Effect of Chronic Near-Ultraviolet Radiation on the Gray Squirrel Lens In Vivo

Seymour Zigman,* Teresa Poxhio,* Thurma McDaniel,* Marjorie F. Lou,† and Nai-Teng Yu‡

The effects of ambient exposure to near-ultraviolet (near-UV) radiation (300–400 nm) on the ocular lens of the diurnal squirrel (Sciurus carolinensis) are reported. Gray squirrels lived in cages illuminated for 12 hr a day with near-UV light (6 mW/cm², 365 nm) for 1 yr. The non-UV-exposed controls were housed separately. In the lenses of UV-exposed animals, anterior pole changes occurred. Central epithelial cells swelled, disappeared, or underwent proliferation. A band of disoriented degenerating fiber cells was seen in the midcortex, with a degree of liquefaction. When lens protein compartments were separated by centrifugation, water-insoluble but urea-soluble fractions were enhanced in the outer and inner cortex and the nucleus. Both high-performance liquid chromatography and polyacrylamide gel electrophoresis revealed that proteins mainly in the midcortex and nucleus were altered considerably. Evidence of a loss of sulfhydryl compounds (by chemical and Raman spectroscopic analyses) and an increase of protein–thiol mixed disulfides (chemically) was also observed. These data prove that repetitive ambient exposure of diurnal animals to near-UV radiation at subsolar levels damages the lens by interfering with the maintenance of epithelial cells and altering the structural proteins; some of this may be due to the conversion of sulfhydryls to mixed disulfides. Invest Ophthalmol Vis Sci 32:1723–1732, 1991

In several previous publications, the biochemistry of squirrel lens proteins and pigments and photochemically induced damage to the proteins and salt balance were described. Although the in vitro experiments provided firm evidence that near-ultraviolet (near-UV) light induced damage, no in vivo experiments using diurnal animals have to date (to our knowledge) provided equally firm evidence. Only in nocturnal animals has in vivo data supported an important role of near-UV radiation in cataract formation. This report provides significant data to prove that near-UV radiation provided repetitively over a long duration—and at lower overall UV radiation levels than those of sunlight—alters lens structure and chemistry and contributes to cataract formation.

Materials and Methods

Animals and UV exposure

Gray squirrels (Sciurus carolinensis) were trapped alive under a New York State license (Department of Environmental Conservation) and housed and maintained in a vivarium as dictated by University Committee on Animal Research agreement (National Institutes of Health). These investigations also conformed to the ARVO Resolution on the Use of Animals in Research. The animals were fed Purina rat chow (Purina Mills Inc., St. Louis, MO) plus assorted fresh vegetables and shelled nuts and seeds.

For UV exposure, the animals were housed individually in mesh cages (dimensions, 11½ × 10½ × 22 inches) placed in Quonset-hut enclosures that were illuminated by 34 BL (black light) (Sylvania-GTE Inc., Danvers, MA) 40-W fluorescent tubes. The radiation level at 365 nm was 6.8 mW/cm² at the position where the squirrel’s eye would normally be. This exposure was continued for 12-hr/day for 454 days, during which the daily exposure was 294 J/cm², and the total exposure was 133,368 J/cm². At the termination of the experiment, the animals were killed humanely by phenobarbital overdose. The controls were kept in ordinary animal room lighting, which consisted of ceiling cool-white fluorescent lighting that was turned...
on for 12 hr a day at the same time that the UV lamps were on for the experimental animals. No UV radiation was detected in the control squirrel cages.

The eyes were enucleated, and the lenses were removed promptly and photographed using a slit lamp. The lenses of six squirrels (three control and three experimental animals) were used for biochemical studies and those of four animals for histology.

Histology

The lenses were fixed at room temperature in 2% glutaraldehyde and 1% paraformaldehyde in 0.05 M phosphate buffer, pH 7.3, with 0.2% tannic acid, 7.3 mM potassium chloride, and 2 mM calcium chloride added. After 1 hour, this fixing solution was changed to 4% paraformaldehyde in 0.05% phosphate buffer, pH 7.3, for 3 days. The lenses were then bisected, infiltrated with glycol methacrylate, and embedded in LR White acrylic (Polysciences, Inc., Warrington, PA) resin in a 60°C oven. Four-micron sections were cut and stained using a 0.2% methylene blue/0.2% azure II stain/0.1% basic fuchsin in 25% methanol, and the periodic acid-Schiff, Feulgen, and alcoholic-fast green reactions.

