Age-Related Lipid Oxidation in Human Lenses

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PURPOSE. To quantify age-related changes in products of lipid oxidation in human lenses and to relate these changes to membrane hydrocarbon chain structure. Deviation from a well-defined membrane-lipid composition and structure could result in alterations in membrane function and disruption of the homeostasis of the cell.

METHODS. Infrared spectroscopy was used to detect lipid compositional and structural changes in human lens membranes associated with age and cataracts.

RESULTS. Lipid oxidation increased linearly threefold relative to total phospholipids in subjects ranging in age between 1 and 85 years, as was evident by increases in trans double bonds, lipid carbonyls, and secondary products. There was no statistical difference between the levels of lipid oxidation in the cortex or nucleus. Lipid hydrocarbon chain order (rigidity) increased from approximately 40% at birth to 70% at 80 years of age. Changes in lipid order correlated with changes in the relative content of membrane phosphatidylcholine and sphingomyelin, and with the level of lipid oxidation.

CONCLUSIONS. Lipid oxidation increased linearly and uniformly throughout the human lens with age. The change in lipid oxidation with age correlated to a change in lipid order. (Invest Ophthalmol Vis Sci. 1998;39:1053–1058)

The physicochemical properties of numerous biologic membranes, including those from the human lens, have been shown to change during aging. We have shown that the hydrocarbon chains of human lens lipids become more structurally ordered (rigid) with age. This increase in order with age correlates to an increase in sphingomyelin and a decrease in phosphatidylcholine. Products of lipid oxidation also have been shown to increase with age and also may influence lipid order. We have shown that oxidation can alter the order of sphingolipids, the predominant phospholipids of the human lens. Choe et al. have suggested that lipid peroxidation plays a dominant role in increasing the rigidity of senescent membranes in numerous systems. Products of lipid oxidation have been reported in clear and cataractous human lenses. Four studies have measured lipid oxidation with age in the human lens. Malondialdehyde (MDA) was shown to increase with age, but the results are controversial because MDA is a poor marker of lipid oxidation. MDA either can bind to proteins and go undetected or, if unbound, can be removed by the aldehyde dehydrogenase present in the lens. Furthermore, the “thiobarbituric acid [TBA] test used to measure MDA content and/or TBA reactivity . . . provides no information on the precise structures of the MDA precursor(s), its molecular origins, or the amount of each formed.” Also, MDA arises from lipid adducts and, especially, from hydroxyl groups, and trans double bonds, or combinations of all. Babi-23-27 has shown that lipophylic fluorophores increase with age in whole lenses. However, when various regions of the lens were examined, no increase in fluorophores relative to phospholipids was detected.

Figure 1 illustrates the mechanism of formation of oxidative products when monounsaturated hydrocarbon chains are oxidized by singlet oxygen. The primary products formed are trans double bonds, hydroxyls, hydroperoxides, and carbonyls. Infrared spectroscopy has been
used to quantify these products for decades in oxidized fats. \(^{20-25}\) In this study infrared spectroscopy and a chemical assay were applied to evaluate primary and secondary products of lipid oxidation in human lenses as a function of age. Because lipid components other than the products of lipid oxidation can contribute to the infrared spectral profiles, the phospholipid composition of the lipid fractions used in this study were quantified using \(^{31}\)P nuclear magnetic resonance spectroscopy, and contribution of changing phospholipid composition to the infrared spectra was assessed.

**METHODS**

Approximately 266 clear human lenses were obtained within 6 hours after death through the Kentucky Lions Eye Bank and the Eye Bank at the Eye Institute at the Milwaukee County Medical Complex. Lenses were excised immediately after removal of the cornea and were frozen in liquid nitrogen to prevent oxidative changes. Spectroscopic studies were performed within an average of 6 months of lens excision. One fourth of the 266 lenses processed were used for this study. The remainder was used in other completed and published studies.\(^{1,5}\) Tissues from the epithelium, cortex, and nucleus were pooled separately. All of the protocols were performed on ice under an atmosphere of argon. All reagents were bubbled with argon before use. The monophasic extraction method\(^{5}\) previously reported was used to extract lens lipids using acetonitrile precipitation. This approach reduced the experimental difficulties associated with the more conventional biphasic methods. All chemicals were obtained from Sigma Chemical (St. Louis, MO).

