Polyunsaturated Fatty Acids and Vitamin E in Rat Rod Outer Segments During Light Damage

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Previous evidence suggests that lipid peroxidation may initiate photoreceptor damage induced by constant light exposure. In order to investigate the role of the antioxidant vitamin E in light damage, Long-Evans (pigmented) rats were atropinized and exposed to constant fluorescent light (Vita-Lite) of 10–20 foot candles for intervals up to 5 days. Following light exposure, retinal rod outer segments (ROS) were prepared and their lipids extracted. Retinas processed in parallel for morphological examination showed progressive ROS deterioration and selective loss of photoreceptor cells at 3 and 5 days of constant light. Similar to previous observations in undilated albino rats, constant illumination resulted in the specific loss of docosahexaenoic acid (22:6\alpha-3) in the ROS. A novel finding in this study was an increase in the content of vitamin E relative to lipid phosphorus, stearic acid, and docosahexaenoic acid in the ROS of constant light-exposed animals. Invest Ophthalmol Vis Sci 27:727-733, 1986

Retina and rod outer segments (ROS) have a greater percentage of their fatty acids as docosahexaenoic acid (22:6\alpha-3) than most other tissues.\(^1\) This polyunsaturated fatty acid is highly susceptible to free radical-induced lipid peroxidation.\(^3\) Several laboratories have presented evidence that lipid peroxidation is associated with, and may play a causal role in, retinal degeneration due to constant illumination. Lipid hydroperoxides were reported to be increased in the retinas of dark-adapted frogs exposed for 30 min to 100 foot-candles (ft-c) of light.\(^4\) We have shown an increase in conjugated dienes associated with the disappearance of 22:6\alpha-3 in isolated ROS from retinas of albino rats exposed to 100–125 ft-c of fluorescent illumination.\(^5\) Kagan et al\(^6\) described an increase in lipid hydroperoxides in retinas of albino rats 24 hr after exposure to 900 ft-c for 3.5 hr. Organisciak et al\(^7\) reported an increase in water soluble peroxydes in retinas of albino rats exposed to 400 ft-c of visible light for 1 hr.

Antioxidants such as vitamin E protect polyunsaturated fatty acids from free radical-induced peroxidation. Since the retina and especially the ROS have high levels of vitamin E,\(^8\) it is possible that this compound may protect the retina from degeneration. Indeed, Hayes\(^10\) observed a decade ago that monkeys deficient in vitamin E developed a macular degeneration characterized by a bilateral, central disruption of the photoreceptor cells. Riss et al\(^11\) made a similar observation in vitamin E-deficient dogs. Albino rats maintained on vitamin E-deficient diets for 20–34 wk underwent a progressive loss of photoreceptor cells. In another study, albino rats deficient in selenium and vitamin E exhibited marked disruption of photoreceptor outer segment membranes, loss of photoreceptor cells, and an increase in lipofuscin granules in the pigment epithelium.\(^13\)

The relationship between vitamin E and retinal degeneration due to constant illumination is not clear. Kagan et al\(^6\) reported a greater decrease in electroretinogram (ERG) amplitudes in vitamin E-deficient albino rats 24 hr after exposure to 900 ft-c for 3.5 hr, compared to vitamin E-supplemented controls. However, Stone et al\(^14\) and Sykes et al\(^15\) showed no effect of vitamin E-deficiency on the susceptibility of albino rats to light damage. Joel et al\(^16\) reported that the total quantity of vitamin E in retina of albino rats transiently decreased with 2 days of constant illumination at 680 ft-c, but returned to control values by day 5. In a recent report, Hunt et al\(^17\) demonstrated that albino rats exposed to constant light (200–250 ft-c) for only 24 hr did not show any significant alteration in the vitamin

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E content of whole retina or ROS when compared to cyclic light controls. The present study was undertaken to investigate the dynamics of polyunsaturated fatty acids and vitamin E in ROS of pigmented rats during exposure to constant light. Pigmented rats were utilized instead of albino rats to minimize the effects of spontaneous light damage to control animals. A preliminary report of this study has been presented.18