Biochemical Preparations

The lenses for biochemical studies were removed and weighed, and then the capsule and epithelium was removed; the outer cortex, inner cortex, and nucleus were separated. The tissues were kept on ice throughout. A microscope was used to dissect the lens layers. The outer cortex was peeled off using spatulas; the remaining lens material was placed in a vial with buffer and a stirring bar and magnetically stirred for 15 min. The inner cortex was in suspension, and the nucleus remained intact. By this method, the nucleus represented 33% of lens’s wet weight. All fractions were homogenized with a Dounce homogenizer in 0.10 M phosphate, 0.5 mM ethylenediaminetetraacetic acid, pH 7.4, and (except for the capsule epithelium) were spun at 16 psi for 10 min using an A-95 rotor in a Beckman airfuge (Beckman Instruments, Richmond, CA). The capsule epithelium was spun at 22 psi for 8 min in a Beckman 18° A 100 rotor in a Beckman air centrifuge. The total soluble proteins (TSP) in the supernatant were removed, and the water-insoluble fraction (pellet) was extracted with 8 M urea diluted to 4 M and spin to obtain urea-soluble and -insoluble (membrane) fractions. Protein levels were determined by the Lowry procedure.

High-Performance Liquid Chromatography (HPLC)

The TSP fractions (30 µg per injection) were separated using a HPLC gel-filtration column (a Bio-Sil TSK-250, Biorad Laboratories, Richmond, CA; 300 x 7.5 mm) and matching guard column (75 x 7.5 mm). The buffer used was 0.10 M phosphate with 0.10 M sodium sulfate and 0.05% sodium azide, pH 7.0, filtered and degassed. A flow rate of 0.40 ml/min was obtained using the Rainin Rabbit 5-ml pump head (Rainin Dist. Co., Woburn, MA). Protein was detected at 280 nm using the LKB 2238 UVICORD SII detector (LKB Biotechnology, Bromna, Sweden). Chromatograms were collected and analyzed using a Shimadzu CR3A Chromatopac (Shimatzu Corp., Kyoto, Japan).

Antibody Preparation

Squirrel TSP was separated on Sephadex G-200 (Pharmacia Corp., Upprada, Sweden); alpha and beta, underwent chromatography again on a G-200 and gamma on a G-75 column. The rabbits were immunized with the purified crystallins to produce antibodies.9

Polyacrylamide Gels

The gels were made using the Bio-Rad MINIPROTEAN II dual-slab cell system (Biorad Laboratories, Richmond, CA). We used 15% acrylamide resolving gels with a 5% stacking gel and a running time of 1.5 hr with the voltage held constant at 200 V. The buffers and solutions were made and used according to Bio-Rad procedures.10 The protein samples usually applied were 5 µg protein/well.

Immunoblotting

Electrophoretic transfer to nitrocellulose sheets was done using the MINI TRANS BLOT system (Bio-Rad) for 1 hr at a constant voltage (100 V). Immunoblots were obtained by using the Bio-Rad IMMUNOBLOT (goat anti-rabbit and horseradish peroxidase) kit. Blots were done on the TSP fraction of all three lens layers. Primary antibody dilutions ranged from 1:200–1:400.

Sulfur Chemistry

Free and protein-bound sulfur-containing compounds were separated initially by precipitating the lens proteins in the homogenate with 10% trichloroacetic acid. Both glutathione and cysteine protein mixed disulfides in epithelium and outer cortex and in inner cortex and nucleus of the lens were separated by ion-exchange chromatography and quantified by methods previously described.10 The glutathione (GSH) and total sulfhydryl (SH) were measured by a modified method of Ellman.11 The Raman measurements of hydroxy (OH) and
SH through the lens were made at an excitation wavelength of 514.5 nm using an argon laser (power, 10 mW) in the laboratory of Dr. T. Yu (assisted by Ms. M-Z Cai). For SH determination, the stretching vibrational mode was observed at 2578 cm\(^{-1}\), and for protein, it was at 2731 cm\(^{-1}\). For OH the value was 3417 cm\(^{-1}\).

**Results**

**Morphology**

When freshly removed squirrel lenses were photographed with a Zeiss stereoscopic microscope (Carl Zeiss, Inc., West Germany), at low magnification, the results in Figure 1 were obtained. Anterior shallow, diffuse, cortical opacities and mild light scattering were observed in a frontal view (Fig. 1A) and by slit lamp (Fig. 1B).

