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UVA Light-mediated Ascorbate Oxidation in Human Lenses

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Abstract

Whether ascorbate oxidation is promoted by UVA light in human lenses and whether this process is influenced by age and GSH levels are not known. In this study, we used paired lenses from human donors. One lens of each pair was exposed to UVA light whereas the other lens was kept in the dark for the same period of time as the control. Using LC-MS/MS analyses, we found that older lenses (41 to 73 years) were more susceptible to UVA-induced ascorbate oxidation than younger lenses (18 to 40 years). Approximately 36% of the ascorbate (relative to control) was oxidized in older lenses compared to ~16% in younger lenses. Furthermore, lenses with higher levels of GSH were less susceptible to UVA-induced ascorbate oxidation compared to those with lower levels, and this effect was not dependent on age. The oxidation of ascorbate led to elevated levels of reactive α -dicarbonyl compounds. In summary, our study showed that UVA light exposure leads to ascorbate oxidation in human lenses and that such oxidation is more pronounced in aged lenses and is inversely related to GSH levels. Our findings suggest that UVA light exposure could lead to protein aggregation through ASC oxidation in human lenses.

Graphical Abstract

This study shows that UVA light (320–400 nm) can oxidize human lens ascorbate and decrease glutathione levels. The combined effects are a decline in ascorbate reduction and an increase in the production of α -dicarbonyl compounds. The latter can react with lens proteins to form advanced glycation end products (AGEs) that can crosslink proteins and could contribute to lens aging and cataract formation.

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SUPPORTING INFORMATION

Supporting Information is available in the online version of this article:

Table S1. AGE and levels of ASC, GSH and α -dicarbonyl compounds (as quinoxaline derivatives) in individual lenses (D/C: dark control, UV: UVA irradiated lens).

Figure S1. UV rig used for irradiation of human lenses.

Figure S2. UVA-induced GSH loss as a function of age in human lenses.

INTRODUCTION

Epidemiological studies have suggested that long-term exposure to UV light increases the risk of cataract formation, which is one of the leading causes of blindness worldwide (1–3). The pathology of cataracts is characterized by yellow-brown pigmentation and clouding caused by chemical modification and protein aggregation. UV light is known to generate reactive oxygen species that can damage proteins or initiate other mechanisms that lead to protein modifications (4). The majority of UVB light (280–320 nm) is filtered by the cornea and may not reach the lens (5). However, Giblin et al. showed that UVA light (320–400 nm) reaches the nucleus of the lens and causes oxidative damage (6).

The human lens contains high levels of ascorbate (ASC; up to 3 mM), possibly to prevent the oxidation of proteins and other lens constituents (7). However, ASC itself undergoes oxidation during aging (8, 9). Upon oxidation, ASC is converted to dehydroascorbic acid (DHA), which can be directly reduced by glutathione (GSH) (10). However, the ASC levels in aged and cataractous lenses are either very low or non-existent, and low ASC levels are associated with higher levels of DHA (9, 11). The loss of GSH along with the loss in the activity of enzymes needed for GSH synthesis and the conversion of GSSG to GSH could favor ASC oxidation in aged and cataractous lenses (12, 9). The DHA that is produced is a strong glycating agent (13–15). First, DHA hydrolyzes irreversibly to 2,3-diketogulonic acid (2,3-DKG), which undergoes further degradation (16, 17). Among the degradation products, α -dicarbonyls, such as 3-deoxythreosone (3-DT), react with proteins and form advanced glycation endproducts (AGEs) (18, 19).

AGEs are heterogeneous in structure, and some AGEs are formed as structures bridging two amino acids in proteins, which can result in the crosslinking of proteins (20). In addition, the extensive glycation of lens proteins leads to their aggregation (15). Thus, there is a strong rationale for AGEs being responsible for protein crosslinking and aggregation in aged and cataractous lenses. This is exemplified by the fact that the AGE levels progressively increase with lens age, and their levels are generally higher in cataractous lenses relative to age-matched non-cataractous lenses (18).

