retinoids from RPE extracts (Gutierrez et al. 2010). Taken together, these experiments provide a new, molecule-specific understanding of *bis*-retinoids and potentially other retinoid metabolites of the visual cycle.

11.2 Materials and Methods

11.2.1 RPE Tissue Preparation

Abca4^{-/-} and *Rpe65*^{-/-} mice were bred from pairs generously provided by Gabriel Travis (UCLA) and Michael Redmond (NEI). *C57bl6* (*wt*) mice were obtained commercially (Harlan, Indianapolis, IN). Six-month-old mice (*n* = 4 each strain, maintained under regular cyclic light) were used. RPE tissue was prepared as described previously for MALDI imaging (Grey et al. 2010) and for absolute quantitation (Gutierrez et al. 2010).

11.2.2 MALDI Imaging

Imaging samples were coated using a thin-layer-chromatography sprayer with 10 mg/mL 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid (Sigma, St Louis, MO) in 10 mL 70:30 ethanol:water (Thermo Fisher, Fair Lawn, NJ). Data were collected in a MALDI-TOF mass spectrometer (Autoflex III; Bruker Daltonics, Billerica, MA). Further details are described elsewhere (Grey et al. 2010).

11.2.3 Quantitation of A2E

For quantitative analysis, dried RPE extracts were diluted to obtain approximately 10 fmol/ μ L solutions in 85% MeOH, 0.1% TFA and injected for LC-MS/MS analysis. Further details followed previous procedures (Gutierrez et al. 2010).

11.3 Results

11.3.1 Spatial Localization of A2E in Murine RPE

The MALDI mass spectra from *Abca4*^{-/-} RPE tissues were dominated by an abundant mass signal at *m/z* 592. The identity of this signal as A2E was confirmed by tandem mass spectrometry (MS/MS): the observed fragmentation pattern collected from the tissue was consistent with expected A2E fragmentation. In addition to A2E, numerous less abundant components were present in the spectra. In this work, we focused our attention to A2E and its oxides.

MALDI images of A2E were generated by plotting the relative intensities of the *m/z* 592 signal over the entire RPE surface (Fig. 11.1). A2E distributions were determined in *wt* (Fig. 11.1a) and *Abca4*^{-/-} (Fig. 11.1b) mice (no A2E was present in the *Rpe65*^{-/-} model, not shown). In both *Abca4*^{-/-} and *wt* mice, A2E accumulated uniformly and in a concentric manner. However, the abundance of A2E was much higher in the *Abca4*^{-/-} model, where A2E occupied a larger concentric area. In the same MALDI imaging datasets, up to three oxidations on A2E were also detected. Figure 11.1 c shows the distribution of singly oxidized A2E (*m/z* 608) in a 6-month-old *Abca4*^{-/-} RPE. The distributions of the three oxidized products closely followed A2E.

11.3.2 Quantitation of A2E

The primary information displayed in the MALDI images is qualitative. To quantitate A2E, an LC-MS/MS-based approach was utilized, which (in comparison with traditional absorption spectroscopic quantitation) is molecularly specific (Gutierrez et al. 2010). This

method relies on a standard curve calculated from various amounts of synthetic A2E. With this method, low femtomole quantities of A2E can be detected, and amounts of A2E from a single *wt* mouse RPE can routinely be quantitated.

Figure 11.2 summarizes the results of A2E quantitation in *Rpe65*^{-/-}, *wt*, and *Abca4*^{-/-} mice. The inset shows two selected ion chromatograms for A2E from a 6-month-old *Abca4*^{-/-} animal representing 30 min of the elution gradient. The upper trace is based on the intact molecular weight (*m/z* 592), while the lower trace indicates the most prominent MS/MS ion (*m/z* 418). The quantitation is based on the area under the lower trace (shaded), as the instrument is more sensitive in MS/MS mode. As seen in the bar graph, there was no A2E in *Rpe65*^{-/-} animals, while compared to *wt* controls (5 ± 3 pmol) the quantity of A2E was elevated in the *Abca4*^{-/-} mice (41 ± 7 pmol).

