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Measurement of Lens Protein Aggregation *in Vivo* Using Dynamic Light Scattering in a Guinea Pig/UVA Model for Nuclear Cataract

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Abstract

The role of UVA radiation in the formation of human nuclear cataract is not well understood. We have previously shown that exposing guinea pigs for 5 months to a chronic low level of UVA light produces increased lens nuclear light scattering and elevated levels of protein disulfide. Here we have used the technique of dynamic light scattering (DLS) to investigate lens protein aggregation *in vivo* in the guinea pig/UVA model. DLS size distribution analysis conducted at the same location in the lens nucleus of control and UVA-irradiated animals showed a 28% reduction in intensity of small diameter proteins in experimental lenses compared with controls ($P < 0.05$). In addition, large diameter proteins in UVA-exposed lens nuclei increased five-fold in intensity compared to controls ($P < 0.05$). The UVA-induced increase in apparent size of lens nuclear small diameter proteins was three-fold ($P < 0.01$), and the size of large diameter aggregates was more than four-fold in experimental lenses compared with controls. The diameter of crystallin aggregates in the UVA-irradiated lens nucleus was estimated to be 350 nm, a size able to scatter light. No significant changes in protein size were detected in the anterior cortex of UVA-irradiated lenses. It is presumed that the presence of a UVA chromophore in the guinea pig lens (NADPH bound to zeta crystallin), as well as traces of oxygen, contributed to UVA-induced crystallin aggregation. The results indicate a potentially harmful role for UVA light in the lens nucleus. A similar process of UVA-irradiated protein aggregation may take place in the older human lens nucleus, accelerating the formation of human nuclear cataract.

Introduction

Cataract, an opacity of the lens of the eye, is the most frequent cause of vision loss in people (1,2), accounting for approximately 50% of all blindness worldwide (3,4). Nuclear cataract, an opacification in the center of the lens, is the most common type in older adults (5,6), and the type most likely to require surgery (3). In the United States, an episode of cataract surgery costs around \$2600 (7). With more than 1.35 million cataract operations performed annually (8), this equates to a total cost of more than \$3.4 billion nationwide (7,9). Finding a means of delaying or preventing maturity-onset human cataract is a major goal of lens and cataract research.

Although the etiology of cataract is still unknown, increasing evidence suggests that oxidation of lens crystallins to form protein aggregates, able to scatter light, is a possible cause (1,10–12). Crystallins present in the center of the lens do not regenerate as do other cellular proteins, making the proteins in this region as old as the individual (13). This may account for the

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accumulation of protein modifications that are known to occur in the human lens with age (10,14). The aging lens also experiences diminishing levels of antioxidants such as glutathione (GSH) (15), and activities of antioxidant enzymes such as GSH reductase and GSH peroxidase (16–18), which are required to protect sulfhydryl (-SH) groups of lens proteins. In advanced nuclear cataracts, >90% of protein thiols and $\geq 45\%$ of methionines have become oxidized to disulfides and sulfoxides, respectively (19,20). The formation of disulfides produces cross-linking of crystallins, which can lead to protein aggregation, protein insolubility and lens opacification (19,21).

Photo-oxidation by UV light is reportedly associated with the formation of maturity-onset cataract (22,23); however, the specific type of cataract that is produced, as well as the mechanism and wavelengths that are involved, and the targets of irradiation, are not well understood. A number of epidemiological studies have linked sunlight exposure with the formation of cataract, but only for the wavelengths of UVB radiation, not UVA (24–26). The exclusion of UVA light as a cause of cataract has been challenged by Dillon (27). The potentially damaging nature of UVA light has been well documented (28), and as we know that, in contrast to UVB, a considerable amount of potentially damaging UVA light can penetrate deep into the human lens (28–30), it is logical to carefully evaluate UVA-induced effects on the lens.

Most, but not all, epidemiological studies, have failed to find a link between sunlight and nuclear cataract (negative association: [24–26]; positive association: [31–34]). A recent study conducted in Australia did find a strong positive association between occupational sun exposure and nuclear cataract (35). It has been suggested that the lens nuclei of only middle-aged and older humans may be susceptible to sunlight (because of GSH loss), and that the process may take place in too short a time for epidemiological studies to detect an association (36,37). It is known that old human lenses, as well as nuclear cataracts, contain a collection of protein-bound UVA sensitizers that produce potentially damaging amounts of H_2O_2 , superoxide anion and singlet oxygen (30,38–40). Changes induced in lens crystallins by singlet oxygen and UVA light are similar to those found in crystallins from human nuclear cataracts (41–43). We have previously established a UVA/guinea pig *in vivo* model for studying early nuclear cataract (44). The guinea pig lens mimics the old human lens by possessing a high level of a protein-bound toxic chromophore—not protein-bound kynurenine as in the old human lens (45), but protein-bound NADPH in the form of zeta crystallin, which makes up 10% of total lens protein in the guinea pig (46,47).

