Lipids in Human Lipofuscin-Enriched Subcellular Fractions of Two Age Populations

Comparison With Rod Outer Segments and Neural Retina

Haydee E. P. Bazan,* Nicolas G. Bazan,*† Lynette Feeney-Burns,‡ and Elaine R. Berman§

The fatty acid composition and content of total phospholipids, free fatty acids (FFA), diacylglycerols (DG), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylethanolamine (PE) were studied in lipofuscin granules of human donors in two age groups, young (less than 40 yr old) and old (more than 47 yr old), and compared with lipids of the photoreceptor rod outer segments (ROS). Neural retina (NR) and retinal pigment epithelium (RPE) also were studied. In both age groups, the lipid composition of the lipofuscin granules differed from that of the ROS, with a decrease in the proportion of phospholipid and an increase in FFA, suggesting very high phospholipase activity in the lipofuscin granules. In ROS, docosahexaenoic acid (22:6) was the predominant FFA, whereas palmitic acid (16:0), arachidonic acid (20:4) and oleic acid (18:1) were the major fatty acids in the lipofuscin granules. The fatty acid compositions of PC, PE, and PS of lipofuscin granules were different from those of the retina. There was proportionally less 22:6 in lipofuscin, and the amounts of saturated and monounsaturated fatty acyl chains such as 16:0, stearate (18:0), and 18:1 were greater than in retina. Compared to ROS, the lipofuscin granules showed a significant decrease in DG containing 20:4 but not 22:6. With aging, there was a decrease in the amount of total polyunsaturated fatty acyl chains (22:6 and 20:4) in the lipofuscin granules. These results show that the lipid composition of lipofuscin is different from that of ingested ROS, probably because of increased phospholipase and peroxidative activities in lipofuscin, directed toward ingested ROS as well as toward other materials from the RPE and blood. 

The relationship between the photosensory retina and the retinal pigment epithelium (RPE) is anatomically and biochemically unique. The two layers are apposed apex-to-apex as a result of embryologic inversion of the optic vesicle to form the optic cup. This establishes an arrangement in the mature retina whereby the apical membranes of the photoreceptor, as they are synthesized and displaced toward the tip of the outer segment, are shed onto the apical surface of the RPE, whereupon they are phagocytosed. Phagocytosis of the shed outer segment membranes is followed by digestion of those membranes within the phagolysosomal system of the RPE cell. However, in aging eyes of all species, and in human eyes most strikingly, lipoidal granules (lipofuscin, or age pigment) accumulate in the RPE. These granules are thought to result from alterations in degradative processes, especially those involving the lipids in the phagosome.

Although outer segment membranes are not the only source of these granules, the strong influence of the photoreceptors in lipofuscin formation has been demonstrated in rats with hereditary photoreceptor degeneration. These animals display a decrease in the amount of lipofuscin in RPE, coupled with the appearance of fluorescent material similar to lipofuscin in the degenerating photoreceptor cells. Moreover, a good correlation between the accumulation of lipofuscin and the loss of photoreceptors in the macula of aging humans has been reported.

One of the unique features of retinal photoreceptors is the very high proportion of polyunsaturated fatty acyl chains of phospholipids, mainly docosa-
hexaenoic acid (22:6) (for reviews see References 7 and 8). This fatty acid is tenaciously retained during dietary deprivation of its 18:3 precursor. Moreover, rod outer segments (ROS) contain dipolyunsaturated molecular species of phospholipids (ie, phospholipid molecules with polyenoic fatty acids at both the sn-1 and the sn-2 position of glycerol).9–12 Although the role of 22:6 and other polyunsaturated fatty acids in the retina is not clear, they are believed to provide a high degree of membrane fluidity for the movement of rhodopsin within the disk membrane. Furthermore, docosahexaenoic acid (22:6) may be enzymatically oxygenated, resulting in the production of docosanoids, which may have biologic properties,13 or it may be peroxidized. Because of this increased polyunsaturated fatty acid content of ROS, it has been proposed that ROS phagocytosed by RPE represent the major precursor for RPE lipofuscin granules.2

Although it is believed that lipofuscin granules contain peroxided lipids,14 the chemical nature of the substance responsible for the fluorescence emitted by the granules is not known. It has been suggested that this fluorescence results from the formation of Schiff bases from products of lipid peroxidation; recently, however, through the use of more refined techniques, the involvement of Schiff bases in the pigment fluorescence has been questioned.15 Furthermore, the lipid composition of the lipofuscin granules is not known.3 Therefore, to more completely understand the molecular events that lead to lipofuscin formation, it is important to analyze the lipid composition of the lipofuscin granules.

