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Glycated Proteins Can Enhance Photooxidative Stress in Aged and Diabetic Lenses

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This study intends to clarify the ability of different carbonyl-containing lens metabolites to form advanced glycation end products, which possess photosensitizer activity and to investigate whether these modified proteins could be implicated in lens photodamage.

Calf lens protein was experimentally glycated with either methylglyoxal, glyoxal, ascorbic acid, or fructose to obtain models of aged and diabetic cataractous lenses. Being exposed to 200 J/cm^2 UVA radiation the model glycated proteins produced 2–3-fold more singlet oxygen compared to the unmodified protein and the superoxide radical formation was 30–80% higher than by the native protein. Ascorbylated proteins demonstrated the highest photosensitizer activity.

Biological responses of glycation-related photosensitizers were studied on cultured lens epithelial cells irradiated with 40 J/cm^2 UVA. Tissue culture studies revealed a significant increase in thiobarbituric acid reactive substances in the culture medium of lens epithelial cells after irradiation and treatment with glycated proteins. Lens proteins had a protective effect against UVA induced cytotoxicity, however, this protective effect decreased with the increasing photosensitizer activity of experimentally glycated proteins.

The documented glycation-related photosensitization could explain the accelerated pathogenic changes in human lens at advanced age and under diabetic conditions.

Keywords: Advanced glycation end products (AGEs); Photosensitization; Singlet oxygen; Superoxide radical; Lens; Lens epithelial cells

Abbreviations: AGE, advanced glycation end product; GO, glyoxal; MGO, methylglyoxal; NADH, reduced nicotinamide adenine dinucleotide; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid reactive substances; UVA, ultraviolet A

INTRODUCTION

Cataract or lens opacity often develops during normal aging and in diabetes. Numerous chemical and physicochemical processes may account for the changes in lens transparency. The aggregation of lens structural proteins (crystallins) into high molecular weight clusters that scatter the light,[1] protein oxidation,[2] changes in lens enzyme activities,[3] altered ion and water content in lens fibers[4] are among the characteristic features in most human cataractous lenses. In many cases various mechanisms for cataract development overlap and intersect with one another.

Biochemical and physical properties of lens proteins are also affected by non-enzymatic glycosylation, i.e. covalent attachment of sugars such as glucose, fructose or pentoses to amino groups e.g. on lysine, arginine residues and to the N-terminus of polypeptide chains. Subsequent oxidation, dehydration, and rearrangements of these glycosylated proteins finally produce a heterogeneous group of compounds, termed advanced glycation end products (AGEs). The latter are originally characterized by their yellow–brown color, a specific non-tryptophan fluorescence (maximum of excitation at 370 nm and emission at 440 nm), and their ability to form crosslinks between protein amino groups.[5,6] Because of their diversity, complexity of the chemical reactions involved and experimental difficulties related to their isolation, only a few AGE structures

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have been chemically characterized. Formation of AGEs is a typical feature during normal lens aging.[7] Their levels are dramatically increased in diabetic cataractous lenses as documented by spectroscopic, fluorescent, chromatographic, and immunochemical methods.[8–11]

AGEs produce reactive oxygen species when exposed to UVA radiation. Such photosensitizer properties have been observed for proteins from human cataractous lenses[12] and have been extensively studied on lens proteins glycated *in vitro* with ascorbic acid.[13]

Ascorbic acid acts as one of the many antioxidants in the lens that can protect biomolecules against damage by free radicals *in vivo*. However, specific AGEs, which could be formed from ascorbic acid *in vitro* and which have been detected in brunescent human lenses suggest that this antioxidant could also play an important role in the normal browning and aggregation of lens crystallins during aging and diabetes.[14,15]

Glucose is regarded as the major glycating agent in the body but its reactivity is very low and of significance only in the case of prolonged hyperglycemia. In the lens elevated glucose levels activate the polyol pathway and as a result fructose levels approach those of glucose.[16] Since fructose glycating reactivity is one magnitude higher than that of glucose, glycation of lens proteins by fructose (fructosylation) could be of prime importance in diabetic lens opacity.[17]