**Preparation of Samples for Infrared Spectroscopy**

The lipidic extracts were dissolved in chloroform and then were layered onto AgCl windows. The chloroform was evaporated under a stream of argon gas, and the lipid sample then underwent lyophilization for approximately 12 hours to remove the chloroform completely.

**Collection and Analysis of Infrared Spectra**

Infrared spectra were acquired with a Fourier transform infrared spectrometer (model 5000; Mattson, Madison, WI) equipped with a deuterated triglycine sulfate detector. Approximately 250 interferograms with an effective spectral resolution of 1.0 cm\(^{-1}\) were recorded, coadded, and apodized with a Happ-Genzel function before Fourier transform.

**Chemical Analysis of Malondialdehyde**

A matching pair of human lenses was homogenized in 10 ml 20 mM TRIS-HCl, pH 7.4, using a Kimatica Polytron (Brinkman Instruments, Westbury NY). The polytron was rinsed in another aliquot of 10 ml 20 mM TRIS-HCl, and the two aliquots were pooled together. A chromogenic reagent (LPO-586; Bioxytech S.A., Bonneuil Marne Cedex, France) was used to measure MDA and hydroxynalkenal. A 650-μl aliquot of freshly prepared 11.4 mM LPO-586 was added to 200 ml lens homogenate. A 150-ml volume of 10.4 M methanesulfonic acid was added to each tube, mixed, and incubated for 40 minutes at 45°C. The absorbance was measured at room temperature at 586 nm. This assay measures MDA and hydroxynalkenal. To measure MDA only, the assay described above was modified by adding 150 ml 37% aqueous HCl instead of 150 ml 10.4 M methanesulfonic acid. No hydroxynalkenal was detected, because there was no significant difference in the absorbance measured by both assays. Standards were supplied in the kit and were correlated linearly within the range of the absorbance values obtained.

**RESULTS**

**Cis/Trans Double-Bond Ratio**

When lipids are oxidized, cis double bonds of the hydrophobic chains rearrange to form trans double bonds (Fig. 1). Trans double bonds do not occur naturally in lipid hydrocarbon chains. Thus, the ratio of the intensity of the trans double bond to the intensity of the cis double bond may be used as an index of lipid oxidation. Evidence for the increase in trans double bonds was shown in the ratio of the intensity of the trans double bond at 981 cm\(^{-1}\) to the intensity of other lipid marker bands, such as the phosphate symmetrical and asymmetrical stretching bands at 1224 and 1087 cm\(^{-1}\), respectively, and the CH stretching bands between 3100 and 2800 cm\(^{-1}\). The age-related increase in trans double bonds relative to lipid marker bands is shown in Figure 2A. This increase with age was significant (Table 1) \((P < 0.001)\) and was almost twice as great in the nuclear lipids than in the cortical ones.

**Lipid Carboxyl Groups**

The carbonyl stretching band near 1734 cm\(^{-1}\) arises from the acyl-linked hydrocarbon chains of lipids with a glycerol backbone, such as phosphatidylcholine or phosphatidylethanolamine, and from products of lipid oxidation. The carbonyl...
FIGURE 2. Age-related correlation of the band areas of (A) the infrared trans double bond at 980 cm⁻¹ and (B) the infrared carbonyl band near 1730 cm⁻¹. Marker bands used for Figure 2 were the phosphate symmetrical and asymmetrical stretching bands, markers for phospholipids, and the CH stretching bands, a marker for total lipid hydrocarbon. The marker bands were normalized to the phosphate symmetrical stretching band area, and standard deviations (n = 3) were less than the size of the symbols. The solid line represents the best fit determined by linear regression analysis for all data points. Lipid structural order is defined as the percentage of trans rotomers in the hydrocarbon chain. The trans rotomers are not to be confused with the trans double bonds in (A). Lipid structural order was correlated to the relative intensity of (C) the infrared trans double-bond band at 980 cm⁻¹ and (D) the infrared carbonyl band near 1730 cm⁻¹. (•) Cortical lipids, (■) nuclear lipids. Error bars represent the SD of the mean. The solid line represents the best fit determined by linear regression analysis for all data points.

band intensity is plotted in Figure 2B against the three lipid marker bands described above. The intensity of the carbonyl exhibited a threefold increase in an age range between 1 and 80 years. The increase was significant (Table 1; P < 0.01), and, unlike the results for the trans double band, there was no significant difference between the nuclear and cortical lipids. An increase in the percentage of glycerophospholipids with increasing age cannot account for an increase in the carbonyl band, because we have shown for these same samples that the relative amount of glycerophospholipids actually decreases with increasing age.² It is more probable that the increase could be caused by an increase in free fatty acids, neutral lipids, which can account for approximately 30% of the lipid carbonyls,²⁰,²¹,²⁴ lipid aldehydes produced from lipid oxidation, or a combination of all.