11.3.3 A2E Oxidation Sites

A major advantage of MALDI imaging is that tracing any molecule within the targeted range does not require a separate experiment. However, the identification of these molecules requires a second MS/MS experiment. The signals of singly (*m/z* 608) and doubly (*m/z* 624) oxidized A2E were confirmed by MS/MS. Figure 11.3 a shows a MS/MS spectrum of singly oxidized A2E from an *Abca4*^{-/-} animal. To see whether the detected oxidation site depends on the utilized sample preparation/instrumentation technique, we also collected MS/MS spectra of oxidized A2E from organic extracts of the RPE (Fig. 11.3b).

There was remarkable agreement between the spectra collected by the two analytical methods. However, they both differed slightly from the published fragmentation patterns for extracted A2E from human eyecups (Avalle et al. 2004), indicating a possible additional site of oxidation in *Abca4*^{-/-} mice. The oxygen can be localized either to the 7, 8/7, 8 -epoxide position (Fig. 11.3c, left) or to the 9, 10/9, 10 -epoxide position (Fig. 11.3c, right).

11.4 Discussion

Lipofuscin accumulation in the RPE is implicated in the development of age-related retino- and maculopathies. The analysis of its components typically requires organic extraction, HPLC separation, and subsequent analysis, which completely preclude spatial localization. A2E is a major component of the chloroform-methanol extract of the RPE. However, without the ability to spatially identify A2E, it is not possible to justify that lipofuscin toxicity is indeed due to A2E accumulation. Because immunohistochemistry cannot be used, it is highly advantageous to utilize the molecularly selective and sensitive MALDI imaging method. With this technique, many compounds can be imaged in a single experiment. In this report, we have focused on A2E and its oxides, but the technique is easily amenable to other *bis*-retinoids and retinoid metabolites.

We have detected A2E and its oxides at 50 μ m resolution in 6-month-old native murine RPE tissue. As expected, there was no A2E present in *Rpe65*^{-/-} animals (which do not produce 11-*cis* retinal), confirming that the visual cycle is required for A2E generation (Katz and Redmond 2001). In the *wt* retina, A2E was mainly localized to the center of the RPE, while in the *Abca4*^{-/-} RPE the distribution became more uniform across the entire RPE. These observations reinforce the rapid accumulation of A2E in the *Abca4*^{-/-} model, and that A2E becomes uniform across the RPE as it accumulates. Thus, the data agree with the hypothesis that A2E and lipofuscin correlate spatially.

Although with an internal standard MALDI data can be utilized for relative quantitation, comparing images between different tissues and strains is rather qualitative. On the other hand, our LC-MS/MS-based method allows absolute quantitation of subfemtomole amounts

of synthetic A2E. The information obtained through our quantitative analysis reinforced the semiquantitative imaging observations (that is, no A2E in the *Rpe65*^{-/-} animals and higher A2E in *Abca4*^{-/-} animals, than in *wt* mice). Moreover, these experiments provided quantitative data not possible by other current methodologies (such as, the level of A2E in 6-month-old *Abca4*^{-/-} mouse RPE is 8-fold over that in the same age *wt* RPE).

In vitro, up to nine oxygens have been identified on A2E; however, we could detect only two oxidation states in most tissues, and no more than three oxidations in all tissues with two fundamentally different analytical methods. The spatial distribution of oxidized A2E followed closely that of A2E, indicating that oxidized forms of A2E remain in the vicinity of their parent molecule. The sites of oxidation detected with the two techniques were identical. In the literature, A2E mono-oxidation has been reported on the 7, 8/7, 8 -epoxide or the 5, 8/5, 8 -furanoid position (Avalle et al. 2004; Jang et al. 2005). However, in the *Abca4*^{-/-} strain, we found that A2E can be mono-oxidized on a mixture of the 7, 8/7, 8 -, and the 9, 10/9, 10 -epoxide positions, indicating the possibility of two native mono-oxidation sites in vivo.