It has already been demonstrated that UVA light oxidizes ASC (21). Recently, Linetsky et al. showed that kynurenines, which are natural lens UV filters, promote UVA-induced ASC oxidation (22). Furthermore, a few AGEs also act as photosensitizers and promote both UVA-induced oxidation and the crosslinking of lens proteins (23, 24). However, whether UVA light can oxidize ASC in intact human lenses and whether this process is dependent on age and GSH content are not known. This study addresses these issues and demonstrates that UVA light-mediated ASC oxidation is higher in aged lenses than in young lenses and is dependent on GSH levels in the lens.

MATERIALS AND METHODS

Chemicals

o-Phenylenediamine, ascorbic acid, Chelex-100, DTPA, EDTA, and GSH were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, formic acid, heptafluorobutyric acid, *N*-ethylmaleimide, 2,2'-bipyridyl, PBS, ferric chloride, trichloroacetic acid sodium phosphate, sodium biphosphate, and PBS were obtained from Fisher Scientific (Waltham, MA). The quinoxalines of DHA, 2,3-DKG, and 3-DT were prepared as previously described (25, 11).

UVA Irradiation of human lenses

All irradiations were carried out with a light source consisting of a 1,000-watt mercury/xenon lamp (Newport Industrial Glass, Stanton, CA) with light passing through a dichroic mirror (280 nm λ 400 nm) and a 335-nm cut-off filter (Kopp 9335; 2-inch-diameter, 3-mm-thick; Newport Industrial Glass, Stanton, CA) (see Supporting Information, Fig. S1). The temperature of the cuvette holder was kept constant at 20°C by using a circulatory cooling unit (NesLab RTE7, Thermo Scientific, Waltham, MA) (Fig. 1S). The UVA light measured was 60 mW/cm² at the cuvette surface. Pairs of individual lenses (fresh and frozen) from human donors were obtained from Saving Sight (Kansas City, MO). Frozen lenses (stored at -80 °C until use), after thawing on ice, were used to study the effect of UVA-light on the levels of ascorbate, GSH and dicarbonyls, and fresh lenses were used to study the formation of AGEs. Lenses were considered fresh when they were shipped on wet ice within 48 h after death; upon arrival they were immediately used in experiments. For irradiation, one lens was placed in a Teflon holder, which was fitted into a 1 cm quartz cuvette. The cuvette was filled with Chelex-100 treated, argon-saturated PBS containing 1 mM each of DTPA and EDTA. The holder was carefully placed in the cuvette to avoid air bubbles and shifting of the lens. Finally, the cuvette was capped, sealed with parafilm and placed in the cooled cuvette holder of the UV apparatus for 2 h of irradiation. The second lens from the same donor was kept in the dark for 2 h as a control. Immediately following the experiment, the lenses were decapsulated, weighed and homogenized in 1 mL argon-saturated PBS containing 1 mM EDTA. Homogenates were stored at -80 °C until being used for the assays described below.

Photometric determination of ascorbate in lens homogenates

The assay was performed as described previously with modifications (26). Briefly, 50 μ L of water was added to 75 μ L of the lens homogenate. The protein was then precipitated with 125 μ L 2 M trichloroacetic acid. After centrifugation at 10,000 g for 5 min, 150 μ L of the supernatant was taken out. Then, 60 μ L 40% phosphoric acid, 60 μ L 4% 2,2'-bipyridyl in 70% ethanol (v/v) and 30 μ L 3% aqueous ferric chloride were added to the supernatant and vigorously mixed before being incubated for 60 min at 37°C. To clarify, the mixture was centrifuged for five min at room temperature and 10,000 g. After centrifugation, 250 μ L was transferred to a 96-well plate, and the absorption was read at 525 nm.

UPLC-MS² analysis for α -dicarbonyls in lens homogenates

α -Dicarbonyls were analyzed as previously described (11).