Lipid cis Band

The number of cis double bonds did not change with age relative to the marker bands (P = 0.47 for cortical and nuclear lipids). There was no significant difference between the number of cis double bonds for nuclear or cortical lipids (Table 2).

Marker Band Changes with Age and Region

The marker bands did not change with age relative to one another (Table 2). This indicates that they are suitable marker bands and that the differences in oxidation bands with age are not the results of changes in marker bands.

Chemical Test for Malondialdehyde

MDA was measured using the chromogenic reagent LPO-586, and a twofold increase was observed between ages 0 and 80 years (Fig. 3). This increase was significant (P < 0.005).

Lipid Order Versus Oxidation

The increase in lipid oxidation with age, as measured by lipid trans, double bonds, and carbonyl bands, correlated with the degree of order in the lipid acyl chains. This correlation is
The data reported herein indicate that products of lipid oxidation and should not be confused with the double band used as an index of lipid oxidation. There was no statistical difference between the nuclear and cortical lipid order versus oxidation curves. The trans CH₂ rotomers used to measure lipid order refer to the conformational arrangement of saturated carbons and should not be confused with the trans double band used as an index of lipid oxidation.

**DISCUSSION**

The data reported herein indicate that products of lipid oxidation increase significantly and linearly with age in the human lens (Figs. 2A, 2B, Table 1). Lipid oxidation was measured in terms of trans double bonds, lipid carbonyls, and MDA.

**Factors That Could Influence Lipid Oxidation Band Intensity**

**Carbonyl Band.** Because lipids with glycerol backbones contribute to the absorbance of the carbonyl band, their contribution must be taken into account to estimate the changes caused by oxidation-induced carbonyls. The infrared spectral data corresponding to pure phosphatidylcholine were used to calculate the carbonyl/marker band ratio as 0.41 at a sphingolipid concentration of 64%, which is the percentage present in the human lens at 7.5 years of age. This value is close to the carbonyl/marker band ratio of 0.38 at 7.5 years of age, which was calculated from the data in Table 2. Thus, at ages below 10 years, little or no contribution to the carbonyl band absorbance is the result of the products of lipid oxidation, but it may be the result of the carbonyls from glycerophospholipids. The increase in carbonyl absorbance with age (Fig. 2B) could not be related to an increase in the glycerolipid content of the membranes; it is actually the sphingolipid concentration that has been shown to increase with age. In particular, in the samples used in this study the content of sphingolipids increased from 64% at 7.5 years to 68% at 85 years of age. Because sphingolipids do not contribute to the carbonyl absorbance at 1680 cm⁻¹ the measured increase in the percentage of sphingolipids would result in a decrease in the carbonyl/marker ratio from 0.41 to 0.37. Instead, an increase was observed. This increase is thus attributed to an increase in products of lipid oxidation, triglycerides, free fatty acids, or combinations of all three (Fig. 1).

**Trans Double-Bond Band.** The number of trans double bonds, a marker of lipid oxidation (Fig. 1) increases with age relative to marker bands (Fig. 2A). Because of the underlying C-C-N⁻² stretching band from the choline-containing phospholipids, the quantification of the trans double bond is difficult. The change in the percentage of sphingolipids with age, as discussed above, would not alter the underlying C-C-N⁻² band intensity, because sphingolipids and the C-N-containing glycerophospholipids have the same C-C-N⁻²/marker band ratio of 0.27. The increase in the trans band intensity with age (Fig. 2A) is proposed to be caused by an increase in the products of lipid oxidation (Fig. 1).

**Lipid Oxidation and Membrane Structure.** In this study we found a direct correlation between lens lipid oxidation and the structural order of lipid acyl chains (Figs. 2C, 2D, Table 1).