In the current work, we studied the acyl composition of diacylglycerols (DG), free fatty acids (FFA), total phospholipids, and phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) of lipofuscin granules of two age groups classified as young (human donors less than 40 yr old) and old (human donors more than 47 yr old). We compared fatty acid composition and content of these granules to those from ROS and neural retina (NR) of the same donors.

Materials and Methods
Collection of Samples
Human eyes were obtained from the Missouri Lions Eye Bank. Table 1 lists the ages of the 28 donors (56 eyes) used in these studies. Postmortem delay varied from 5–36 hr, during which eyes were kept in the dark at 4°C until dissection.

The eyes were bisected at the ora serrata; the vitreous was removed; and the retina was peeled off, immersed in 0.32 M sucrose in a vial, and stored frozen until shipped. The posterior eyecup was rinsed twice with 0.32 M sucrose to remove broken outer segments, and the RPE cells were brushed into 0.32 M sucrose. RPE homogenates and lipofuscin granules were obtained as described previously.2 Tissues were kept frozen until sample procurement was complete. The samples were thawed in ice, lightly vortexed, pooled, and centrifuged for 10 min at 1500 rpm in order to pellet the cellular fraction. The cells were diluted to 2.25 ml with distilled water and homogenized by 10 strokes of a motor-driven Teflon™ pestle. An aliquot of whole homogenate was set aside, and the remaining homogenate was centrifuged for 1 min at 1000 rpm to remove nuclei and most melain.

The resulting supernatant was layered onto a discontinuous sucrose gradient of 1.08, 1.18, and 1.29 g/ml. The gradient was centrifuged for 5 min at 9000 rpm in a swinging bucket rotor. When viewed with ultraviolet light, three fluorescent bands were seen.8 The middle band (B2), containing relatively uncontaminated lipofuscin granules, was collected at the 1.08/1.18 g/ml interface of the sucrose gradient. In order to obtain enough material for lipid analysis, the specimens were pooled as listed in Table 1.

The purity of the bands for each sample was confirmed by electron microscopic examination (Fig. 1 and Reference 2).

Retinas were kept at −70°C in 0.32 M sucrose until ROS were isolated. Ten retinas per tube were vortexed at low speed for 1 min in 6 ml sucrose-buff ered 5 mM Tris-acetate (pH 7.4) containing 65 mM NaCl and 0.2 mM MgCl2, and were spun at 4000 rpm for 4 min. The supernatant was decanted into an ice-cold graduated cylinder. This procedure was repeated once, and the supernatant containing ROS was diluted with 2 volumes of 10 mM Tris-acetate (pH 7.4). The pellet containing the (ROS-free) NR was diluted with 6 volumes of 10 mM Tris-acetate buffer (pH 7.5) and centrifuged for 10 min at 10,000 rpm.

The ROS suspension was centrifuged at 5000 rpm for 4 min, and the pellet was resuspended in 0.77 M

<table>
<thead>
<tr>
<th>Table 1. Human eyes analyzed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group of eyes according to age</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Young</td>
</tr>
<tr>
<td>Young</td>
</tr>
<tr>
<td>Old</td>
</tr>
<tr>
<td>Old</td>
</tr>
<tr>
<td>Old</td>
</tr>
<tr>
<td>Old</td>
</tr>
</tbody>
</table>

* Pairs of eyes.

The number in parentheses refers to number of samples. ROS, rod outer segments; B2, purified lipofuscin fraction; RPE, retinal pigment epithelium; NR, neural retina (ROS-free retina, including inner segment).
sucrose buffered with 10 mM Tris-acetate buffer (pH 7.5) and 0.1 M MgCl₂. Purified ROS were obtained by overlaying the suspension onto a discontinuous sucrose gradient as described previously.

Extraction and Isolation of Lipids

The final ROS and NR fractions were transferred to glass tubes with Teflon™ caps, and the lipids were extracted under N₂ with 20 volumes of chloroform:methanol, 2:1 by volume. The lipid extract was washed first with 0.05% CaCl₂ and second with Folch theoretical upper phase (chloroform:methanol:0.05% CaCl₂ [3:48:47 by volume]) to eliminate contaminants. Fraction B₂ and RPE homogenate were kept at −70°C until extracted as above.

The purified lipid extracts were dried under N₂ at 35°C and resuspended in a small quantity of chloroform:methanol (2:1 by volume). Phospholipids, DG, and FFA were separated by thin-layer chromatography. The origin of the plate containing the phospholipids was covered, and the plate was sprayed with 0.2% 2′-7′-dichlorofluorescein in methanol. DG and FFA were visualized under ultraviolet light, scraped into tubes, and converted to methyl esters of fatty acids with 14% BF₃ in methanol prior to gas-liquid chromatographic analysis.