UPLC-MS² analyses of GSH in lens homogenates

First, 10 μ L of the lens homogenate was incubated with 50 μ L aqueous 20 mM *N*-ethylmaleimide at room temperature for 10 min. The protein was precipitated with 40 μ L 2 M trichloroacetic acid. After centrifugation at 10,000 g, the supernatant was diluted tenfold and 1 μ L was injected into a Waters Acquity UPLC system (Milford, MA) connected to a Sciex 4500 QTrap (Redwood City, CA). Chromatographic separations were carried out on an ACQUITY BEH C18 peptide column (100 $\times$ 2.1 mm, 1.7 μ m, Waters, Milford, MA) connected to a guard column using a flow rate of 0.5 ml/min. Water (solvent A) and 80% acetonitrile (solvent B, (v/v)) were used as eluents. Heptafluorobutyric acid (0.12%, v/v) was added to both eluents. Analyses were performed at a column temperature of 40°C using gradient elution: 10% B (0 to 0.25 min) to 15 % B (1.5 min) to 35 % B (3 min) to 100% B (5 to 6.5 min). The column was equilibrated at 10% B for 1 min prior to the next analysis. Detection of the analytes was achieved using multiple reaction monitoring in a Sciex QTRAP 4500 mass spectrometer. The ion source was run under the following conditions: temperature: 500°C, ion spray voltage; 3 kV, curtain gas; 35 ml/min, nebulizer gas; 50 ml/min, heating gas; 50 ml/min. The declustering potential was set to 40 V. The MRM parameters for the GSH-*N*-ethylmaleimide derivatives (t_R =2.8 & 2.9 min) were as follows (Q1 $\rightarrow$ Q3 [m/z], collision energy [eV], cell exit potential [V]; Quantifier: 433 $\rightarrow$ 304, 20, 12; Qualifier 1: 433 $\rightarrow$ 201, 29, 14; Qualifier 2: 433 $\rightarrow$ 287, 30, 15. Quantitation was performed using the standard addition method.

Statistics

All samples were analyzed in triplicate. The data were analyzed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA). P values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The dosage of UVA light that we used (60 mW/cm² or 432 J/cm² for a 2 h exposure time) in our experiments is similar to what has been used previously (22, 21), but is higher than what is typically encountered in the middle of a sunny day. For example, about 10 mW/cm² of UV solar radiation were estimated to reach the surface of the earth at 40°N latitude (central US) (27). The UV solar radiation is stronger in regions closer to the equator. In Riyadh, Saudi Arabia (25°N latitude), the maximum UV solar radiation was found to be up to 28 mW/cm² at noon (28). Sabburg et al. found a whole day average of about 1 mW/cm²/day in Toowoomba, Australia (28°S latitude), but unfortunately did not indicate the maximum UV solar radiation (29). Zigman reported 1 mW/cm² reaching the lens in Maryland, United States (27). Even though the average human is generally not exposed to full solar irradiation (30), UVA light is known to produce cumulative damage during the regular life span of human lenses (1–3). Thus, the present setup could mimic the cumulative damage to the lens that usually occurs over a lifetime.

A total of 28 lenses were subjected to UVA irradiation. To minimize the variations between dark control and UVA-irradiated lenses, paired lenses from individual donors were used in all experiments. Following UVA irradiation, lenses were immediately homogenized to exclude any non-UVA-induced oxidation of ASC. Homogenates were used for the analysis of ASC, GSH, and selected α -dicarbonyls. The results are presented in Table 1 (see Supporting Information for the levels in individual lenses, Table S1).

The levels of all analytes varied widely among lenses, even when they were of similar age. This could be attributed to the different physiological and environmental conditions of the donor. Further, we cannot rule out effects of thawing (of frozen lenses) itself, if any, on the levels of analytes.

Because of individual differences, percent ASC oxidation was examined as a measure of the impact of UVA light, with the assumption that baseline levels were approximately equal in a pair of lenses from the same donor. Figure 1 shows the UVA-induced ASC oxidation in individual lens pairs.