**Table 1. Linear Regression Analysis Parameters for Lipid Oxidation Band Curves**

<table>
<thead>
<tr>
<th>Curve</th>
<th>Intercept</th>
<th>Slope</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonyl band vs marker bands</td>
<td></td>
<td></td>
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<tr>
<td>Cortical lipids</td>
<td>0.35 ± 0.06</td>
<td>0.0039 ± 0.0010</td>
<td>6</td>
<td>0.004*</td>
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<td>Nuclear lipids</td>
<td>0.35 ± 0.08</td>
<td>0.0042 ± 0.0014</td>
<td>5</td>
<td>0.014*</td>
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<tr>
<td>Cortical and Nuclear lipids</td>
<td>0.35 ± 0.06</td>
<td>0.0040 ± 0.0007</td>
<td>11</td>
<td>0.000*</td>
</tr>
<tr>
<td>Trans band vs marker bands</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortical lipids</td>
<td>0.037 ± 0.03</td>
<td>0.0028 ± 0.0005</td>
<td>6</td>
<td>0.000*</td>
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<tr>
<td>Nuclear lipids</td>
<td>0.007 ± 0.04</td>
<td>0.0041 ± 0.0007</td>
<td>5</td>
<td>0.001*</td>
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<tr>
<td>Cortical and nuclear lipids</td>
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<td>0.0034 ± 0.0042</td>
<td>11</td>
<td>0.000*</td>
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<tr>
<td>Carbonyl/marker bands vs lipid order</td>
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<td></td>
</tr>
<tr>
<td>Cortical lipids</td>
<td>−0.13 ± 0.10</td>
<td>0.012 ± 0.006</td>
<td>6</td>
<td>0.053</td>
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<tr>
<td>Nuclear lipids</td>
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<td>0.013 ± 0.007</td>
<td>5</td>
<td>0.050*</td>
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<tr>
<td>Cortical and nuclear lipids</td>
<td>−0.082 ± 0.10</td>
<td>0.011 ± 0.004</td>
<td>11</td>
<td>0.010*</td>
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<tr>
<td>Trans/marker bands vs lipid order</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Cortical lipids</td>
<td>−0.44 ± 0.04</td>
<td>0.0106 ± 0.0026</td>
<td>6</td>
<td>0.003*</td>
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<tr>
<td>Nuclear lipids</td>
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<td>0.013 ± 0.005</td>
<td>5</td>
<td>0.017*</td>
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<tr>
<td>Cortical and nuclear lipids</td>
<td>−0.43 ± 0.06</td>
<td>0.011 ± 0.002</td>
<td>11</td>
<td>0.000*</td>
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</tbody>
</table>

*P < 0.05 indicates linear relationship is statistically significant. Regression order = 1; ± SEM.

shown in Figures 1C and 1D and in Table 1 (P < 0.05). Lipid order was defined as the ratio of trans and CH₂ rotomers, a measure of structural order (rigidity). There was no statistical difference between the nuclear and cortical lipid order versus oxidation curves. The trans CH₂ rotomers used to measure lipid order refer to the conformational arrangement of saturated carbons and should not be confused with the trans double band used as an index of lipid oxidation.

**Table 2. Parameters for Infrared Lipid Marker Bands**

<table>
<thead>
<tr>
<th>Lipid Type</th>
<th>CH Stretching bands/Asymmetrical PO₂-stretching band</th>
<th>CH Stretching bands/Symmetrical PO₂-stretching band</th>
<th>Symmetrical PO₂-stretching band/Asymmetrical PO₂-stretching band</th>
<th>cis/Assymetric PO₂-stretching band</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical Lipids</td>
<td>6.57 ± 0.92</td>
<td>2.0 ± 0.2</td>
<td>3.3 ± 0.2</td>
<td>0.018 ± 0.006</td>
</tr>
<tr>
<td>Nuclear Lipids</td>
<td>9.1 ± 0.93</td>
<td>2.5 ± 0.2</td>
<td>3.6 ± 0.1</td>
<td>0.021 ± 0.008</td>
</tr>
<tr>
<td>P</td>
<td>0.56</td>
<td>0.002*</td>
<td>0.014*</td>
<td>0.52</td>
</tr>
</tbody>
</table>

*P < 0.05 indicates a statistically significant difference between the parameters measured for nuclear and cortical lipids. n = 6; ± SD.
FIGURE 3. Malondialdehyde in whole lens homogenates determined chemically. (--->) 95% confidence interval. The solid line represents the best fit determined by linear regression analysis for all data points.