The total phospholipids were removed from the origin of the plate and extracted three times from the silica gel with 4 ml chloroform:methanol:acetic acid:water (50:39:1:10 by volume), followed by an extraction in methanol. This procedure allows the quantitative recovery of the phospholipid fraction.

One fifth of the total phospholipids of each sample was derivatized to methyl esters of fatty acids. Four fifths of each sample were resolved into individual phospholipids using two-dimensional thin-layer chromatography. After spraying the plates with 0.2% 2′-7′-dichlorofluorescein in methanol, PC, PE, and PS were visualized under short-wave ultraviolet light. Phosphatidylinositol (PI) in RPE and particularly in the B₂ fraction did not give reproducible results for the individual fatty acid compositions, although we used the PI values obtained to calculate the total amount of phospholipids in each fraction. Solvent blanks were subjected to the same steps as the samples. Silica gels containing the individual phospholipids and blanks were scraped and transferred quantitatively into Teflon™-screw-capped test tubes, and fatty acid methyl esters were obtained. Proteins were determined by the method of Lowry et al.

Fatty Acid Methyl Ester Analysis

Fatty acid methyl esters of the different lipids were separated and quantitated with methylheptadecanoate as an internal standard in a Varian 6000 chromatograph equipped with a hydrogen flame-ionization detector and a 2-mm × 1.80-cm glass column packed with 10% SP 2330 on 100/200 mesh Chromasorb W-AW (Supelco, Relafonte, PA). The temperature was programmed from 185°C (15 min) to 210°C (50°C/min) and held for 5.7 min, after which...
it was increased to 230°C (50°C/min) and held for 20 min. Solvent blanks were subjected to the same steps as the samples and subtracted. Identification was based on comparison with retention times of known standards analyzed under identical conditions and by catalytic hydrogenation followed by gas-liquid chromatography.22 Plots of logs of retention times vs carbon numbers were made to verify the identity of the long-chain polyunsaturated fatty acids.

Electron Microscopy

An aliquot of the lipofuscin band was removed and placed in an aldehyde fixative4 in BEEM capsules for 1 hr at room temperature. The particles were pelleted, postfixed in osmic acid, dehydrated, and embedded in epoxy resin. The pellets were oriented perpendicular to the centrifugal force for thin sectioning. After being stained with uranyl acetate and lead citrate, the samples were photographed by transmission electron microscopy.

Statistical Analysis

Statistically significant differences (P < 0.05) between ROS and B2 and between young and old samples for lipofuscin B2 and ROS fractions were evaluated by the student t-test as well as by three-way analysis of variance (ANOVA) and Duncan’s multiple-range test.

Chemicals

Chemicals used were from Sigma Chemical Co., St. Louis, Missouri, unless otherwise noted.

Results

Electron Microscopy

Electron microscopic examination of B2 from the sucrose density gradient revealed osmiophilic granules of fairly uniform size (0.9–1.5 μm) that closely resembled lipofuscin granules found in RPE cells in situ3,4 (Fig. 1). Although the normal limiting membrane of the granules was often missing or ruptured, presumably because of the osmotic effects of the isolation procedure, the contents of the granules, including surface blebs, did not appear to be disturbed (Fig. 1, inset).

Lipids

Aging produces a 2-fold increase in the total lipid content of lipofuscin (Table 2). The term “lipid” in this study refers to phospholipids, DG, and FFA. Lipids such as cholesterol and triacylglycerol were not measured. In frog, rat, and other species,8 phospholipids represent 90–95% of the ROS total lipids, but the proportion of phospholipids in the lipofuscin granules is unknown. The amount of proteins in lipofuscin (B2) also increases with age. Human ROS contain quantities of lipid and protein an order of magnitude higher than those in lipofuscin. A decrease in both fractions was observed with aging, resulting in a comparable lipid/protein ratio in ROS of both age groups.

There were significant differences in the lipid composition between ROS and lipofuscin (Table 3). In ROS of younger samples, PC was the major phospholipid. In older samples there was a significant decrease in the proportion of PC and a relative increase in PE and PS, although in neither age group did the total amount of the lipids analyzed change. However, in the lipofuscin fraction there was a decrease (expressed as milligrams of protein) in the amount of PC, PS, and PE, compared to ROS. PS and PE are the two lipids containing the highest proportion of 22:6 in the photoreceptors. PI, or a lipid spot with the same Rf as PI reference standards in two-dimensional thin-layer chromatography, was found in variable and (in two samples) very high proportions in lipofuscin of younger donors—3%, 7%, and 15% of the total phospholipids in the samples analyzed. This phospholipid requires more detailed investigation. Neutral lipids, eg, DG and especially FFA, were in higher proportions in lipofuscin than in ROS.