In the current study, we have used dynamic light scattering (DLS) to investigate UVA-induced protein aggregation in the guinea pig lens *in vivo*. We have previously employed this technique to evaluate lens protein aggregation in hyperbaric oxygen-treated guinea pigs (21). DLS has been used for nearly three decades to study aggregation of lens proteins, both *in vitro* and *in vivo* (48–50). This technique, which employs a weak incident laser beam to measure Brownian movement of proteins (51), is emerging as a valuable diagnostic tool for early detection of ocular diseases such as cataract (52,53). The results of our study provide *in vivo* evidence to link UVA-induced protein aggregation with an increased level of nuclear light scattering (NLS) in the guinea pig lens.

Materials and Methods

Animals

Male Hartley guinea pigs, initially 17 months old, were obtained from Kuiper Rabbit Ranch (Indianapolis, IN). The animals were held for 4 weeks before the start of UVA light treatment. The guinea pigs were 18 months old when UVA light treatment began. Before the start of the study, lenses of the animals were examined by slit-lamp biomicroscopy, and guinea pigs with

cortical or nuclear opacities were excluded. The diet used throughout the study was Purina guinea pig Chow 5025 (Purina Mills, Richmond, IN), containing 0.1% ascorbic acid. Animal care and work performed in this study conformed to the US Department of Agriculture standards and the ARVO statement for the use of animals in ophthalmic and vision research.

In vivo exposure of guinea pigs to UVA light

The procedure for exposing guinea pigs to UVA light was modeled after Giblin *et al.* (44). Briefly, UVA irradiation of animals was conducted with use of Sylvania black light blue fluorescent lamps (F15T8/BLB; Osram Sylvania, Inc., Danvers, MA) with a spectral distribution of 340–410 nm wavelength, and maximum intensity at 365 nm. The lamps were about 5 cm above the caged animals. The irradiance on the animal corneas was approximately 0.5 mW cm^{-2} , and the lamps were kept on nearly continuously for 5 months. The room temperature was 20°C and the temperature inside the cage was $23\text{--}24^\circ\text{C}$. Guinea pigs kept under these conditions showed no hair loss or damage to the skin or cornea. There was slight drying of the outer ears which was treated once per week with a small amount of moisturizing sun block lotion (Coppertone SPF50; Schering-Plough, Inc., Memphis, TN) (control animals were not treated similarly). Age-matched controls were kept under normal lighting conditions. Lens transparency of control and UVA-exposed guinea pigs was assessed using Zeiss slit-lamp photo-microscopy after full dilation of the eyes with 1% tropicamide and 10% phenylephrine.

Light scattering measurements

Static light scattering (SLS) and DLS were performed *in vivo* on eyes of control and experimental guinea pigs using a fiber optic laser probe. Procedures for this measurement have been described in detail by Simpanya *et al.* (21). Initially, the eyes of guinea pigs were dilated with drops of 1% solution of tropicamide (Alcon Pharmaceutical Co., Fort Worth, TX), and the animals were anesthetized intramuscularly with xylazine (8 mg kg^{-1}) and ketamine (40 mg kg^{-1}), administered 10 min apart. A laser power of $100 \mu\text{W}$ with a wavelength of 640 nm was employed for the measurements. Guinea pigs were positioned in such a way that the laser beam passed through the center of the eye along the optical axis of the lens. Scattered light was collected at 154° . The corneal surface was kept moist by topical application of 0.9% saline.

To conduct SLS measurements, the cornea was first detected by moving the laser light probe toward the eye and detecting reflected light from the cornea. At a threshold of 10,000 counts s^{-1} , the movement of the motorized probe came to an automatic stop. The cornea surface was used as the origin for SLS. The laser probe was moved at a velocity of 0.01 mm s^{-1} from the cornea to the posterior capsule of the lens, a total distance of approximately 6 mm.

To determine the relative sizes of proteins in lenses of experimental animals compared with controls, DLS analysis was conducted immediately at the end of each SLS scan in the same position within the eye. The anterior capsule was used as the zero position for DLS analysis. DLS measurements were conducted every 0.1 mm across the $\sim 5.0 \text{ mm}$ optical axis of the guinea pig lens for a total of approximately 50 measurements per lens. At the 50 locations in the lens, the instrument recorded 10–15 measurements of protein diameter in 5 s per location. The instrument was calibrated against suspensions of polystyrene beads with 80 nm diameter. Measured protein sizes are presented as relative sizes using arbitrary units to compare protein sizes in various regions of lenses of control and UVA-treated animals. The reason for this is that in order to measure absolute protein sizes, proteins must be present in dilute solutions of $\leq 0.1\%$ by weight, with no interactions (54). The presence in an intact lens of highly packed proteins at concentrations $>300 \text{ mg mL}^{-1}$ (21) causes in addition to ionic and hydrophobic interactions, significant slowing of the diffusion of proteins, increasing their apparent size. For these reasons, it is not possible to measure absolute protein sizes accurately in intact lenses (54).