Recent research has shown that physiological glycation processes also include the modification of proteins by reactive α-dicarbonyl compounds. Methylglyoxal (MGO) and glyoxal (GO) have been proposed to be formed by degradation of glucose or from both Schiff's bases and Amadori products in early glycation.[18] Despite the much lower concentrations of these metabolites in the lens as compared to glucose and ascorbic acid, they could also contribute to modifications of lens proteins since they are much more reactive than the parent carbohydrates and their binding to proteins is irreversible. Specific biomarkers of glycation with these α-oxoaldehydes found in cataractous lenses confirm their involvement in modification of lens proteins.[11,19,20]

Reflecting the high incidence of diabetes in today's western societies and the increased levels of AGEs formed by ascorbic acid, fructose and α-oxoaldehydes in aged and diabetic lenses, the demand to characterize their photosensitizer activities becomes evident. This then outlined the first aim of these investigations: quantification of the primary products of types I and II photodynamic reactions (superoxide radical O_2^- and singlet oxygen $^1O_2$) produced by lens proteins glycated with different carbonyl compounds.

The link between photochemical formation of active states of molecular oxygen and biological responses is generally assumed but insufficiently characterized. Thus the second aim of this study was to quantify whether photodynamic reactions of AGEs increase UVA induced cytotoxicity and hence enhance lens photooxidative damage. Therefore we investigated the effect of AGE-modified lens proteins on bovine lens epithelial cells after UVA irradiation. The extent of cell photodamage was assayed in terms of lactate dehydrogenase leakage and formation of thiobarbituric acid reactive substances (TBARS).

MATERIALS AND METHODS

Materials

Reagents of highest quality available were obtained from Sigma-Aldrich Chemie GmbH (Germany) and ICN Biomedicals GmbH (Germany), unless otherwise indicated. MGO was prepared by hydrolysis of 1,1-dimethoxypropanone and purified by fractional distillation under reduced pressure. GO was obtained from glyoxal trimer dihydrate after distillation over P_2O_5. MGO and GO concentrations were determined as described by McLellan and Thornalley.[21]

Glycation of Lens Proteins

Total calf lens protein was isolated as described elsewhere.[22] Briefly: Dissected calf lenses were homogenized with 4°C-cold distilled water (3 ml for mg lens fresh weight) and homogenates were centrifuged in Beckman J-21 centrifuge for 30 min at 4°C and 32,000 *g*. The supernatant representing the water-soluble alpha, beta and gamma crystallins, was lyophilized. AGE-modified proteins were obtained after incubation of 10 mg/ml protein with either 25 mM ascorbic acid, MGO, GO, or 500 mM fructose in 100 mM sodium phosphate buffer pH 6.9, containing 1 mM diethylenetriaminepenetaacetic acid as a chelator of heavy metal ions to prevent the oxidation of carbonyl compounds. The solutions were sterilized through 0.2 μm Minisart filters (Sartorius, Germany) and incubated at 37°C. In order to obtain proteins with varied extents of modification, two incubation periods (2 and 3 weeks) were applied for the reactions with ascorbic acid, MGO, and GO, while fructosylated protein was analyzed after 6 weeks of incubation. Control proteins were treated alike, except that no carbonyl compound was added. All incubations were set up in triplicates.

At the end of the incubation periods the low molecular weight compounds were removed by extensive dialysis against 30 mM NH_4HCO_3 at 4°C.

The resulting AGE-modified proteins were lyophilized and analyzed. Samples of the three incubation lots were pooled and stored at −20°C for utilization in later irradiation and tissue culture experiments.

UV absorption of glycated lens proteins (1 mg/ml in 7 M urea) was measured using a Beckman DU 650 spectrophotometer. Fluorescent readings of protein solutions (0.25 mg/ml in 7 M urea) were recorded on a Shimadzu spectrofluorimeter RF-5000 in a mirrored cuvette. Isoelectric focusing was carried out as described elsewhere[23] and proteins were stained with Coomassie Blue R 250.

Cell Culture System

Cultured bovine lens epithelial cells were initiated and maintained as previously reported[24] and were grown in a humidified incubator at 37°C in 5% CO_2 atmosphere. Confluent cells, passage II, were used for irradiation studies. Prior to irradiation cell layers were washed with 1 ml of phosphate-buffered saline (PBS) and left in either 1 ml of PBS (for the controls) or 1 ml of protein solution (4 mg/ml in PBS). Non-irradiated control cells were treated likewise and kept wrapped in black paper over the same period of time and under the same conditions as the irradiated cells.