Diacylglycerols

There were significant differences between lipofuscin granules and retina in the fatty acid composition of DG (Fig. 2). The DG of NR and ROS contained mostly arachidonic acid (20:4) and stearic acid (18:0). The proportion of 20:4 was significantly higher in ROS of both age groups than in the corresponding lipofuscin fractions. In contrast, DG of RPE and of the lipofuscin granules contained higher proportions of docosahexaenoic acid (22:6) than did NR and ROS. The 22:6/20:4 ratio, 0.90, for DG in the lipo-

Table 2. Lipid and protein content of B2 and ROS fractions from the eyes of humans of different ages

<table>
<thead>
<tr>
<th>Component</th>
<th>Young B2</th>
<th>Old B2</th>
<th>Young ROS</th>
<th>Old ROS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid (μg/eye)</td>
<td>5.2 ± 2</td>
<td>11 ± 1*</td>
<td>213 ± 33</td>
<td>181 ± 35</td>
</tr>
<tr>
<td>Protein (μg/eye)</td>
<td>8 ± 2</td>
<td>33 ± 10</td>
<td>265 ± 5</td>
<td>210 ± 10*</td>
</tr>
</tbody>
</table>

Lipid content was calculated from the moles of fatty acid contained in the total phospholipids, diacylglycerols, and free fatty acid pool in B2 and ROS fractions, and represent mean ± SE. The average molecular weights used were: phospholipids, 800; diacylglycerols, 600; and free fatty acids, 230. B2, lipofuscin granule fraction; ROS, rod outer segment fraction.

Young samples from donors less than 40 yr old; Old samples from donors more than 47 yr old.

* Significant differences (P < 0.05) between young and old specimens in each fraction.
Lipid composition of human ROS and lipofuscin fractions of two age populations

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Young</th>
<th>Old</th>
<th>B2</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>43 ± 2</td>
<td>34.7 ± 1.2</td>
<td>30 ± 6</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>PE</td>
<td>34.5 ± 0.2</td>
<td>35.9 ± 5.6</td>
<td>13.3 ± 5*</td>
<td>13.2 ± 0.4†</td>
</tr>
<tr>
<td>PS</td>
<td>11.7 ± 1.1</td>
<td>16.2 ± 4</td>
<td>4.2 ± 0.6†</td>
<td>3.7 ± 1.0t</td>
</tr>
<tr>
<td>PI</td>
<td>4.2 ± 0.7</td>
<td>4.6 ± 1.1</td>
<td>8.2 ± 6</td>
<td>6.2 ± 2.0</td>
</tr>
<tr>
<td>DG</td>
<td>1.3 ± 0.7</td>
<td>1.8 ± 0.9</td>
<td>3.3 ± 0.4†</td>
<td>3.9 ± 1.3</td>
</tr>
<tr>
<td>FFA</td>
<td>5 ± 1.6</td>
<td>6.5 ± 2.4</td>
<td>36.4 ± 20.4</td>
<td>44.8 ± 6.2‡</td>
</tr>
<tr>
<td>Total nmoles/mg protein</td>
<td>1187 ± 361</td>
<td>1357 ± 246</td>
<td>770 ± 29</td>
<td>407 ± 64*‡</td>
</tr>
</tbody>
</table>

The values, expressed as percent of total (mean ± SE) of three samples in each group, are calculated from the total nmoles of fatty acids in each lipid. ROS, rod outer segments; B2, enriched lipofuscin fraction; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidyserine; PI, phosphatidylinositol; DG, diacylglycerol; FFA, free fatty acids.

* Significant decrease in older samples in ROS (P < 0.05) and B2.
† Significant differences (P < 0.05) in young samples between ROS and B2.
‡ Significant differences (P < 0.05) in old samples between ROS and B2; in FFA, P < 0.01.

Free Fatty Acids

A large pool of FFA was present in the lipofuscin bands of young and old samples, and although the amount of lipid per milligram protein in B2 declined with aging (Table 3), the proportion of FFA, with respect to the other lipids, remained high. In contrast to the high proportion of free 22:6 in human NR and ROS, the predominant unsaturated FFA in the lipofuscin bands were 20:4 and oleic acid (18:1) (Table 4). The saturated fatty acids also are different. Palmitic acid (16:0) is found in higher proportion than stearic acid (18:0) in lipofuscin, in contrast to the retina and ROS, where there is more 18:0 than 16:0. In all of the fractions analyzed, the percentage of polyunsaturated FFA was higher in the older samples. The proportion of free arachidonic acid increased with age in the lipofuscin granules from 15.9 ± 2.7 to 22.4 ± 2.2 nmoles percent of total FFA.