In conclusion, we have recently developed two new methods for the analysis of A2E and other *bis*-retinoids in RPE tissue from models of retinal degenerations: a MALDI-based technique for multiplex and molecularly specific imaging; and an LC-MS/MS-based technique for sensitive quantitation. Each method has its own advantages, therefore utilizing both approaches has a great potential for addressing disease pathogenesis in models of retinal degenerations. Together, they will provide valuable new information on distinct spatial regions in normal and diseased tissues.

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References

- Avalle LB, Wang Z, Dillon JP, et al. Observation of A2E oxidation products in human retinal lipofuscin. *Exp Eye Res.* 2004; 78:895–898. [PubMed: 15037123]
- Caprioli RM, Farmer TB, Gile J. Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. *Anal Chem.* 1997; 69:4751–4760. [PubMed: 9406525]
- Delori FC, Dorey CK, Staurenghi G, et al. In vivo fluorescence of the ocular fundus exhibits retinal pigment epithelium lipofuscin characteristics. *Invest Ophthalmol Vis Sci.* 1995; 36:718–729. [PubMed: 7890502]
- Eldred GE, Lasky MR. Retinal age pigments generated by self-assembling lysomotropic detergents. *Nature.* 1993; 361:724–726. [PubMed: 8441466]
- Grey AC, Crouch RK, Koutalos Y, et al. Spatial localization of A2E in the retinal pigment epithelium. *Invest Ophthalmol Vis Sci.* 2010; 52:3926–3933. [PubMed: 21357388]
- Gutierrez DB, Blakeley L, Goletz PW, et al. Mass spectrometry provides accurate and sensitive quantitation of A2E. *Photochem Photobiol Sci.* 2010; 9:1513–1519. [PubMed: 20931136]
- Jang YP, Matsuda H, Itagaki Y, et al. Characterization of peroxy-A2E and furan-A2E photo-oxidation products and detection in human and mouse retinal pigment epithelial cell lipofuscin. *J Biol Chem.* 2005; 280:39732–39739. [PubMed: 16186115]
- Katz ML, Redmond TM. Effect of Rpe65 knockout on accumulation of lipofuscin fluorophores in the retinal pigment epithelium. *Invest Ophthalmol Vis Sci.* 2001; 42:3023–3030. [PubMed: 11687551]
- Sakai N, Decatur J, Nakanishi K, et al. Ocular age pigment “A2E”: an unprecedented pyridinium *bis*-retinoid. *J Am Chem Soc.* 1996; 118:1559–1560.

Winkler BS, Boulton ME, Gottsch JD, et al. Oxidative damage and age-related macular degeneration. Mol Vis. 1999; 5:32. <<http://www.molvis.org/molvis/v5/p32/>>. [PubMed: 10562656]

Fig. 11.1.

Spatial localization of A2E from murine eyecups. MALDI images of A2E (m/z 592) are shown in RPE tissue from a 6-month-old *wt* (a) and *Abca4*^{-/-} (b) animal. (c) MALDI image of singly oxidized A2E (m/z 608) from a 6-month-old *Abca4*^{-/-} animal. Bar = 1 mm

Fig. 11.2.

Quantitative analysis of A2E via mass spectrometry. Quantitation of A2E is shown for *Rpe65*^{-/-}, *wt*, and *Abca4*^{-/-} mice, all of age 6 months. The inset illustrates the method of quantitation. AUC (shown *shaded*) was determined for the most intense fragment ion (*m/z* 418, *inset bottom*), of the intact A2E (*m/z* 592, *inset top*) in an *Abca4*^{-/-} animal

Fig. 11.3.

Oxidation of A2E. MS/MS spectra of singly oxidized A2E (m/z 608) obtained directly from tissue used for MALDI imaging (a) and from organic extracts used for LC-MS/MS analysis (b). (c) The MS/MS spectra support a mixture of one oxygen in either the 7, 8/7, 8 position (left) or 9, 10/9, 10 position (right)