Materials and Methods
Female Long-Evans (pigmented) rats weighing between 180–200 grams (Simonsen Laboratories; Gilroy, CA.) were acclimated for at least 2 wk in our vivarium under cyclic light (12 hr light/12 hr dark) of intensity less than 2 ft-c (as measured with a Tektronix J16 Photometer [Beaverton, OR] using a cosine corrected remote illuminance probe) at the inside front of the cage nearest the fluorescent overhead room lights. On the first day of exposure, both eyes of each animal were dilated with a topical application of 2% atropine sulfate, and the animals were placed in constant light chambers. The light chambers are plexiglass cylinders (8 in diam) that have been sandblasted to diffuse incoming light. The floor of each cylinder is wire mesh (0.25 in). Droppings are collected in pans below the animal’s field of view. Ten cylinders are set in a horizontal plexiglass sheet in a circular pattern surrounded by a white formica reflecting surface. A central bank of five Vita-Lite fluorescent 40 watt lamps (Duro-Test Corp.; North Bergen, NJ) serves as the light source. The spectral energy distribution of the Vita-Lite corresponds closely to that of natural outdoor light. Each lamp is covered with a thin black mesh screen to attenuate light intensity to the desired level. The side of each cage facing the light also is covered with a thin black mesh screen. This lighting configuration results in an illumination range of 10–20 ft-c in all possible directions within the cylinders. The temperature within the cylinders is 20 ± 1°C.

Rats were exposed for 0, 2, 3, 4, or 5 days under the above conditions and were allowed access to water and food ad libidum. Rats were atropinized every 2 days to maintain full pupil dilation throughout the course of an experiment. In order to minimize variation, animals were placed in the light chamber at different times so that biochemical analysis could be carried out the same day on retinas from animals exposed to various periods of constant illumination. Rats maintained under cyclic light in our vivarium but not treated with atropine served as controls. The use of rats in the present study conforms to the ARVO Resolution on the Use of Animals in Research.

Retinal dissection, preparation of ROS membranes, and all biochemical analyses of lipid and protein components were performed under typical laboratory lighting (30–50 ft-c). All solutions were saturated with argon to minimize lipid peroxidation during membrane preparation and lipid extraction procedures.

ROS membranes were isolated by discontinuous sucrose gradient centrifugation. Retinas from the left and right eyes of individual rats were dissected in mammalian Ringers, pooled, and homogenized gently in 2 ml of 1.175 g/ml sucrose buffered with 10 mM Tris-acetate (pH 7.4) containing 70 mM NaCl, 2 mM MgCl₂, and 0.1 mM EGTA. Homogenization was achieved by five strokes of a motor-driven (1600 rpm) teflon pestle (#S20) in a glass homogenization tube (#S30, TRI-R Instruments; Inc.; Rockville Centre, NY). Clearance between pestle and tube was 0.11–0.15 mm. The homogenate was transferred to a 5 ml cellulose nitrate centrifuge tube and overlaid sequentially with 2 ml of 1.140 g/ml sucrose, followed by 1 ml 1.115 g/ml sucrose. These latter two sucrose solutions were buffered with 10 mM Tris-acetate (pH 7.4) and contained 0.2 mM MgCl₂ and 0.1 mM EGTA. The sucrose density gradient tube was spun at 82,000 x g for 1 hr at 4°C. The sucrose interfacial band (1.115/1.140) containing the ROS membranes was removed and lipids were extracted with chloroform–methanol partitioning of the aqueous suspension according to the procedure of Bligh and Dyer.19 The chloroform extract (lipid phase) was washed once with 0.2 volume of the theoretical Folch upper phase.20 The lipid phase was made to a known volume with chloroform and aliquots taken for (1) determination of vitamin E content by high-pressure lipid chromatography (HPLC), (2) fatty acid methyl ester determination by gas-liquid chromatography (GLC), and (3) quantitation of lipid phosphorus.21 In several experiments, aliquots of the ROS preparation were evaluated for purity by polyacrylamide gel electrophoresis.22

Vitamin E was measured by a high pressure liquid chromatography method modified from that of Bieri et al.23 To the aliquot taken for vitamin E analysis, a known amount of vitamin E acetate was added to serve as an internal standard. Butylated hydroxytoluene (BHT, 200 µg) was added and the mixture evaporated to dryness under argon and immediately taken up in 100% methanol and injected on a 5-µm Lichrosorb 5RP8 column (Spectra–Physics; Santa Clara, CA.). Separation of vitamin E and vitamin E acetate was achieved with a mobile phase of methanol–water (96: 4 v/v) at a flow rate of 2.0 ml per min and monitored at 288 nm with a Kratos Spectra flow 773 (Ramsey, NJ) set at 0.001 absorbance units full scale. Vitamin
Fig. 1. Morphological changes in the retina of Long-Evans rats exposed to constant light. Immediately following light exposure, retinas were dissected in Ringer's in a manner identical to that for the ROS isolation and were transferred to 2.5% glutaraldehyde-1% formaldehyde fixative. The sections for each time point were taken from the central superior region of the retina. a, cyclic light, b, 3 days constant light, and c, 5 days constant light (all, ×1080).