Phospholipids

Table 5 shows the fatty acid composition of total phospholipids in different fractions. The most striking differences were an increase in 18:1 and a lower proportion of 22:6 in the lipofuscin granules of both young and old samples, compared to ROS. As a consequence, the degree of unsaturation (calculated as explained above) and the ratio of 22:6 to 20:4 for total phospholipids were lower in the granules than in the corresponding photoreceptors. There were no age-related changes observed in the fatty acid composition of the total phospholipids.

Because some changes can be undetectable in a pool as complex as total phospholipids, we analyzed the fatty acyl groups of the three major lipids—PC, PE, and PS—in the different fractions, in order to determine whether changes could be observed with aging.

The main fatty acyl groups of PC in ROS were 22:6 (24%), 16:0, 18:0, and 18:1; these comprised approximately 60% of the total fatty acids in this phospholipid (Fig. 3). ROS of older samples contained an increased proportion of 22:6 (32.5%, P < 0.05) and a decrease in 18:1 from 32.5% to 13.5%, (P < 0.05). The NR (also containing inner segments of photoreceptors and some undetached ROS) contained a lower proportion of 22:6 than did ROS. The composition of PC in the lipofuscin band was different from that in the retina. The amount of 22:6 in PC in the lipofuscin was less than one third of that in ROS of both young and old samples. There was, however, a significant increase in the proportion of 18:1 in older samples and in linoleate (18:2) in the lipofuscin granules of both age groups, compared to ROS. This, of course, produced a decrease in the degree of unsaturation in lipofuscin granules compared to ROS. Enriched lipofuscin granules of older samples contained a larger quantity of 16:0 and 18:2, and a significantly smaller quantity of 18:0, than did those of young samples.

There was no difference between the two age groups in 22:6 of PE in ROS (Fig. 4). In contrast with PC, there was a higher proportion of 20:4 in PE of the lipofuscin granules than in ROS. Docosapentaenoate (22:5, n-3), a precursor of 22:6, also was found in
appreciable amounts (3.2 ± 0.5 nmole percent) and increased with age (7.1 ± 1.1, P < 0.05) in the lipofuscin fraction. The presence of these two predominant polyunsaturated fatty acids, 20:4 and 22:5, accounts for the significant difference in the ratios of 22:6/20:4 in PE of lipofuscin granules and ROS, even though the degree of unsaturation of PE in these granules was greater than that of PC and, in the older samples, was the same as that of ROS.

The composition of PS in human ROS is unique (Fig. 5). Longer and more highly unsaturated fatty acids were found: 24:4, 24:5, and 24:6 comprise more than 11% of the total fatty acids in this phospholipid. There were no detectable amounts of these fatty acids in the NR or in the RPE fractions. The fatty acids were identified by their retention times compared to standards and by catalytic hydrogenation followed by gas-liquid chromatography. As in the other phospholipids studied, there was also a high proportion of 22:6 in PS of ROS. The highly unsaturated components of PS resulted in a degree of unsaturation in human ROS of more than 400, compared to 200 for PC and 300 for PE. PS in the lipofuscin granules was found to contain a significantly lower proportion of

Fig. 2. Fatty acid composition of diacylglycerols of human neural retina (NR), rod outer segments (ROS), homogenates of retinal pigment epithelium (RPE), and lipofuscin granules (B2) in young (A) and old (B) samples. 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 20:4, arachidonic acid; 22:6, docosahexaenoic acid. Values represent the mean percentage (±SE) of two or three samples. ** and *** represent statistically significant differences (P < 0.05) between lipofuscin fraction B2 in comparison to ROS for a particular fatty acid.
Table 4. FFA composition of B2 and other retina fractions in human eyes of two different age populations