Results

Increased lens NLS was apparent in animals exposed to UVA light for 5 months, compared with age-matched controls, as evidenced by slit-lamp biomicroscopy (Fig. 1). The increase in NLS appeared as a nuclear haze, which produced a clear separation between the lens cortex and nucleus, compared with the control.

Analysis of control guinea pig eyes *in vivo* by SLS showed clearly the anatomic arrangement of the eye, *viz.* the cornea, aqueous humor, three regions of the lens (anterior cortex, nucleus, posterior cortex) and the vitreous humor (Fig. 2A). The cornea showed a high level of light scatter due to the reflection of laser light to the detector from this tissue. The control lens showed a low level of SLS for the entire axial diameter (~5.0 mm) (Fig. 2A). In contrast, lenses of guinea pigs treated with UVA radiation for 5 months showed an increased level of SLS, especially in the center of the nucleus, 2.0–3.5 mm from the surface of the cornea (Fig. 2B).

To assess changes in lens protein size *in vivo* after UVA radiation of guinea pigs, DLS analysis was performed. Measurements were made every 0.1 mm across the optical axis of the guinea pig lens. Figure 3 shows a representative DLS profile of average protein sizes across the lens of a control and UVA-exposed animal. Guinea pigs kept under UVA light showed larger apparent protein sizes in the lens nuclear region, compared with proteins in the lenses of control animals; however, the increased sizes were not always uniform across the entire lens nuclear region, as is shown in the representative example of Fig. 3. For this reason, apparent protein diameters were averaged for two separate regions of the lenses: anterior cortex (0–1.0 mm from the anterior capsule) and nucleus (1.1–4.4 mm from the anterior capsule) (Fig. 4). The total numbers of measurements made for each region are stated in the legend of Fig. 4. The average apparent size of proteins in the lens nucleus of UVA-irradiated guinea pigs was 2.8 times larger than comparable proteins in the lens nucleus of age-matched controls ($P < 0.001$) (Fig. 4). The anterior lens cortex showed no significant difference in average protein size between control and UVA-exposed animals ($P > 0.1$).

A more detailed analysis of protein size distribution was conducted at two specific locations in the lenses of control and UVA-exposed guinea pigs, located in the anterior cortex and in the approximate center of the nucleus (Table 1). The intensity (level) of small diameter proteins (<50 arbitrary units) at one location in the lens nucleus was 28% lower for UVA-exposed animals compared with controls (67% intensity compared with 93%, $P < 0.05$). In contrast, the level of large diameter proteins (>50 arbitrary units) at the same location in the nucleus was nearly five-fold higher for UVA-exposed animals compared with controls (33% intensity compared with 7%, $P < 0.05$). In addition, the size of small diameter proteins and the size of large diameter proteins were three-fold and 4.5-fold larger, respectively, in the nucleus of UVA-exposed animals, compared with controls (Table 1). No significant changes in either intensity or size of small diameter or large diameter proteins were observed in the lens anterior cortex as a result of UVA exposure (Table 1).

Discussion

A major finding of this study was that chronic exposure of guinea pigs to a low level of UVA light produced aggregation of proteins in the center of the lens with a concomitant increase in lens NLS. The intensity of UVA light employed, 0.5 mW cm^{-2} , was about one-tenth the level of ambient UVA present in sunlight in New York City at noon on a clear day in August (55). Presumably, the observed protein aggregation was related to the absorption of UVA light by the UVA chromophore NADPH which is present in the guinea pig lens at high concentration (100 times higher than the human lens [46,47]). NADPH is known to produce reactive oxygen species including singlet oxygen, superoxide anion and H_2O_2 when irradiated with UVA light

in the presence of oxygen (56,57), and we have detected trace amounts of oxygen (about 1 mmHg) present in the guinea pig lens nucleus (F. J. Giblin, unpublished result). Much of the NADPH present in the guinea pig lens is bound to the protein zeta crystallin (accounting for 10% of total guinea pig lens protein [46,47]), which has been reported to be in higher concentration in the guinea pig lens nucleus compared with the cortex (58). This presence of a toxic protein-bound UVA filter in the guinea pig lens nucleus mimics the aging human lens nucleus which contains various UVA-absorbing kynurenine compounds bound to crystallins (59). The binding of kynurenines to lens crystallins has been shown to produce lens crystallin damage (45), and it has been suggested that this binding may accelerate the progression of human nuclear cataract (10,12).