E absorbs maximally at 292 nm and vitamin E acetate at 284 nm. Mixtures of known amounts of these two compounds dissolved in methanol were run routinely at 288 nm to establish detector response factors that could be used to quantitate the levels of vitamin E in our samples. This procedure can quantify as little as 5–10 ng of vitamin E per injection.

A known amount of heneicosanoic acid (21:0) and 200 μg BHT were added to the aliquot taken for fatty acid methyl ester analysis. Methyl esters were prepared with 14% BF₃-methanol and purified on silica gel HR plates using the solvent system hexane:ethyl ether (70:30, v/v). The methyl ester fraction was recovered from the plates and quantitated by GLC using the 21:0 internal standard as described previously.

Retinas from each exposure time were taken also for histological examination by light microscopy. Immediately following light exposure, retinas were dissected in mammalian Ringers in a manner identical to that for the ROS isolation and subsequently transferred to 2.5% glutaraldehyde and 1% formaldehyde buffered to pH 7.35 with 0.09 M sodium cacodylate. After an initial fixation period of 2 hr, the tissue was rinsed several times in cacodylate buffer and postfixed in 1% OsO₄ for 1 hr. This was followed by dehydration in a graded ethanol series, clearing with propylene oxide, and embedding in Epon–Araldite. Orientation of retinal areas according to quadrants was determined during initial dissection of the retina from the eye cup. Thick sections (1-μm) were mounted on glass slides and stained with toluidine blue for light microscopy.

Results

Histological examination of the retinas of pigmented rats exposed to constant light showed a time-dependent photoreceptor degeneration when the pupil was maintained at maximal dilation with atropine. Figure 1 shows the progression of photoreceptor degeneration in dissected retinas following 3 (Fig. 1b) and 5 (Fig. 1c) days of constant light compared to cyclic light controls (Fig. 1a). Three days of illumination (Fig. 1b) resulted in degenerative changes in the photoreceptor cells of the superior retina including nuclear pyknosis, outer segment disorganization, and darkening of cytoplasmic regions presumably due to necrosis. Other quadrants of the retina showed these same morphological changes to a lesser degree. After 5 days exposure, there was a generalized damage to photoreceptor cells throughout the retina, and the outer nuclear layer in
the superior region (Fig. 1c) was reduced in thickness by about one-third, indicating the destruction and removal of photoreceptor cells. Outer segments in the superior retina were reduced to only a small fraction of their original length. After 5 days, there was no region of the retina that did not show pyknotic nuclei and outer segment disruption.

ROS were prepared from retinas that were removed from the eyecup. No attempt was made to recover the debris that accumulated in the subretinal space. The yield of ROS membranes per retina recovered by gradient centrifugation declined with length of time of constant illumination and by 5 days was 50% of that from retinas of the cyclic light controls. The decreased yield of membranes may be due in part to degeneration and in part due to loss of lipid from the membranes, which resulted in increased membrane density and subsequent alternation of flotation properties on the sucrose gradient. We have observed an increase in membrane yield at the 1.140/1.170 g/ml sucrose interface of light-damaged retinas as well as an increase in the relative amount of a protein with the molecular weight of opsin.

The polyacrylamide gel tracings of the ROS membranes shown in Figure 2 demonstrate that opsin was the dominant protein, comprising 85–90% of the proteins staining with Coomassie blue. As previously reported for albino rats whose retinas were damaged at more intense illumination, we observed that a protein in the range 53K–55K molecular weight binds to ROS membranes of both control and light-damaged rats during isolation. It is clear from these electrophoretic patterns that the ROS membranes prepared after 3 or 5 days of constant illumination and analyzed for lipid changes were not significantly contaminated with other membranes.