<table>
<thead>
<tr>
<th>FFA*</th>
<th>Young</th>
<th>Old</th>
<th>Young</th>
<th>Old</th>
<th>Young</th>
<th>Old</th>
<th>Young</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>16.2</td>
<td>7.2</td>
<td>15.7 ± 0.8‡</td>
<td>8.4 ± 3‡</td>
<td>22</td>
<td>17.9</td>
<td>22.2 ± 0.7</td>
<td>17.3 ± 1.2†</td>
</tr>
<tr>
<td>18:0</td>
<td>35</td>
<td>22.6</td>
<td>22.5 ± 1.5</td>
<td>16.6 ± 4</td>
<td>24.2</td>
<td>12.9</td>
<td>15.5 ± 3.1</td>
<td>10.3 ± 2.3</td>
</tr>
<tr>
<td>18:1</td>
<td>10</td>
<td>11.9</td>
<td>12.1 ± 0.8†</td>
<td>12.7 ± 0.6</td>
<td>19.8</td>
<td>15.8</td>
<td>18 ± 0.6†</td>
<td>16.9 ± 0.2</td>
</tr>
<tr>
<td>18:2</td>
<td>1.2</td>
<td>2.1</td>
<td>2 ± 0.5†</td>
<td>2.3 ± 0.1†</td>
<td>6</td>
<td>7.4</td>
<td>5 ± 0.4†</td>
<td>6.3 ± 1.3†</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.6</td>
<td>0.9</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>0.6</td>
<td>0.9</td>
<td>0.5 ± 0.1</td>
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</tr>
<tr>
<td>20:4</td>
<td>16.2</td>
<td>22.4</td>
<td>16.4 ± 0.8</td>
<td>18.4 ± 1.1</td>
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<td>21.4</td>
<td>15.9 ± 2.7</td>
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<tr>
<td>22:4</td>
<td>2.1</td>
<td>1.8</td>
<td>2.3 ± 0.3</td>
<td>3.9 ± 0.8</td>
<td>1.8</td>
<td>5.3</td>
<td>2.7 ± 1.7</td>
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<tr>
<td>22:5</td>
<td>1.3</td>
<td>1.3</td>
<td>1.5 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>3.1</td>
<td>2.1</td>
<td>2.4 ± 1.5</td>
<td>2.9 ± 0.1</td>
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<tr>
<td>22:6n-3</td>
<td>13.9</td>
<td>26.7</td>
<td>22.9 ± 1.8†</td>
<td>31.5 ± 6.4†</td>
<td>3.1</td>
<td>10.3</td>
<td>11.3 ± 4.2†</td>
<td>14.8 ± 1.2†</td>
</tr>
</tbody>
</table>

The values (mean of two or three samples ± SE) are expressed as nanomole percent of the total FFA.

Table 5. Fatty acid composition of total phospholipids of young and old human retina and RPE fractions

<table>
<thead>
<tr>
<th>Fatty acid*</th>
<th>Young</th>
<th>Old</th>
<th>Young</th>
<th>Old</th>
<th>Young</th>
<th>Old</th>
<th>Young</th>
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<tbody>
<tr>
<td>16:0</td>
<td>18.9</td>
<td>17.6</td>
<td>13.0 ± 2.0</td>
<td>14.0 ± 1.1</td>
<td>15.2</td>
<td>12.6</td>
<td>11.3 ± 1.9</td>
<td>15.1 ± 1.1</td>
</tr>
<tr>
<td>18:0</td>
<td>25.5</td>
<td>25.1</td>
<td>20.0 ± 2.8</td>
<td>23.1 ± 1.7</td>
<td>19.5</td>
<td>14.1</td>
<td>17.5 ± 1.2</td>
<td>16.1 ± 2.5</td>
</tr>
<tr>
<td>18:1</td>
<td>16.4</td>
<td>18.3</td>
<td>14.0 ± 1.0†</td>
<td>10.4 ± 3.3§</td>
<td>17.0</td>
<td>16.4</td>
<td>24.6 ± 0.2†</td>
<td>28.8 ± 1.6§</td>
</tr>
<tr>
<td>18:2</td>
<td>1.5</td>
<td>1.9</td>
<td>2.0 ± 1.0‡</td>
<td>1.0 ± 0.1‡</td>
<td>4.0</td>
<td>7.5</td>
<td>6.1 ± 0.1‡</td>
<td>8.8 ± 1.1‡</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.3 ± 0.5</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.8</td>
<td>0.6</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>1.4</td>
<td>0.6</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>20:4</td>
<td>11.6</td>
<td>12.3</td>
<td>10.8 ± 5.4</td>
<td>6.7 ± 1.3</td>
<td>17.4</td>
<td>21.2</td>
<td>12.7 ± 0.5</td>
<td>9.8 ± 1.2</td>
</tr>
<tr>
<td>22:4</td>
<td>2.7</td>
<td>2.5</td>
<td>2.9 ± 0.4</td>
<td>2.4 ± 0.2</td>
<td>3.0</td>
<td>3.2</td>
<td>3.1 ± 0.3</td>
<td>2.7 ± 0.9</td>
</tr>
<tr>
<td>22:5</td>
<td>1.0</td>
<td>0.9</td>
<td>1.7 ± 0.1</td>
<td>1.9 ± 0.6</td>
<td>1.5</td>
<td>2.2</td>
<td>1.7 ± 0.3</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>22:6</td>
<td>17.8</td>
<td>16.4</td>
<td>30.0 ± 1.1‖</td>
<td>35.0 ± 5.0‖§§</td>
<td>14.0</td>
<td>9.9</td>
<td>8.5 ± 0.6‖</td>
<td>6.6 ± 0.5‖§§</td>
</tr>
</tbody>
</table>