UVA Irradiation

The UVA radiation source was a Black-Ray Lamp UVL-56 (Upland, CA, USA). This lamp provided broadband UVA emission between 340 and 400 nm with a maximum at 365 nm. Plastic Petri dishes (35-mm in diameter and containing either solutions of glycated protein or cell layers covered with protein solutions) were positioned on a black surface to avoid light reflection. The lamp was set directly on the plastic dish covers to eliminate any UVB irradiation (UVB fluence rate was less than 0.01 mW/cm^2). The density of radiant flux was measured by UV intensity meter (Waldmann, Germany).

The irradiation of protein solutions (1.5 ml) was carried out on a water-cooled plate over 7 h. During that time the temperature of the solutions did not exceed 20°C. Lens epithelial cells received 40 J/cm^2 UVA radiation dose delivered over a period of 1.5 h. Temperatures measured in the cell dishes during experimental irradiation were not higher than 27°C.

Quantitation of the Produced Reactive Oxygen Species after UVA Irradiation of Glycated Proteins

Singlet oxygen and superoxide radical productions were measured as described elsewhere.[12,25] The buffers used for both assays were treated with Chelex 100 resin to remove traces of transition metal ions. Low-soluble modified proteins were sonicated prior to the experiments.

For quantitation of singlet oxygen solutions of either native or modified protein (2 mg/ml in 50 mM potassium phosphate buffer pH 6.9, containing 1 mM diethylenetriaminepentaacetic acid) were irradiated in the presence of 2 mM carnosine, 50 μM *N*,*N*-dimethyl-4-nitrosoaniline (Aldrich Chemical Company, Milwaukee, USA) with or without 10 mM sodium azide. The absorbance decrease at 440 nm due to bleaching of *N*,*N*-dimethyl-4-nitrosoaniline by singlet oxygen was used to calculate the amount of produced $^1O_2$. A decrease of 0.084 absorption units was considered to be equivalent to the generation of 1 μmol/ml singlet oxygen.[26]

Solutions of either native or modified protein (2 mg/ml in 50 mM potassium phosphate buffer pH 8.5, containing 50 μM diethylenetriaminepentaacetic acid and 120 μM cytochrome C) were irradiated in two experimental sets: (i) in presence and (ii) in absence of 300 U superoxide dismutase for quantitation of generated superoxide radical. Aliquots of catalase solution were added every hour during irradiation to a total concentration of 480 U. The superoxide formation was calculated by the superoxide dismutase-dependent increase in absorbance at 550 nm due to reduction of cytochrome c ($\varepsilon_{max} = 2.1 \times 10^4$ M/cm).

TBARS Assay for Lipid Peroxidation

Immediately after irradiations PBS supernatants were collected, 500 μl were mixed with 500 μl thiobarbituric acid reagent,[27] and heated at 100°C for 30 min. After cooling and centrifuging, the absorbance of solutions at 532 nm was read against reagent blank, containing thiobarbituric acid and PBS. An extinction coefficient of 1.56×10^5 M/cm was used to calculate the concentration of malondialdehyde-thiobarbituric acid adduct. The readings for sample blanks (protein solutions in PBS) were subtracted from the readings of supernatants. The cells were lysed with 1 ml 0.5% sodium dodecyl sulfate and protein concentration was measured using bicinchoninic acid,[28] with calf lens protein as the standard. TBARS values were expressed as nmol malondialdehyde equivalents per microgram cellular protein. Each TBARS measurement was carried out in duplicate with four dishes per data point.

Lactate Dehydrogenase Assay for Cell Viability

The method applied by Tebbe and colleagues[29] was adapted to the specific conditions of our experiments. After irradiation cell dishes were left in the incubator at 37°C for 2 h. Thereafter the culture medium was collected and stored at −20°C prior to

the analysis of released lactate dehydrogenase. Replacement medium contained 1 ml 0.5% Triton X-100 (Merck, Darmstadt, Germany) for cell lysis. The supernatant, containing lysed cells, was collected after a period of 45 min in the incubator and stored at −20°C prior to measurement of intracellular lactate dehydrogenase.