Three lenses showed levels below 0 (−0.4 to −7.5 %); we interpreted those as indicating the absence of ASC oxidation. However, this could also be attributed to slightly different starting levels of ASC in control and UVA-irradiated lenses. However, in the majority of the lenses, UVA light promoted ASC oxidation, and it reached 94% in one lens. Interestingly, there was a trend suggesting that aged lenses were more susceptible to UVA-induced ASC oxidation than younger lenses. However, there were a few exceptions; some aged lenses showed no or very mild ASC oxidation after UVA exposure. For instance, one 73-year-old lens showed ASC oxidation at only 6%, whereas an age-matched lens (72 years) showed 64% ASC oxidation. A similar absence or very low level of ASC oxidation was also encountered in a few younger lenses. Such heterogeneity in ASC is not unexpected because the individual donor's lifestyle, genetic/epigenetic makeup and lens constituents could possibly contribute to the susceptibility of ASC to oxidation.

To the best of our knowledge, ours is the first study that derivatized/stabilized GSH before LC/MS² analysis. Tsentalovich et al. used LC-MS for measuring GSH in human lenses (55 to 70 years) (9), but GSH was not derivatized unlike in our study. Furthermore, in that study GSH levels in individual lenses were not given and no lenses younger than 55 were used. However, the mean value of GSH in their study was 3.3 nmoles/mg lens, which is somewhat similar to this study (4.0 nmoles/mg lens). In our study, the GSH levels in control lenses ranged from 0.1 to 12.9 nmoles/mg lens and the highest value of 12.9 nmoles/mg lens was observed in a 18 year old lens. The upper levels in our study are considerably higher than those previously reported (31, 32). However, one publication reported even higher values than we found (33). In these studies, GSH was determined by colorimetric methods. Thus, we believe that the differences in GSH levels are probably due to differences in methods used. In general, mass spectrometric quantification provides better accuracy than colorimetric methods.

In contrast to ASC oxidation, there was no correlation between age and the UVA-induced GSH loss (see Supporting Information, Fig. S2). In general, the GSH levels decreased after

UVA exposure. It is known that high levels of GSH have a protective effect against UVA-induced ASC oxidation (22). The levels of GSH versus ASC oxidation in individual lenses are shown in Figure 2.

We assumed that the starting levels of GSH in the UVA-exposed lens were comparable to those in control lenses. There was a negative correlation between the GSH level and the percent of ASC oxidized. The lenses that had relatively high levels of GSH showed lower UVA-induced ASC oxidation than did lenses with low levels of GSH. In addition, ASC oxidation can be influenced by age-dependent decline in transportation of GSH to the nucleus of the lens (34). These observations provide further evidence that GSH protects against ASC oxidation in the lens. However, there were a few instances in which low levels of GSH did not result in high ASC oxidation (e.g. lens #8, Table S1). It is reasonable to assume that there may be additional inhibitory factors of ASC oxidation in the lens, such as cysteine or dehydroascorbate reductase (35). Further studies are required to clarify this issue. However, it is prudent, based on the findings for ASC oxidation and the GSH levels, to propose that the difference in ASC oxidation at relatively similar GSH levels might explain why cataract onset varies so much among humans.

We also analyzed the levels of selected ASC oxidation products, DHA, 2,3-DKG and 3-DT as stable quinoxaline derivatives. The interdependence of ASC oxidation to these α -dicarbonyl products is shown in Figure 3.

The total levels of α -dicarbonyls showed a strong correlation with ASC oxidation (Fig. 3A); however, the total levels of α -dicarbonyls were lower than the total ASC oxidation. This can be explained by the fact that quinoxaline derivatization of DHA is not quantitative, and our assay did not measure other ASC oxidation products, such as methylglyoxal and threosone (36, 16).