Degree of unsaturation

Total phospholipids were isolated from the origin of the monodimensional plate, extracted, and converted to fatty acid methyl esters before gas-liquid chromatography analysis. The degree of saturation was calculated as explained in the text. NR, neural retina (ROS-free retina, including inner segment); ROS, rod outer segments; RPE, retinal pigment epithelium; B2, purified lipofuscin fraction.

Discussion

This paper presents the first analyses of the lipid composition of human lipofuscin granules. Our data show that the lipid pattern is complex, and that the total quantity of phospholipids in the lipofuscin
Fig. 3. Fatty acid composition of PC of young (A) and old (B) human retinas and RPE fractions. NR, human neural retina; ROS, rod outer segments; RPE, homogenates of retinal pigment epithelium; B2, lipofuscin granules. 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 20:4, arachidonic acid; 22:6, docosahexaenoic acid. The values correspond to the average (±SE) of two or three samples. Only the most abundant fatty acids are shown. Significant differences \((P < 0.05)\) in fatty acid composition (*, **, ***, and ****), and degree of unsaturation (open circles) or 22:6/20:4 ratio (filled circles) between ROS and the B2 fraction are noted in the figure. Also, significant effects attributed to age between the same fraction (filled triangles) are noted.

Fig. 4. Fatty acid composition of PE of human neural retina (NR), outer segments (OS), homogenates of retinal pigment epithelium (RPE), and lipofuscin granules (B2) in young (A) and old (B) samples. 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 20:4, arachidonic acid; 22:6, docosahexaenoic acid. Values represent the mean percentage (±SE) of two or three samples. *•**, *** and **** represent statistically significant differences \((P < 0.05)\) between lipofuscin fraction B2 in comparison to ROS for a particular fatty acid.
granules is lower than in ROS, whether results are expressed per eye or per milligram protein (Tables 2, 3). In agreement with previous studies,4 we found an increased lipid content in lipofuscin with age. The lipid/protein ratio in the B2 fraction changes from 2.6 ± 1.1 for young samples to 1.2 ± 0.3 for older samples, and represents an accumulation of both lipids and protein in lipofuscin with aging. Furthermore, the lipid composition in the lipofuscin granules is different from that of the retina.

Table 6. Total amount of docosahexaenoic acid and arachidonic acid in ROS and B2

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Age of sample donor</th>
<th>ROS (nmoles/mg protein)</th>
<th>B2 (nmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22:6</td>
<td>Young</td>
<td>480 ± 50</td>
<td>182 ± 15</td>
</tr>
<tr>
<td></td>
<td>Old</td>
<td>716 ± 100</td>
<td>85 ± 14*</td>
</tr>
<tr>
<td>20:4</td>
<td>Young</td>
<td>155 ± 11</td>
<td>220 ± 30</td>
</tr>
<tr>
<td></td>
<td>Old</td>
<td>149 ± 20</td>
<td>96 ± 20*</td>
</tr>
</tbody>
</table>

Values are calculated from the sum of docosahexaenoic acid (22:6) and arachidonic acid (20:4) content in total phospholipids, diglycerides, and the free fatty acid pool.

* Significant (P < 0.01) decrease in older samples.

The composition of the DG pool is similar in human ROS and NR, with a large percentage of 20:4 and 18:0 and a relatively low percentage of 22:6. This composition is similar to that described previously in bovine ROS DG.23 The B2 fraction of RPE, on the contrary, contains less arachidonoyl DG than do the photoreceptors—34% of the total fatty acids for ROS, compared to 17% in B2 of the young eyes, and to 7% in the B2 fraction of older eyes. This loss is compensated by an increase in palmitate and stearate in DG.

One possible explanation for these results is that an increase of peroxidative activity in the granules with age produces a depletion of arachidonate in DG. This peroxidation could occur nonenzymatically by way of free radicals, or by the action of enzymes such as lipoxygenase, cyclooxygenase, or the cytochrome P-450-dependent monoxygenase system. Previous studies24 have shown no increase in the cyclooxygenase and lipoxygenase reactions of RPE of dogs with ceroid lipofuscinosis, a disease model of age-related lipofuscin accumulation.