Enzyme activity was spectrophotometrically determined by measuring the reduced nicotinamide adenine dinucleotide (NADH) disappearance rate. Briefly: 450 μl of either PBS supernatant or suspension with Triton-lysed cells were added to 500 μl of 1 mM sodium pyruvate in 200 mM Tris–HCl buffer pH 7.4. Fifty microliters of 4 mM NADH in the same buffer were added and the absorbance at 340 nm was read over period of 3 min. ΔA_{340}/min was recorded from the initial linear portion of the resulting curve. All samples were assayed in duplicates at 25°C. The readings for sample blanks (protein solutions in PBS) were subtracted from values found for subsequent supernatants. Cell viability was calculated as equal to

$$\frac{\text{Extracellular lactatec dehydrogenase activity}}{\text{Total (intracellular + extracellular) lactate dehydrogenase activity}} \times 100$$

Data shown are means ± SD of four dishes for every experimental condition.

Statistical Analysis

Differences between groups of modified proteins were analyzed by ANOVA using SPSS software. Paired or unpaired *t*-test as appropriate was applied to compare production of reactive oxygen species by different glycated proteins. Unpaired *t*-test was used to evaluate the effect of glycated proteins on lens epithelial cells. *P* values of less than 0.05 were considered significant.

RESULTS

Characterization of Glycated Proteins

ANOVA-test for differences between triple incubations with one and the same carbonyl compound but for different incubation periods revealed statistically significant variations only for ascorbylated proteins. MGO and GO produced maximally modified protein after 2-week period and further incubation did not significantly alter the level of protein modification. The characteristics of incubated controls (2 and 6 weeks) demonstrated no differences beyond experimental error and resembled non-incubated control.

The isolated native lens protein practically did not absorb above 300 nm. In contrast, glycated proteins exhibited a broad absorption in the UVA region (Fig.1). MGO- and GO-glycated proteins possessed new maxima at about 325 nm, while a wide shoulder of the maximum at 280 nm developed in the spectrum of ascorbylated protein. A negligible increase in the absorbance above 300 nm was observed for fructose-modified protein. This broadband absorption of glycated proteins is considered to result from formation of a number of chromophores during glycation reactions. Since the UV lamp used did not emit radiation below 340 nm, the absorbance at 365 nm (the wavelength of maximal lamp emission) was recorded as a measure for the relative ability of studied carbonyl compounds to form chromophores absorbing at this wavelength. The UV characteristics of different AGE-preparations are listed in Table I.

The fluorescent emission at 440 nm, excited at 370 nm, was appreciably enhanced in glycated proteins, indicating that AGEs were formed (Table I). The highest quantum yields showed fluorophores produced by MGO and GO. No increase in fluorescence was observed with longer incubation periods for these AGEs. However, the longer incubation period considerably enhanced the intensity of non-tryptophan fluorescence of ascorbic acid-modified proteins.

Isoelectric focusing (Fig. 2) revealed a dramatic decline in the positive charge for MGO-glycated protein followed by GO- and ascorbic acid-modified proteins, respectively. Fructosylated protein was the closest to the native one. This sequence was consistent with the relative ability of different carbonyl compounds to form fluorophores (Table I, column 4).

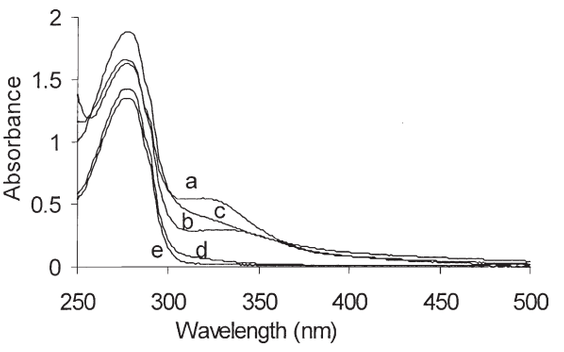

FIGURE 1 UV spectra of lens proteins incubated with: (a) 25 mM methylglyoxal for 3 weeks; (b) 25 mM glyoxal for 3 weeks; (c) 25 mM ascorbic acid for 3 weeks; (d) 500 mM fructose for 6 weeks; (e) shows the spectrum of native lens protein. Spectra are scanned using 1 mg/ml solutions in 7 M urea.