Distinct peaks and troughs were seen for DLS profiles for the lens nuclear regions of the UVA-treated guinea pigs. For example, in Fig. 3, particle sizes in certain regions of the UVA-exposed lens nucleus are two to three times higher than those in other regions of the nucleus. This was typically seen for each of the UVA-exposed lenses, and was also observed previously for the lens nuclei of hyperbaric oxygen-treated guinea pigs (21). The reason for this is not clear, but it is possible that early UVA-induced protein aggregation in the lens nucleus may not progress homogeneously, resulting in the presence of larger aggregates in certain regions and smaller aggregates in others. The peaks and troughs do not appear to be due to the presence of lens membranes or sutures as they are not observed during analysis of control lenses. It is also possible, as discussed previously (21), that if aggregates become too large, multiple light scattering (a single photon scattering many times in the lens) may occur, causing absorption of light, diffusion broadening and the appearance of apparently lower particle sizes.

Although not measured in the current study, protein disulfide was presumably a major constituent of UVA-induced high molecular weight aggregates detected in the guinea pig lens nucleus. We have previously shown the presence of increased levels of protein disulfide in the lens nucleus of UVA-exposed guinea pigs (44). In addition, Barron *et al.* (60,61) have also shown an increase in lens nuclear disulfide in guinea pigs exposed to UVA light for months, and this increase in disulfide was also linked with an increase in lens NLS. Each of the guinea pig lens crystallins, and particularly the beta, gamma and zeta crystallins, possess high levels of -SH groups, contributing to the high level of -SH present in the guinea pig lens nucleus of nearly 50 mM. Disulfide-crosslinked aggregates have also been found in increased amounts in the lens nucleus of oxygen-treated guinea pigs (21) as well as in human nuclear cataracts (19,20). Thus, protein disulfide appears to be a common feature associated with loss of transparency of the lens nucleus. The similarity between UVA-induced lens effects observed in this study and previously determined O₂-induced lens effects (21) is consistent with a role for oxidation in the UVA irradiation of guinea pig lenses.

We have shown previously that about 85% of UVA light that strikes the guinea pig cornea is able to reach the lens, and that UVA radiation can penetrate deep into the guinea pig lens nucleus (44). Although considerably more UVA light is absorbed in the guinea pig lens cortex than in the nucleus (44), we observed in the present study that UVA-induced aggregate formation occurred only in the nucleus. One reason for this is most likely the relatively low level of reduced GSH present in the guinea pig lens nucleus (<3 mM) compared with the cortex (nearly 25 mM). Long-term exposure of guinea pigs to UVA light has been shown to decrease the level of GSH in the lens nucleus by about 30%, with no significant effect on GSH levels in the cortex (44). GSH is known to be protective against UVA light *in vitro* (62,63), possibly due to its ability to scavenge reactive oxygen species. The low level of GSH in the guinea pig lens nucleus, combined with trace amounts of molecular oxygen present in this region, creates a favorable environment for UVA-induced oxidation of protein -SH groups to form disulfide-crosslinked aggregates. The older human lens nucleus also suffers from a low level of GSH, approaching 1 mM (10,64), leading to the potentially damaging binding of UVA-absorbing

kynurenine compounds to lens crystallins as mentioned above (10,12). The results of the present study conducted with guinea pigs suggest that the lens nuclei of older humans may be more susceptible to UVA-induced aggregation of lens crystallins, possibly leading to nuclear cataract.

Based on our previous gel electrophoresis data involving the UVA/guinea pig model (44), we would expect that the composition of the aggregates observed in this study would include each of the lens crystallins, including alpha, beta, gamma and zeta. All of the lens crystallins except alpha-B crystallin contain -SH groups, and thus can participate in disulfide crosslinking. Zeta crystallin would be expected to be a major component of the aggregates as we have found that it is highly susceptible to both disulfide-crosslinking and oxidation-induced precipitation (21) and, in addition, zeta is the guinea pig lens crystallin that contains the bound UVA chromophore, NADPH. It also cannot be ruled out that portions of certain cytoskeletal proteins, such as actin or the α - or β -tubulins, may also have been included in the aggregates as we have demonstrated previously a nearly complete UVA-induced loss of lens nuclear cytoskeletal proteins after long-term exposure of guinea pigs to UVA light (44).