**Histology**

Although control squirrel lenses had evenly spaced, uniform epithelial cells anterior to the columns of uniform hexagonally arranged cortical fiber cells (Fig. 2A), the UV-exposed squirrel lenses had numerous defects. Occasional swollen (Fig. 2B), missing, and proliferative epithelial cells (Fig. 2C) were seen anterior to slightly swollen superficial cortical fiber cells which were interrupted with occasional vacuoles. The

---

Fig. 1. The anterior surfaces of squirrel lenses to demonstrate the anterior central subcapsular opacities that resulted from ambient near-UV radiation (BLB lamps at 6 mW/cm\(^2\)) for 1 year. (A) Control. (B) UV-exposed (original magnification X25). Slit lamp view of the lenses. (C) Control (D) UV-exposed (original magnification X25).
midcortex had a band of degenerating fibers (Fig. 2D).
No disorganization was seen in the deep cortex, the
perinuclear, or the nuclear regions.

**Biochemistry**

Figure 3 shows the degree of aggregation of the TSP
fractions of control and UV-exposed squirrel lens
layers into water-insoluble proteins. In every layer,
the aggregated (water-insoluble) proteins were present
at greatly elevated levels (significant at $P = 0.05$ for
$n = 6$).

In Figure 4, the HPLC elution profiles of the TSPs
of control and UV-exposed animals of the outer and
inner cortex and nucleus are shown. The HPLC profiles
showed only the following subtle UV-induced
changes: a loss of the 32.9-min band in the outer cor-
tex, a loss of the same band in the inner cortex, a drop
in void volume material, and an increase of the
25.4-min material and a decrease of 37-min material
in the nucleus. Figure 5 provides the integrated per-
centage of the TSPs in the peaks of UV-exposed and
control squirrel lens layers. The integrator counts
shoulders in the area of a peak as a separate peak;
EFFECT OF CHRONIC NEAR-UV RADIATION / Zigman et al

Therefore the 37-min eluted material would be counted even if it was a shoulder. Data on six control and six UV-treated lenses were used to obtain this figure. In the outer cortex, decreases in peaks at approximate molecular weights of 130 and 37 kD were related to UV exposure. The void volume proteins were decreased in UV-treated lenses in the inner cortex and nucleus, and the 290- and 250-kD peaks were increased by UV exposure only in the nuclear layer. Significant increases in the nuclear protein peaks at 130 kD and increases at 60 kD in both lens layers were observed due to UV exposure. The 37-kD peaks decreased in both layers in the UV-treated squirrel lenses compared with the controls. The 22- and 18-kD bands and an increase in the 21-kD band. The <10-kD material represents free peptides, amino acids, and pigments in the lens, and these increased in the inner cortex of the UV-exposed lenses.

Figure 6 illustrates the alterations in the soluble lens crystallins resulting from near-UV exposure of the squirrels as observed by polyacrylamide gel electrophoresis (PAGE). No significant alterations in PAGE TSP protein profiles were observed in the epithelium (not included). A diminution (due to UV exposure) of the βH peptides with a molecular weight of 32 kD was seen in the outer and inner cortex; this did not occur in the nucleus. In the nuclear TSP, however, a decrease of 30-kD material was observed, and there was more diffuse heavy molecular weight peptide material. In the urea-soluble fractions of the outer and inner cortex, the noticeable changes due to UV exposure were additional diffuse staining in both higher molecular weight and lower molecular weight regions of the gels. In the nucleus, greater levels of diffuse staining in the high molecular weight regions of the gels were seen in the UV-exposed profile than in the control profile. An increase of peptide material of approximately 19 kD was also seen in the UV-exposed profiles of the nucleus. No changes in the urea-insoluble PAGE gels were significant, and they are not shown here.

Densitometric analysis of the TSP PAGE profiles are shown in Figure 7. These data were from six UV-exposed and six control lenses. No changes in the lens epithelial cell soluble proteins were significant at \( P < 0.05 \), and they are not shown. The outer cortical analysis showed decreases of 32-, 27-, and 19-kD bands and an increase of 28-kD material. In the inner cortex, UV exposure caused significant decreases in the 33- and 27-kD peptides and an increase in 28-kD peptides. The only statistically significant UV-induced changes in the TSP PAGE profile of the nuclear region was a decrease in the 19-kD band. Statistically significant \(( P < 0.05)\) PAGE findings in the urea-soluble extracts due to UV exposure were as follows. In the outer cortex, there were decreases were in the 49- and 25-kD bands and an increase in the 21-kD band. In the inner cortex there were increases in nonspecific staining at 46 to 37 kD and in the 17-kD regions of the gels. In the nucleus, increased staining at 46 to 37 kD and at 15 to 14 kD was observed; an 18-kD band also was absent.