TABLE I Characteristic data of model AGE preparations

Carbonyl compound	Incubation period (weeks)	Optical density at 365 nm (absorption units/mg)	Non-tryptophan fluorescence (em. 440/ex. 370 nm) arbitrary units/mg)
Control	0	0.003 ± 0.000	7.7 ± 0.3
Control	2	0.003 ± 0.000	7.6 ± 0.2
Control	6	0.005 ± 0.001	7.4 ± 0.1
Fructose	6	0.013 ± 0.002*	8.2 ± 0.4
Methylglyoxal	2	0.176 ± 0.007*	25.5 ± 0.8*
	3	0.190 ± 0.011*	24.9 ± 0.5*
Glyoxal	2	0.163 ± 0.007*	25.8 ± 0.3*
	3	0.167 ± 0.006*	24.1 ± 0.6*
Ascorbic acid	2	0.168 ± 0.004*	15.1 ± 0.1*
	3	0.180 ± 0.006*,†	20.7 ± 0.6*,†

*$P < 0.01$ vs. non-incubated control. †$P < 0.05$ vs. the protein incubated with ascorbic acid for 2 weeks.

Production of Reactive Oxygen Species by Glycated Proteins upon UVA Irradiation

For better comparison between photosensitizer properties, glycated lens proteins were assayed for singlet oxygen and superoxide radical production upon UVA dose of 200 J/cm^2. The control protein produced 0.92 μmol $^1O_2$ per mg protein (Fig. 3A, bar 1). Similar amount of singlet oxygen was generated by fructosylated protein (1.17 μmol/mg). A significant increase in singlet oxygen formation resulted from other glycated proteins. MGO-modified protein produced 77–93% more singlet oxygen and *ca.* 2–3-fold higher concentrations in comparison to control were generated by GO- and ascorbic acid-glycated proteins. No incubation period dependence on the produced $^1O_2$ was observed for MGO- and GO-glycated proteins. In contrast ascorbylated proteins obtained after different incubation periods, which had different AGE characteristics, produced statistically distinguishable quantities of singlet oxygen (Fig. 3A).

All glycated proteins produced higher quantities of superoxide radical than native protein (Fig. 3B). However, the changes in photosensitizer activity were not that large as observed for singlet oxygen production. Quantity of superoxide radical was increased by *ca.* 80% in ascorbylated and MGO-modified proteins (incubation period of 3 weeks) when compared to the control. Although fructose did not cause significant alterations in the protein as shown in Table I and Figs. 1 and 2, fructosylated protein depicted commensurable production of superoxide radical with other glycated proteins.

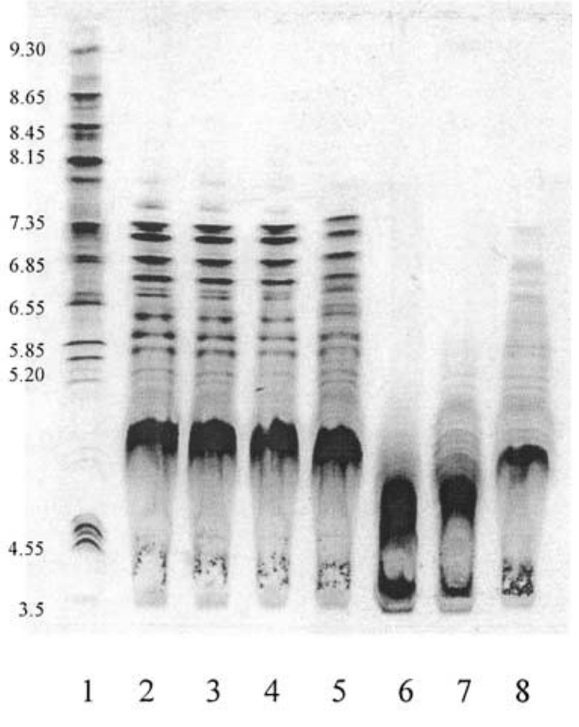

FIGURE 2 Isoelectric focusing of glycated lens proteins. Lane 1, broad range pI markers (Amersham Pharmacia Biotech, UK); lane 2, non-incubated control; lane 3, incubated control (2 weeks); lane 4, incubated control (6 weeks); lane 5, fructose-modified protein; lane 6, methylglyoxal-modified protein (3 weeks); lane 7, glyoxal-modified protein (3 weeks); lane 8, ascorbic acid-modified protein (3 weeks).