The DHA and 2,3-DKG levels increased with ASC oxidation (Fig. 3B), but the 2,3-DKG levels were higher than the DHA levels (Fig. 3C). This could be due to the short half-life of DHA under physiological conditions of about 30 min and the irreversibility of 2,3-DKG to DHA or ASC (37). Although 3-DT can also be derived from glucose, we recently showed that it is mainly derived from 2,3-DKG in the human lens (11). An increase in the 3-DT levels also coincided with an increase in ASC oxidation, although this increase was modest (Fig. 3D), further suggesting that 2,3-DKG degraded relatively slowly and could give rise to other products in the lens. α -Dicarbonyls are major precursors of AGEs (38), they primarily react with lysine and arginine residues to generate AGEs and can also form crosslinking adducts between arginine and lysine residues in proteins (18). Thus, our results suggest that UVA-mediated ASC oxidation in the human lens could lead to lens protein modification by AGEs (18, 11). However, we were not able to demonstrate AGE formation from ASC oxidation in the lens because frozen lenses are not suitable for longer incubation after irradiation, which is necessary for AGE formation. To address this hindrance, we used freshly harvested lenses (harvested within 48 h after death) that were shipped in wet ice. We irradiated those lenses (5 lenses in total, for 2 hrs with UVA light as above and subsequently incubated at 37°C in artificial aqueous humor for a week). However, after one week, we unexpectedly found that ASC was completely degraded in control lenses that were not

irradiated, similar to UVA-irradiated lenses (data not shown). This demonstrated that human lenses harvested in this way are not suitable for ASC-mediated AGE synthesis studies. A further refinement in tissue harvesting, reduction in post-mortem time or improvement of culture conditions are needed for future studies.

In conclusion, we demonstrated that ASC could be oxidized by UVA light *ex vivo* in human lenses. Aged lenses in general were more susceptible to UVA-induced ASC oxidation than young lenses. Because the GSH levels decreased with age, ASC oxidation increased, it is reasonable to conclude that GSH is necessary for maintaining ASC redox equilibrium in the lens. However, for some lenses, no such correlation was found. It is likely that additional factors influence UVA-mediated ASC oxidation, e.g., individual levels of kynurenines in the lens, diet, environmental conditions, lifestyle and/or systemic diseases such as diabetes. Our results further underscore the importance of protecting eyes from UV light by wearing sunglasses to limit damage to the lens. Based on our results it is tempting to speculate that replenishing lenses with fresh antioxidants, such as GSH, might limit ASC oxidation and possibly inhibit/delay cataract formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The following abbreviations are used

AGEs	advanced glycation end products
GSH	reduced glutathione
GSSG	oxidized glutathione
MRM	multiple reaction monitoring
3-DT	3-deoxythreosone
2,3-DKG	2,3-diketogulonic acid
DHA	dehydroascorbic acid
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
WW	wet weight

Figure 1. UVA-induced ASC oxidation as a function of age in human lenses. 95% confidence interval is shown.

Figure 2.
Relationship between UVA-mediated ASC oxidation and GSH in human lenses. WW, wet weight.

Figure 3. Relationship between ASC oxidation and change in the levels of α-dicarbonyl compounds in UVA-exposed lenses to control lenses. Sums of DHA, 2,3-DKG and 3-DT are given in A. Individual graphs for DHA, 2,3-DKG, and 3-DT are given in B, C, and D, respectively. WW, wet weight.

Table 1

Levels of ascorbate, GSH and α -dicarbonyl compounds (DHA, 2,3-DKG, 3-DT) in control and UVA-exposed lenses. WW, wet weight.

Number of lenses (age range)		28 (18 to 73 years)		
Parameter	Dark Control		UVA Exposed	
	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range
Ascorbate (nmoles/mg lens WW)	0.59 $\pm$ 0.34	0.07 – 1.36	0.45 $\pm$ 0.34	n.d. – 1.21
GSH (nmoles/mg lens WW)	4.0 $\pm$ 3.4	0.10 – 12.9	2.4 $\pm$ 2.1	0.07 – 8.0
DHA (pmoles/mg lens WW) *	24.7 $\pm$ 16.7	0.9 – 68.8	36.5 $\pm$ 27.9	0.8 – 108.9
2,3-DKG (pmoles/mg lens WW) *	38.5 $\pm$ 36.1	1.2 – 111.1	72.4 $\pm$ 74.1	0.6 – 250.3
3-DT (pmoles/mg lens WW) *	0.31 $\pm$ 0.18	0.09 – 0.69	0.36 $\pm$ 0.25	0.10 – 0.95

n.d. - not detectable

* - as quinoxaline derivative