Table 1 shows that constant illumination did not affect the concentration of stearic acid (18:0) in ROS phospholipids as illustrated by the similar ratios of 18:0 to lipid phosphorus. Since stearic acid is not susceptible to peroxidation, the ratio of this fatty acid to lipid phosphorus remained constant over five days of continuous illumination. In sharp contrast, the ratio of 22:6ω3 to lipid phosphorus and the ratio of 22:6ω3 to 18:0 decreased after 5 days of constant light. The latter ratio is more accurate, since values for 18:0 and 22:6ω3 were obtained from the same chromatogram. There was no apparent loss of arachidonic acid (20:4ω6), the second most abundant polyunsaturate in ROS, as evidenced from no change in the ratio of 20:4ω6 to 18:0.

Table 2 shows that the vitamin E in the ROS membranes, expressed as the mole ratio of vitamin E to lipid phosphorus, increased at day 5 to a level significantly higher than that present in cyclic light controls (P < 0.005). Also shown in Table 2 are the mole ratios of vitamin E to 18:0 and 22:6ω3, respectively. Vitamin E was elevated relative to these two fatty acids at day 5 when compared to the cyclic light control.

Discussion

These studies demonstrate that pigmented rats with dilated pupils may be readily light damaged with rel-
E does not act in ROS as a free radical scavenger. The presumptive that either lipid peroxidation is not a factor in the reactions involved in light damage. This is supported by observations of vitamin E levels in light-stressed photoreceptor membranes. First, vitamin E may not participate in the reactions involved in light damage. Second, vitamin E is not scavenged by vitamin E present in large concentrations in photoreceptor outer segments.

An unexpected finding of the present study was that vitamin E in ROS did not decrease during the course of the degeneration, but rather appeared to increase at day 5 to levels significantly higher than those present in the cyclic light controls. During this time, however, there was a clear loss of 22:6 relative to vitamin E. There are several possible explanations for the conservation of vitamin E levels in light-stressed photoreceptor membranes. First, vitamin E may not participate in the reactions involved in light damage. This would not be scavenged by vitamin E present in large concentrations.

### Table 1. Fatty acids in rod outer segments of Long-Evans rats following constant light

<table>
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<tr>
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<tbody>
<tr>
<td>Cyclic (10)</td>
<td>0.45 (0.03)</td>
<td>0.89 (0.09)</td>
<td>0.147 (0.017)</td>
<td>1.96 (0.20)</td>
</tr>
<tr>
<td>2-day-CL (7)</td>
<td>0.43 (0.01)</td>
<td>0.86 (0.08)</td>
<td>0.166 (0.016)</td>
<td>1.98 (0.22)</td>
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<tr>
<td>4-day-CL (4)</td>
<td>0.44 (0.02)</td>
<td>0.87 (0.02)</td>
<td>0.151 (0.003)</td>
<td>1.95 (0.10)</td>
</tr>
<tr>
<td>5-day-CL (9)</td>
<td>0.45 (0.03)**</td>
<td>0.66 (0.18)§</td>
<td>0.146 (0.048)**</td>
<td>1.45 (0.34)§</td>
</tr>
</tbody>
</table>

* The retinas from the left and right eye of individual rats were pooled. Cyclic: cyclic light (12L:12D) in vivarium; 2-, 4-, or 5-day-CL: days of constant light (CL). Number of independent determinations is indicated in parenthesis. † Mole ratio (mol/mol) of 18:0 to 22:6 to lipid phosphorus (LP) and the mole ratio (mol/mol) of 20:4 to 22:6 to 18:0 in the ROS membranes. Values are reported as mean (SD). Statistical comparison was made using a two-tailed student t-test. Significantly different from cyclic: ¥P < 0.002, §P < 0.001, **not significant.

At low intensity fluorescent light. Changes in retinal morphology were evident by 3 days. As reported previously by others,23 the earliest changes were most notable in the photoreceptors of the superior retina, although after 5 days of constant illumination, degenerative changes were evident in photoreceptors across the entire retina. The outer segments were distended and fragmented. However, when the retina was dissected free from the pigment epithelium, much of the outer segment material remained attached to the inner segment. ROS prepared from retinas of animals exposed for 3 or 5 days to constant illumination had similar SDS polyacrylamide protein patterns as the cyclic light controls.

An unexpected finding of the present study was that vitamin E in ROS did not decrease during the course of the degeneration, but rather appeared to increase at day 5 to levels significantly higher than those present in the cyclic light controls. During this time, however, there was a clear loss of 22:6 relative to vitamin E. Hunt et al17 exposed albino rats to light of 250 ft-c for 24 hr. Although they did not report data for fatty acids, they observed that the vitamin E concentrations in photoreceptor outer segments were significantly different from cyclic.