The average diameter of protein aggregates found in the lens nucleus of the UVA-exposed guinea pigs (638 arbitrary units, Table 1) was 70 times greater than that of control small diameter proteins (9 arbitrary units, Table 1). If it is assumed that the control small diameter proteins were 5 nm in diameter (about 40% of this fraction would be composed of 10–12 nm diameter alpha crystallin), it can be estimated that the diameter of the UVA-induced lens nuclear aggregates would be about 350 nm. Aggregates of this size would have produced significant scattered light (48) in the guinea pig lens nucleus, as was observed in the current study (Fig. 1). Proteins of similar sizes, 5 nm for small proteins and nearly 700 nm for large aggregates, have been found to be present in young and old human lenses, respectively, using light scattering techniques (65,66). Electron microscopic analysis of protein aggregates in human cataractous lenses have shown sizes of 200–500 nm (67,68), while similar studies of X-irradiated rabbits have shown lens nuclear protein aggregates of up to 1000 nm in diameter (69).

In summary, we have shown by DLS analysis *in vivo* that exposure of guinea pigs to a physiologically relevant level of UVA light can induce increased protein aggregation in the center of the lens. Presumably this result is related to the high level of UVA chromophore (NADPH bound to zeta crystallin) present in the guinea pig lens nucleus, along with trace amounts of molecular oxygen, leading to UVA-induced generation of reactive oxygen species. A similar process of UVA-induced protein aggregation may take place in the older human lens nucleus, possibly accelerating the formation of human nuclear cataract.

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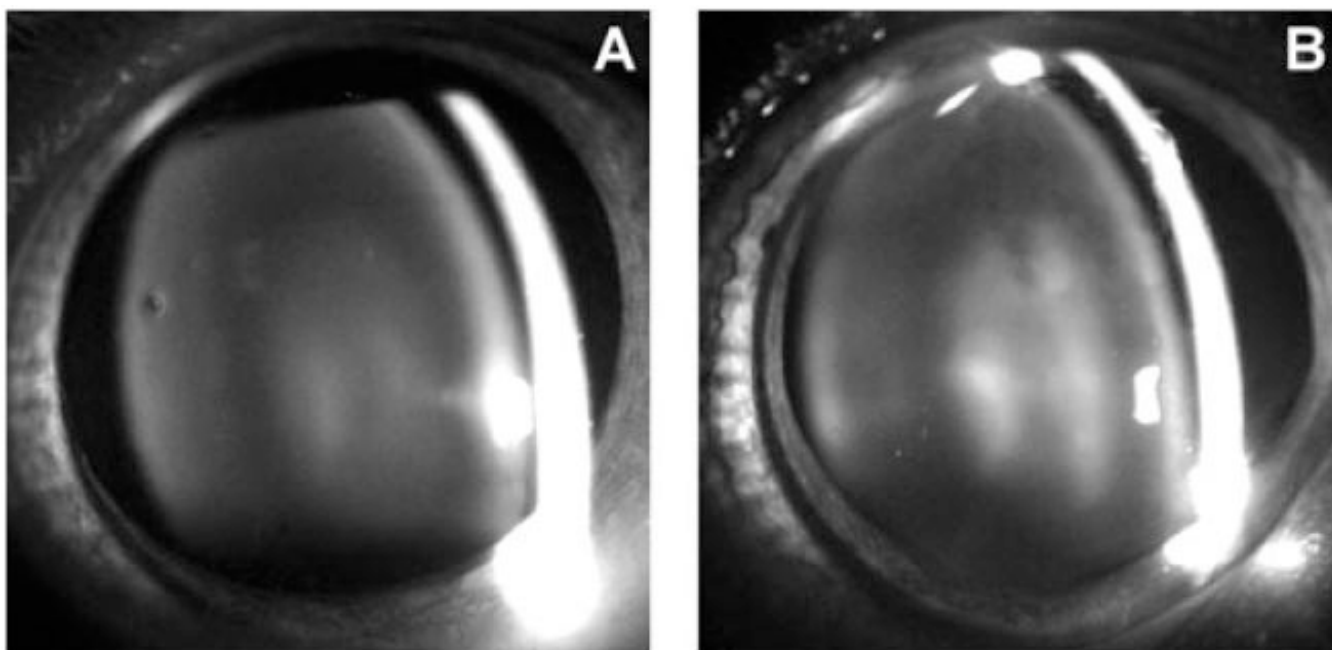


Figure 1. Typical slit-lamp biomicroscopy photographs of guinea pig eyes of a control (23 months old) and UVA-treated animal (23 months old) after 5 months of *in vivo* exposure. Note the increased lens nuclear light scattering in the UVA-treated lens (B), compared with the control (A), particularly in the center of the nucleus.