There also were changes in the crystallins observed by immunoblotting (Fig. 8). Antibody to alpha in the outer cortex appeared as two bands at 23 and 21 kD. Some nonspecific staining also occurred in the β-crystallin region of the gel. No difference in alpha bands was seen in the outer cortex. In the inner cortex and the nucleus, alpha blots showed diminished 23-kD bands in the UV-exposed samples. Antibody to βH blots showed two equivalent bands at 32 and 30 kD in the outer cortex. The 32-kD band was reduced in the inner cortex, and in the nucleus, it was entirely absent. Antibody to βL blots contained strong anti-βL reactions, and these are not included here. The antibody to the gamma band appeared at 21 kD. In blotted samples of outer cortex and inner cortex, the 21-kD band was less distinct in the UV than the control samples. Greater immunologic reactivity occurred in the nucleus, but no changes due to UV exposure were found.

Definite changes in sulfur chemistry due to UV exposure were observed and are documented in Figure 9. As shown in Figure 9A, the total SH content in the outer layers and in the nucleus decreased in the UV-treated lenses. No changes were detected in the inner cortex. In the outer cortex, decreased SH groups were mainly those of proteins (Fig. 9B). The UV-induced
changes in protein-thiol mixed disulfides are shown in Figures 9C (cysteine protein mixed disulfide) and 9D (GSH protein mixed disulfide). Both cysteine and GSH protein mixed disulfides increased in the outer and inner lens layers. Although the cysteine protein mixed disulfide increased in the nucleus, the GSH protein mixed disulfide decreased. The overall balance of SH and disulfide was in the direction of a loss of SH and GSH and an increase of mixed disulfides.

When examined by Raman spectroscopy, the SH and OH contents were also affected (Table 1). The UV exposure led to a drop of SH groups both in the cortex and the nucleus. The OH, which represents water content, increased slightly in both portions of the lens. If the ratios of the contents of SH to OH were calculated, a definite drop in SH concentration (based on water content) was found in both the nucleus and cortex.

Discussion

This report shows that long-term, broad-band ambient near-UV radiation at lower levels than found in sunlight can lead to cataracts in the diurnal gray squirrel. It also supports and extends the findings of previous studies using nocturnal species,\textsuperscript{7,8} photochemical methods,\textsuperscript{12-14} and in vitro procedures.\textsuperscript{4,6} These show that there are many target sites where damage can occur.

One target appeared to be the central lens epithelial cells and subcapsular cortex. Swollen epithelial cells and a band of fiber degeneration were observed histologically. We found well-defined opacities which appeared to be related to epithelial cell failure to control water balance by Na/K adenosine triphosphatase. This conclusion is supported by in vitro findings from our laboratory.\textsuperscript{5,6}
Other targets appeared to be the crystallins themselves, although natural sensitizers (tryptophan and kynurenine) may play a role in their UV-induced alterations. Direct absorption of UVA by the crystallins is slight. That UV exposure causes crystallin cross-linking relative to singlet-oxygen production has been shown.\(^\text{15}\)

We found that UV exposure stimulated increased levels of water-insoluble proteins when comparing samples from lens periphery with the nucleus. As the lenses were growing throughout the experiment, it is not surprising to find an accumulation of the cross-linked proteins toward the nucleus, even though the major UV absorption takes place in the anterior cortex. Only about 6% of UVA reaches the squirrel lens nucleus.

The HPLC data showed that statistically significant changes in the levels of crystallins occur more frequently near the nucleus. Degradative changes in the crystallins and a buildup of peptides with molecular weights lower than 20 kD were observed, particularly in the inner cortex. The PAGE of the lens proteins also found subtle alterations. Because PAGE requires the proteins to be denatured using sodium dodecyl sulfate and dithiothreitol, some bonding interactions between the protein chains that may have been induced by UV could have been broken by these reagents. The most significant changes observed in the soluble proteins were enhancement of a 28-kD chain and decreases in 27- and 19-kD chains in the outer and inner cortex.