Choe et al.\(^3\) have suggested that lipid peroxidation plays a dominant role in increasing the rigidity of senescent membranes in numerous systems. Other investigators have demonstrated that lipid oxidation orders the hydrophobic chains.\(^26-35\) Our earlier study on the oxidation of sphingomyelin\(^4\) showed that oxidation has a dual effect on lipid order. Mild oxidation was found to order the lipid hydrocarbon chains, whereas strong oxidation was found to disorder them. The ordering may be partially attributed to the formation of trans double bonds,\(^34,36\) which, as shown in this study, increase with age (Fig. 2A). Also, the increases in sphingomyelin content with age contribute toward the ordering of lens lipids with age, perhaps because of the strong lipid-lipid interactions established between interface regions and high hydrocarbon chain saturation.

**Threshold of Lipid Oxidation and Cataract Hypothesis.** Despite the measured increase in products of lipid oxidation with age, all the lenses examined in this study were clear. This suggests that the membranes are able to function normally even at the levels of lipid oxidation (15%–50%) measured in this study. Because products of lipid oxidation are known to be deleterious to most systems, however, it is possible that a threshold level of lipid oxidation could exist above which the lens membranes could no longer function normally and cataractogenesis would be initiated. Primary products of lipid oxidation can cause cataractans in animal models,\(^3,5,37,38\) and the majority of studies,\(^7,9,12-59\) with one exception,\(^17\) indicate that lipid oxidation may occur at higher levels in cataracts. We hypothesize that there is a threshold of lipid oxidation above which the biochemical events leading to opacification may be triggered. This threshold could be surpassed earlier in some subjects predisposed to cataract formation.

**References**


Changes in Visually Guided Behavior of Royal College of Surgeons Rats as a Function of Age: A Histologic, Morphometric, and Functional Study

David A. DiLoreto, Jr., 1 Constancia del Cerro, 1 Christopher Cox, 2 and Manuel del Cerro 1

PURPOSE. To compare the changes in visually guided performance as a function of age between Royal College of Surgeons (RCS) dystrophic and congenic rats and to correlate photoreceptor cell number with visually guided performance in age-matched populations of RCS dystrophic rats.

METHODS. The visually guided performances of RCS dystrophic (n = 6) and congenic (n = 7) rats were studied from 0.75 to 12 months of age using a water escape paradigm that tested their ability to find a submersed, randomly placed platform that used a light source as a clue. The time to find the platform (latency) was recorded. In age-matched dystrophic RCS rats, histopathologic changes were described and the number of photoreceptor cell nuclear profiles per mid sagittal retinal section was counted. Changes with age in visually guided behavior and photoreceptor cell populations of RCS dystrophic rats then were compared.

RESULTS. The latency of RCS dystrophic rats increased significantly beyond that of congenic rats after 6 months of age. Photoreceptor cell number in dystrophic rats precipitously decreased through 6 months of age, stabilized at 9 months, and decreased further at 12 months. Two unexpected results were seen in the dystrophic animals: At 6 months of age, as few as 22 ± 3 photoreceptor cell nuclei per mid sagittal section provided similar latencies as at 2 months when there were as many as 400. Although the number of photoreceptor cells remained stable from 6 to 9 months of age, functional vision significantly deteriorated.

CONCLUSIONS. Two important phenomena were observed. First, the RCS rats performed very well in the water escape test even while their photoreceptor cell population was being decimated. Second, once a low threshold was reached, a dramatic deterioration of visually guided behavior occurred without a further reduction in photoreceptor cell numbers. (Invest Ophthalmol Vis Sci. 1998;39:1058—1063)

The Royal College of Surgeons (RCS) rat is a well-known animal model of retinal degeneration. 1 The recessive hereditary defect within the retinal pigment epithelium of the dystrophic rat causes a form of retinal degeneration, attributed primarily to the failure of the phagocytosis of shed rod outer segments. 2 Because the dystrophic rat differs from its congenic counterpart by only one gene locus, the two strains provide ideal experimental groups for the study of retinal degeneration. Hence, it has been used to study a wide range of topics in retinal cell biology, including pigment epithelium—photoreceptor cell interaction, 3 growth factor therapy, 4 and even adult human retinal transplantation. 5

Original histopathologic studies reported that the RCS dystrophic retina was completely devoid of rod photoreceptor cell nuclei by 9 weeks of age. 6 Subsequently, some photoreceptor cell nuclei were shown to be present for 2 years. 6