The retina, like other parts of the central nervous system, contains a small FFA pool25,26 in which a
selective increase of 20:4 and 22:6 is observed after anoxia or depletion of energy.\textsuperscript{27,28} These fatty acids are released from membrane phospholipids by phospholipases A. Our results show that the most prevalent free polyunsaturated fatty acid in human ROS is 22:6, whereas in the B\textsubscript{2} fraction, the pool of FFA is much larger and free 20:4 is the predominant polyunsaturated FFA. Although this large pool of FFA in B\textsubscript{2} may be due in part to hydrolysis of membrane lipids during postmortem delay and isolation of the samples, the observed lack of large pools of FFA in other fractions, such as ROS, suggests the presence of very active lipases and phospholipases in lipofuscin.

Phospholipase A\textsubscript{2} releases fatty acids from the sn-2 position of membrane lipids. Unsaturated fatty acids such as 20:4 and 22:6 are esterified predominantly to the sn-2 position. Once these unsaturated fatty acids are released, they can become substrates for lipid peroxidation. Lipid peroxidation is initiated by free radicals; enzymatic scavenging systems such as superoxide dismutase and natural antioxidants such as vitamin E are involved in controlling these reactions. Melanin offers protection from the attack of free radicals, but it decreases in human RPE with aging.\textsuperscript{29} Therefore, the combined effects of degradative lipid enzymes (such as phospholipase A\textsubscript{2}) and a decrease in the defense mechanism against peroxidation can contribute to the decrease of 22:6 and 20:4 observed in lipofuscin granules in human RPE with aging.

We also found a complex phospholipid pattern among these fractions. PS composition in ROS presents a very distinctive pattern, with longer, more unsaturated fatty acids than the other phospholipids: 24:4, 24:5, and 24:6 are detected consistently in photoreceptors of humans (current study) and cattle,\textsuperscript{11} but not in the other fractions. Very long (22–36 carbons) and highly unsaturated fatty acids have been described in retinas of other species.\textsuperscript{10,11,30} The fatty acid compositions of choline, ethanolamine, and serine phosphoglycerides of human brain gray matter with different forms of ceroid lipofuscinosis show a reduction of 22:6 almost exclusively in PS,\textsuperscript{31} without affecting the other lipids. In the current study, the decrease in total 22:6 in the lipofuscin granules with aging (Table 6) may be due to the decrease in the general proportion of lipids, expressed in terms of protein concentration. It is important to emphasize that we have studied a purified fraction of lipofuscin granules, in contrast to the heterogeneous tissue analyzed in the above studies.\textsuperscript{31} The decrease in phospholipids in the lipofuscin granules, concomitant with the increase in the proportion of FFA compared to ROS, strongly indicates the presence of very active phospholipases in the B\textsubscript{2} fraction.

The photoreceptors and the lipofuscin granules have a different phospholipid composition; the main phospholipid found in the lipofuscin granules is PC, in agreement with previous work on the composition of purified bovine RPE,\textsuperscript{32,33} which also contains a very high proportion of PC (54%) compared to PE\textsuperscript{34} and a very low proportion of 22:6 (only 2.3% of plasma membrane lipids). The phospholipids PE and PS, which contain higher proportions of 22:6 than PC in ROS, are found in lower proportions in the lipofuscin granules. Since, as mentioned, polyunsaturated fatty acids are very susceptible to conversion to lipid peroxides, 22:6 may be peroxided more rapidly in the granules than is arachidonic acid, producing a different composition of phospholipids in ROS than in lipofuscin. A difference in 22:6 composition between phospholipids of bovine ROS and RPE, with lower levels of 22:6 in the RPE, has also been reported.\textsuperscript{35} The different lipid composition in RPE may reflect not only the degradation of ingested ROS in lipofuscin, but also the presence in this fraction of other lipid components from the blood and the RPE itself. Morphologic changes in the interaction between choroid and RPE have been reported in older rats and are attributed to a less efficient exchange of materials between the choroid and the RPE.\textsuperscript{35} Furthermore, another minor but important lipid, DG, contains predominantly 20:4 in the photoreceptor, whereas 22:6-containing DG appear more abundantly in RPE. Another possible mechanism to explain the difference in composition between ROS and B\textsubscript{2} is that, in the course of degradation in the phagosome, certain lipid components, eg, 22:6, instead of being incorporated into lipofuscin granules, may become noncovalently bound to interphotoreceptor matrix proteins\textsuperscript{36} and transported back from the RPE to the NR, where they can be reutilized in various processes, including ROS membrane assembly. Clearly, a better understanding of these mechanisms will be important in the elucidation of the many factors involved in the aging process.

Key words: aging, diacylglycerols, free fatty acids, human lipofuscin, phospholipids

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