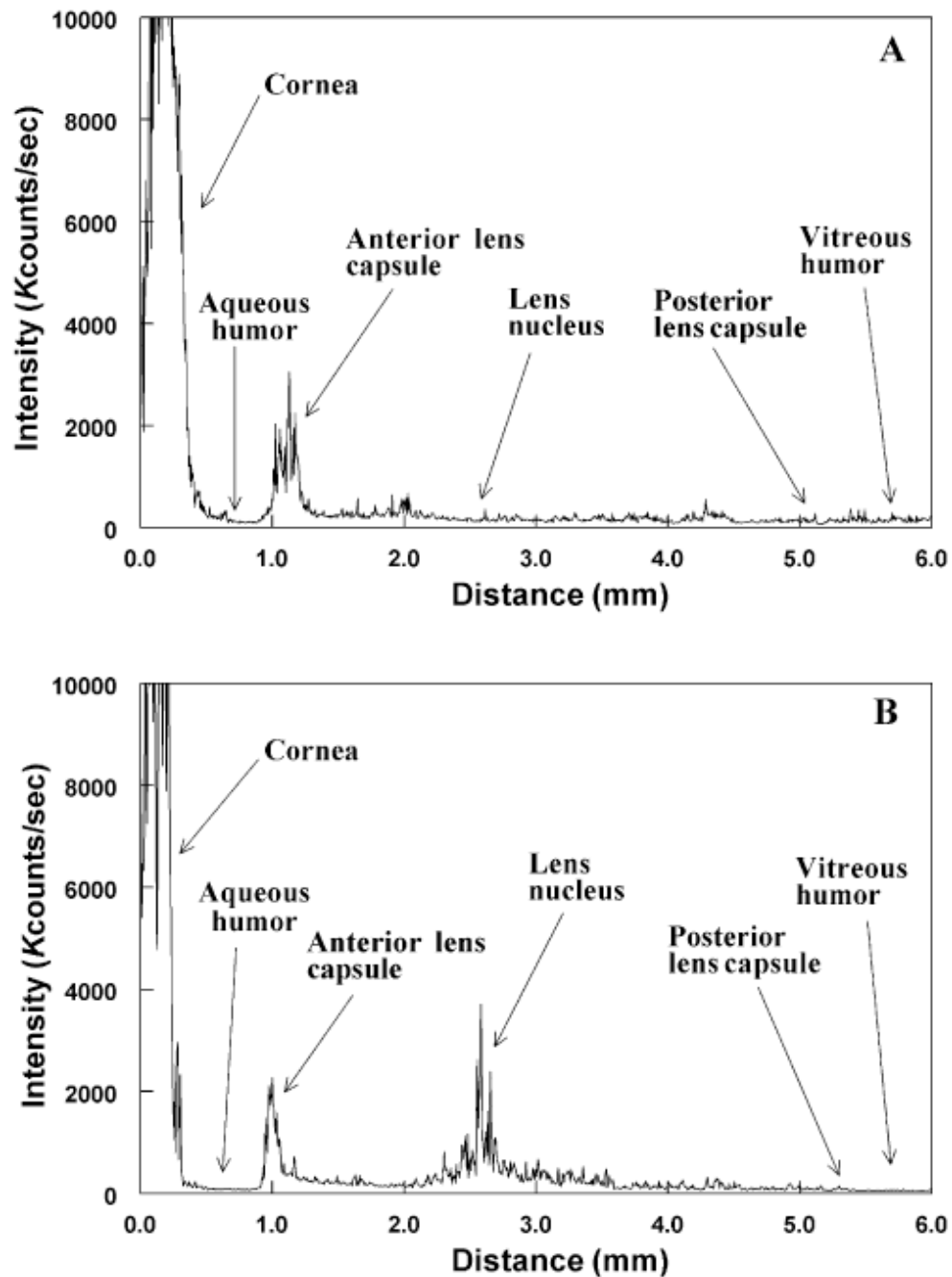


Figure 2. Typical static light scattering (SLS) analyses *in vivo* of eyes of control and UVA-treated guinea pigs. Each analysis was conducted along the optical axis of the lens, measuring 4.5–5.0 mm in diameter. Kcounts/s: 1000 photon counts per second of collected light intensity. (A) Control, 23-month-old untreated animal; (B) animal treated over a period of 5 months with UVA light. Note the increase in SLS intensity in the UVA-exposed lens, mainly in the central region, compared with that of an age-matched control animal.