Effect of Glycated Proteins on Lens Epithelial Cells after UVA Irradiation

TBARS, indicating lipid peroxidation, were found at a concentration of only 36 ± 7 nmol/μg cellular protein in the medium of control non-irradiated cells but at 114 ± 7 nmol/μg after UVA irradiation (Fig. 4A). Supplementation of PBS medium with native lens protein did not change significantly the TBARS level relative to the irradiated control, indicating neither an enhanced lipid peroxidation nor a protective effect against it. Irradiation of the cell layers covered with solutions of glycated proteins remarkably accelerated lipid peroxidation. The presence of ascorbylated proteins in culture medium led to approximately 6 fold higher TBARS concentration compared to the irradiated control.

Effects of the described experimental settings on cell survival are demonstrated in Fig. 4B. When an UVA radiation dose of 40 J/cm^2 comparable to the daily quantum reaching the eyes *in vivo*[30] was

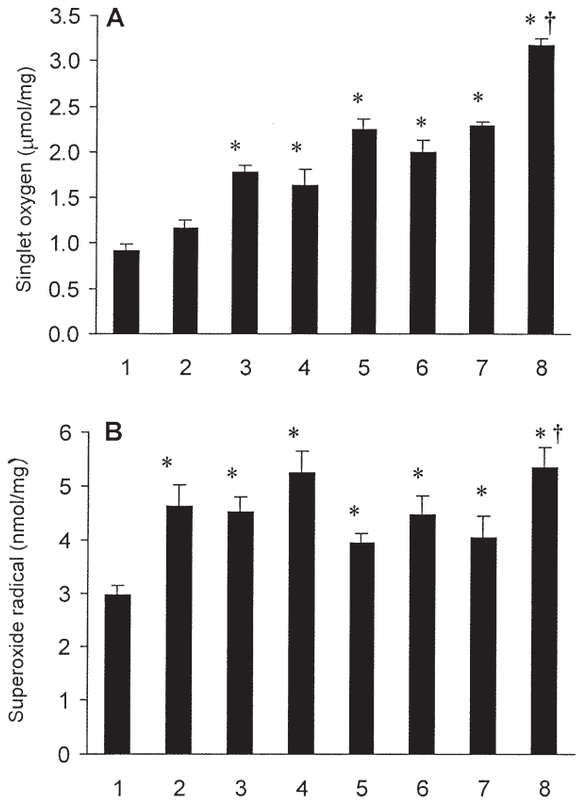

FIGURE 3 Reactive oxygen species produced by glycated proteins: (A) singlet oxygen (μmol/mg protein), and (B) superoxide radical (nmol/mg). 1, Control native protein; 2, fructose-modified protein; 3–4, methylglyoxal-modified proteins (incubated for 2 and 3 weeks, respectively); 5–6, glyoxal-modified proteins (incubated for 2 and 3 weeks, respectively); 7–8, ascorbic acid-modified proteins (incubated for 2 and 3 weeks, respectively). Values are means of three measurements ±SD. *, denotes significant difference vs. control protein; †, denotes significant difference vs. protein incubated with the same glycating agent for 2 weeks.

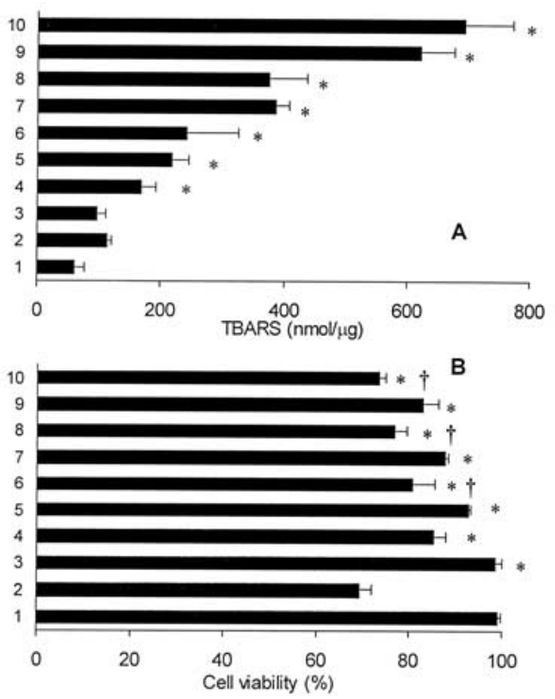

FIGURE 4 Lipid peroxidation and cell viability of lens epithelial cells after UVA irradiation evaluated by means of: (A) released TBARS (nmol/μg) into cell culture medium and (B) LDH leakage. 1, non-irradiated cells; 2, cells irradiated through PBS; 3, cells irradiated with PBS containing native protein; 4, cells irradiated with fructose-modified protein; 5–6, cells irradiated with methylglyoxal-modified proteins (2- and 3-week incubation period, respectively); 7–8, cells irradiated with glyoxal-modified proteins (2- and 3-week incubation period, respectively); 9–10, cells irradiated with ascorbylated proteins (2- and 3-week incubation period, respectively). Values shown are means ± SD of four dishes for every experimental condition. *, denotes significant difference vs. cells irradiated with PBS alone. †, denotes significant difference vs. protein incubated with the same glycating agent for 2 weeks.