Whether lipid peroxidation is causal in light-induced retinal degeneration has not been proven. However, there is evidence that lipid peroxidation occurs during light damage to the retina.4-7 Based on the known antioxidant properties of vitamin E, it seems highly unlikely that free radicals generated in the lipid bilayer would not be scavenged by vitamin E present in large concentrations in photoreceptor outer segments.

The second possible explanation for our results is that during the course of light damage vitamin E is destroyed while providing protection against lipid peroxidation reactions, but is replaced. The light-stressed outer segments may sequester vitamin E from other retinal cells, the pigment epithelium, or the retinal circulation to maintain vitamin E at levels found prior to light stress.

A third possible explanation for our results is that vitamin E does scavenge unpaired electrons from free radicals generated in the light-damaged photoreceptor lipids, but is regenerated by transferring the unpaired electron to a second antioxidant such as ascorbic acid.

### Table 2. Vitamin E in rod outer segments of Long-Evans rats following constant light

<table>
<thead>
<tr>
<th>Light Group*</th>
<th>Vit E/LP</th>
<th>Vit E/18:0</th>
<th>Vit E/22:6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic (10)</td>
<td>1.72 (0.18)</td>
<td>3.80 (0.38)</td>
<td>1.94 (0.31)</td>
</tr>
<tr>
<td>2-day-CL (7)</td>
<td>1.64 (0.42)</td>
<td>3.79 (1.04)</td>
<td>1.93 (0.62)</td>
</tr>
<tr>
<td>4-day-CL (4)</td>
<td>1.89 (0.18)</td>
<td>4.22 (0.17)</td>
<td>2.15 (0.23)</td>
</tr>
<tr>
<td>5-day-CL (9)</td>
<td>2.24 (0.48)§</td>
<td>5.02 (1.46)§</td>
<td>3.75 (1.86)**</td>
</tr>
</tbody>
</table>

* The retinas from the left and right eyes of individual rats were pooled. Cyclic: cyclic light (12L:12D) in vivarium; 2-, 4-, or 5-Day-CL: days of constant light (CL). Number of independent determinations is indicated in parenthesis. † Mole ratio (mmol/mol) of vitamin E (Vit E) to lipid phosphorus (LP) in the ROS membranes. Vitamin E was measured on the same ROS preparation used to quantitate fatty acids.

Statistical comparison was made using a two-tailed student t-test. Significantly different from cyclic: ¥P < 0.002, §P < 0.001, **not significant.
Changes in ascorbic acid have been observed in light damage. Organisciak et al.31 showed that the levels of ascorbic acid decreased in the retina of albino rats during constant illumination. The injection of ascorbic acid albino rats prior to light stress decreased the loss of photoreceptor cells32 and 22:6omega-33 in the retina compared to noninjected controls. In addition, Tso and colleagues observed loss of ascorbic acid from the retina compared to noninjected controls. In addition, Tso and colleagues observed loss of ascorbic acid from the retina of albino rats prior to light stress decreased the loss of photoreceptor cells32 and 22:6omega-33 in the retina compared to noninjected controls. Moreover, guinea pigs made deficient in ascorbic acid were found to be more susceptible to light damage than controls.36 These data would indicate that ascorbic acid decreased in the retina of albino rats during constant illumination. This suggestion is strengthened by in vitro studies showing that ascorbic acid acts synergistically with vitamin E to prevent lipid peroxidation37 and that oxidized vitamin E may be regenerated to the reduced state by ascorbic acid.38

Tappel39 suggested in 1968 that vitamin E, ascorbic acid, and glutathione act synergistically as redox agents to terminate lipid peroxidation in cells. Since all three compounds are present in relatively large amounts in the retina, it seems reasonable to suggest that they may serve a similar function in this tissue. Thus, in studies on light damage and other retinal degenerations that may involve lipid peroxidation, it is necessary to consider the overall antioxidant potential of the tissue.

Key words: retinal degeneration, photoreceptor membranes, light damage, vitamin E, docosahexaenoic acid, lipid peroxidation.

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References

27. Rapp LM and Williams TP: A parametric study of retinal light damage in albino and pigmented rats. In The Effects of Constant