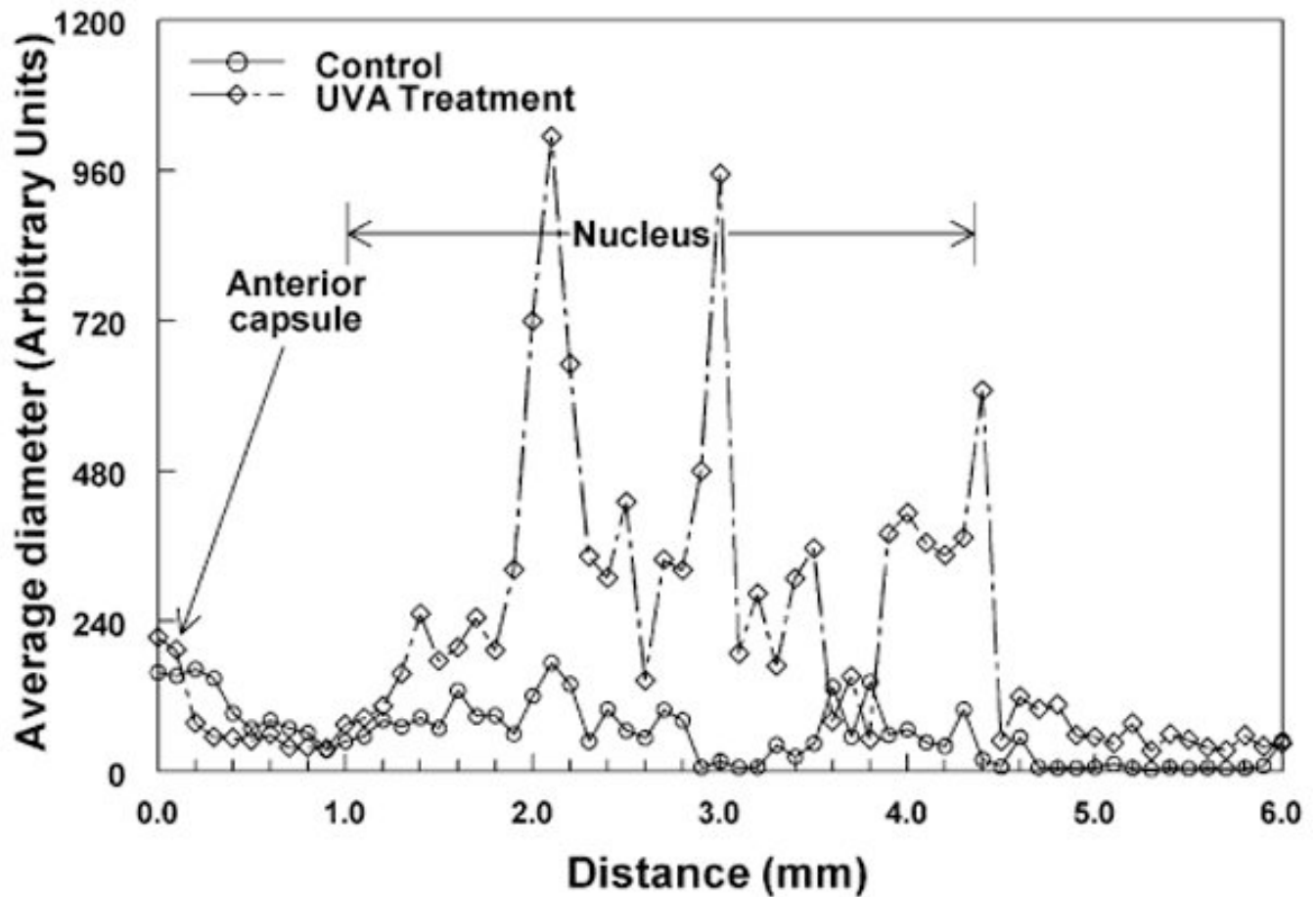


Figure 3.

Representative dynamic light scattering (DLS) analysis profiles *in vivo* across lenses of control and UVA-treated guinea pigs. Fifty measurements of average protein diameter were made every 0.1 mm across the optical axis of each lens. The instrument made 10–15 measurements of protein diameter at each location and provided an average value. DLS data are not expressed as absolute sizes of lens proteins but as arbitrary units of diameter (see Materials and Methods for an explanation). (○) 23-month-old control animal; (◊) 23-month-old experimental animal after 5 months of UVA light exposure.