applied to lens epithelial cells covered with PBS alone, cell viability was reduced to 69.17 ± 2.78%. In contrast cell survival was close to that of non-irradiated cells when the cell layer was covered with PBS solution containing native lens protein (Fig. 4B, bar 3). Similarly, the experimentally glycated proteins demonstrated significant protective effects on cell viability compared to cells irradiated through PBS alone. However, the protective effect of glycated proteins was significantly reduced in correlation with their photosensitizer activity. The strongest producer of reactive oxygen species (protein incubated with ascorbic acid over a 3-week period, see Fig. 4B, bar 10), reduced cell viability to 73.25 ± 1.65%.

DISCUSSION

For proper evaluation of UV induced impacts on our visual organ, especially in lens aging and causes of cataract, a better understanding of photodynamic reactions of intrinsic crystalline-bound photosensitizers is required.

As shown in Fig. 3A,B 0.92 μmol/mg singlet oxygen and 2.97 nmol/mg superoxide radical were produced by unmodified protein. Glycation of the same protein led to significant increases in the amount of produced activated oxygen species. We intended to establish the correlation between the level of protein glycation and photosensitizer activity as evidence that the generated singlet oxygen and superoxide radical originate from AGE-related structures. This assumption was in part supported by the presented results. The good correlation ($r = 0.828$) found between the absorbance at 365 nm and the quantity of released singlet oxygen suggests that newly formed chromophore(s) participate in type II photodynamic reaction. Distinguishable quantities of generated oxygen species were observed only for ascorbylated proteins obtained

after different periods of incubation, which varied in their AGE characteristics, but were not observed for MGO- and GO-glycated proteins.

The basic lysines and arginines are recognized to be the amino acid residues modified during the glycation reaction. Thus, shifts in the net charge of glycated proteins to more negative values are proportional to the number of the basic groups involved in glycation. Increased acidity also may be indicative of the formation of AGEs containing carboxyl group, e.g. N_ε-(carboxymethyl)lysine and N_ε-(carboxyethyl)lysine, but all of the studied carbonyl compounds are capable of producing such AGEs depending on their reactivity. Neither the singlet oxygen nor the superoxide radical formation correlated with the changes in isoelectric point of glycated proteins, which indicates that the concentrations of AGE-related structures *per se* are not decisive for the demonstrated photosensitizer activity. Most likely the differences in photosensitizer activities are due to variations in chemical structures of AGEs involved in photodynamic reactions. The relatively high concentration of superoxide radical released by fructosylated protein assumes that fructose might form a specific photosensitizer participating in type I reaction.

Both $^1O_2$ and O_2^- productions do not correlate well with the intensity of non-tryptophan fluorescence ($r = 0.532$ and 0.480, respectively). This finding was not unexpected because intensively fluorescing compounds have higher singlet and lower triplet quantum yield, and thus are poor photosensitizers.

The role of ascorbic acid in the lens is not well understood.[31] It appears that it acts as an antioxidant protecting against oxidative damage but experimental data indicate that it can also participate in oxidative modification of lens proteins during aging, e.g. through formation of photosensitizers in the lens. Ascorbylated proteins did not possess the highest level of glycation among the proteins tested here as shown in Fig. 2 but they produced the highest quantity of activated oxygen species after UVA irradiation. Apparently ascorbic acid forms a specific UVA absorber or even more than one capable of producing $^1O_2$ and O_2^- with high quantum yield (Fig. 3A,B, bars 7 and 8).

However, the glycation of lens proteins with MGO, GO and fructose may also be implicated in photosensitizer activity of aged and cataractous lens since AGE-like structures formed by these carbonyl compounds are capable of producing singlet oxygen and superoxide radical upon UVA irradiation (Fig. 3A,B, bars 3–6).