By PAGE, most of the UV-induced alterations of soluble proteins were found in the outer cortical and inner cortical regions. No significant changes in PAGE data for the urea-insoluble fraction were seen, and no presentation of these gels was made. However, in the urea-soluble fraction, the greatest protein...
changes appeared in the inner cortical and nuclear regions of the lens. Aside from the increases and decreases of particular protein species, there was evidence of an increase in crosslinking and degradation of crystallins. The differences between the results of HPLC and PAGE analysis may be due to the effects of the denaturing agents used only during electrophoresis.

Immunoblotting also revealed small changes in the levels of soluble crystallins due to ambient near-UV exposure. Some nonspecific staining appeared in the 32 to 30-kD regions of the anti-α and -γ blots, which identify βγ crystallins. This is probably a result of incomplete separations of the crystallins to be used for antibody production by gel-filtration columns. In the βΓ blots, there was an abundance of reactions to βγ bands, and only small reactions with the 23- and 25-kD βs. No changes due to UV exposure were found, and these data were not presented. After UV exposure, notable decreases in the higher molecular weight band of βγ crystallins (ie, 32 kD) were seen in samples from all three regions examined. It is possible that reduction of the 32-kD βγ and the 23-kD band indicates that they became part of the aggregated protein pool.

To determine whether interactions of SH and disulfide compounds in the squirrel lens were influenced by UV exposure in vivo, measurements of compounds with SH groups and the presence of disulfide bonds and mixed disulfides in several regions of the lens were made. A major loss of GSH and total SH in the outer portion of the lens was observed; in the same lens layers, both GSH and cysteine mixed disulfides increased.
Lanes 1 & 2: Outer Cortex, 1=C, 2=UV. Lanes 3 & 4: Inner Cortex, 3=C, 4=UV. Lanes 5 & 6: Nucleus, 5=C, 6=UV.

Fig. 8. Immunoblots of the PAGE gels shown in Figure 6.

Fig. 9. Sulfur chemistry of the squirrel lens of (A) total SH, (B) GSH, (C) GSH-protein mixed disulfide, and (D) cysteine-protein mixed disulfide. epi = lens epithelium; OC = outer cortex; IC = inner cortex; N = nucleus. Solid bars are the control, and filled or hatched bars are the UV-exposed values. Limit bars represent standard errors.
Table 1. Raman spectroscopic analyses of SH and OH in squirrel lenses

<table>
<thead>
<tr>
<th>Squirrel lenses</th>
<th>Normal</th>
<th>UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mg)</td>
<td>129.7</td>
<td>126.3</td>
</tr>
<tr>
<td>Laser focused point</td>
<td>102.9</td>
<td>102.2</td>
</tr>
<tr>
<td>SH 1-2578/1-2730</td>
<td>1.17</td>
<td>1.31</td>
</tr>
<tr>
<td>OH 1-3417/1-2936</td>
<td>0.37</td>
<td>0.48</td>
</tr>
<tr>
<td>Ratios of SH/OH</td>
<td>3.16</td>
<td>2.73</td>
</tr>
</tbody>
</table>

By comparing the data points above, the intensity ratio for both SH and OH of cortex are greater than nucleus. Comparison of the SH concentration of normal and UV-affected lenses: UV-affected lens has less SH concentration for both nucleus and cortex than the normal lens. Comparison of the OH concentration of normal and UV-affected lenses: UV-affected lens has greater OH concentration for both nucleus and cortex than the normal lens.

Raman spectroscopy showed that signal intensities of SH and OH of the cortex were greater than those of the nucleus. The SH intensity became lower in the nucleus and cortex of UV-exposed lenses than in the control lenses. Thus, the SH concentration based on water content (ie, OH content) was decreased due to UV exposure. This result is similar to that reported earlier for UV effects on the mouse lens.16

Therefore UV exposure created conditions in the squirrel lens that rendered the SH groups more reactive or oxidizable. This was probably not due to direct UV radiation effects on the SH groups because they do not absorb light at 365 nm. Sensitized photochemical reactions are thus indicated. Substances such as superoxide anion, singlet oxygen, and other unidentified radicals generated in the lens by light17 could oxidize the SH groups of cysteine, glutathione, and proteins and cause them to be linked with disulfide bonds.18 Another contributing factor may be a reduction in the activity of lens glutathione reductase; this is inactivated under in vitro conditions by UV light exposure.19

Key words: near UV light, squirrel lens, in vivo, cataract, lens proteins

References