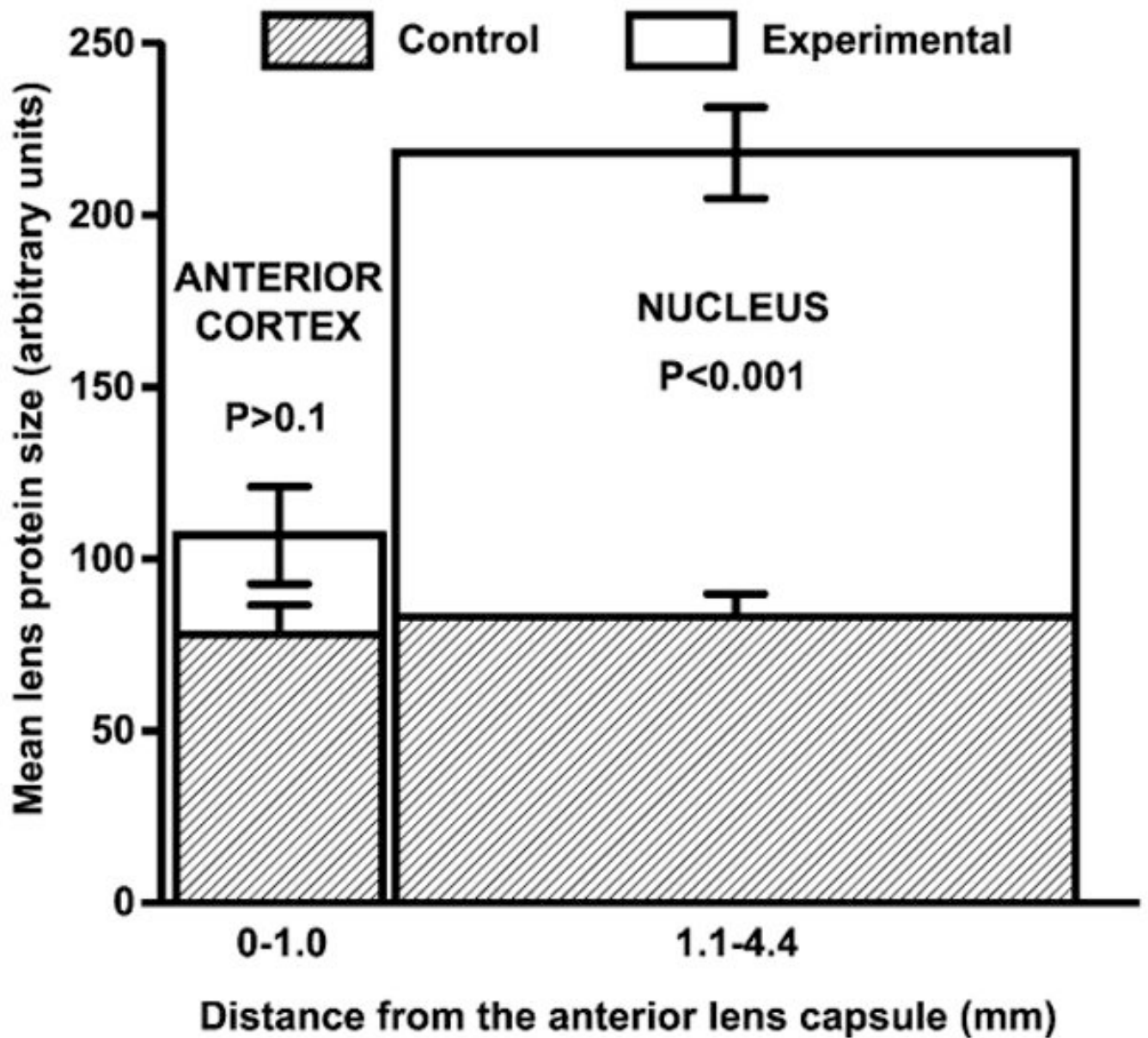


Figure 4. Analysis of average *in vivo* dynamic light scattering data for measurements taken across the lenses of control and UVA-treated guinea pigs. Measurements of average protein diameter were made every 0.1 mm across the optical axis of each lens (see legend of Fig. 3). These data were then averaged for two regions of each lens for control animals and animals exposed to UVA light. Analyses were conducted at 3 and 5 months of UVA exposure and the data combined ($n = 6$, control; $n = 5$, UVA-exposed). The total numbers of measurements made for each region were: control anterior cortex: 66; experimental anterior cortex: 88; control nucleus: 204; and experimental nucleus: 270. Each result is expressed as the mean \pm SEM.

Table 1
Protein size distribution analysis conducted *in vivo* for lenses of control and UVA-exposed guinea pigs.

	Intensity (%)		Protein diameter (arbitrary units)		P-value
	Control	UVA	Control	UVA	
Nucleus					
Small diameter	93 ± 7	67 ± 26	9 ± 5	26 ± 9	<0.01
Large diameter	7 ± 7	33 ± 26	140 ± 147	638 ± 678	>0.1
Anterior cortex					
Small diameter	96 ± 6	93 ± 7	12 ± 4	12 ± 4	>0.1
Large diameter	4 ± 6	6 ± 7	158 ± 298	201 ± 356	>0.1

In vivo dynamic light scattering analyses were conducted in lenses of guinea pigs exposed to 0.5 mW cm^{-2} of UVA light. Analyses were conducted at 3 and 5 months of UVA exposure and the data combined. Data were collected at one specific location each in the anterior cortex and in the approximate center of the nucleus for UVA-irradiated ($n = 5$) and age-matched control animals ($n = 6$). Small and large diameter proteins were classified as being <50 or >50 arbitrary units, respectively (see Materials and Methods for an explanation of why the data are expressed as arbitrary units of protein diameter). Results are expressed as the mean ± SD.