The possibility that some reactive oxygen species are released from pre-existing protein bound photosensitizers cannot be excluded. Keeping with this concept are the measurable concentrations of singlet oxygen and superoxide ion produced by non-modified lens protein. The alterations in chemical structure caused by glycation result in conformational changes that might expand the number of light-reactive structures on the protein backbone (e.g. tryptophan residues) and hence make them more susceptible to photooxidation. Since there is no quantitative measure for changes in the crystallin conformation, it is difficult to determine the contribution of this effect to the entire photosensitizer activity of the glycated proteins.

Singlet molecular oxygen and superoxide radical can harm living systems by oxidizing critical organic molecules. In our *in vitro* experiments the photodynamic reaction II was favored because generated superoxide radical was about three orders of magnitude less than singlet oxygen. The former produces hydrogen peroxide (H_2O_2) by spontaneous or enzymatic dismutation. The O_2^- and H_2O_2 may in turn participate in Haber-Weiss or Fenton reactions to produce the very harmful hydroxyl radical. Thus, a minor production of O_2^- cannot be excluded as an important contributor to photooxidative injury.

Under experimental irradiation of lens epithelial cells glycation-related photosensitizers produced extracellular reactive oxygen species. Prime targets of their damaging effects are the cell membranes and the triggered lipid peroxidation is most often assessed by TBARS assay. A good positive correlation ($r = 0.930$) of measured TBARS concentrations with the quantity of singlet oxygen produced by different AGE preparations prompted us to consider $^1O_2$ as the main initiator of lipid preoxidation upon UVA irradiation. This assumption is in agreement with another studies on the involvement of singlet oxygen in the UVA radiation-dependent formation of TBARS.[32,33]

UVA cytotoxic effect has been studied on many types of cells including lens epithelial cells[34] and could be substantiated by our studies as well. Exposure of lens epithelial cells to physiological levels of UVA reduced cell viability by 30% (Fig. 4B, bar 2). As detailed in Fig. 4A,B, cell viability does not follow the slope of membrane peroxidation. An explanation for this result could be that TBARS levels were measured immediately after irradiation and they indicate the direct and rapid effects of produced reactive oxygen species. In contrast, the lactate dehydrogenase leakage reflects the state of cultured cells 2 h after irradiation.

The protective effect of lens proteins on cell survival observed in our experiments has been attributed to α-crystallin that was present in our preparations as well. This protein is believed to act as a molecular chaperone *in vitro* supporting lens epithelial cell resistance to UVA stress.[35] However, glycation decreases this chaperone function.[36] In the light of our investigations this implies that ascorbylated proteins, which are less modified than

MGO- and GO-glycated proteins, might have better chaperone-like properties although they are more active as photosensitizers. Thus the reduction in cell viability presented in Fig. 4B can be considered as a sum of overlapping and to some extent opposing effects.

Lens epithelium *in vivo* has one of the highest concentrations of reduced glutathione among all investigated cell types.[37] It also contains a large number of antioxidant enzymes, demonstrating adequate activity to protect the cells against oxidative stress. The here presented results demonstrate that reactive oxygen species produced by glycated proteins via photodynamic reactions can cause significant oxidative injury even on the well-protected lens epithelial cells. Although UVA dose reaching the lens core is lower than that at the lens surface, this damage might be more pronounced in the deeper fiber cells i.e. in the older part of the lens where highly glycated proteins are localized in maximal concentrations and in which glutathione concentration[38] and antioxidant enzyme activities[3] are lower than in the lens epithelium.

It is now generally accepted that many changes associated with lens opacity in the elderly, under diabetic and other conditions result from oxidative stress within the lens and especially so in the outer components of the lens.[39] This study details that AGEs formed from most abundant carbonyl metabolites in the lens are sources of reactive oxygen species being exposed to UVA radiation. AGE structures may exert deleterious effects not only through crosslinks between crystallins but also by rendering them into active photosensitizers. Photosensitization triggers lipid peroxidation and thus causes abnormal fluidity in the cell membranes, which in turn disrupts vital functions, such as maintenance of selective permeability to ions or the signal transduction. Aged and diabetic lenses with a reduced antioxidant defense system would be especially prone to these pathways.

In conclusion then, it appears conceivable to assume that the reaction of the lens proteins with reactive oxygen species derived from AGE-related photosensitizers could be of prime causative importance for accelerated pathogenic changes in the elderly and under diabetic conditions.

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