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Article

Oxidative Modifications in Crystallin Proteins and Lens Epithelial Cells Associated with Photosensitized Reactions Mediated by the Major Chromophore Arising from Glucose Degradation

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Deleterious effect mediated by glucose degradation, as a parallel pathway to Maillard reaction, was analyzed in terms of the feasibility of inducing photo-crosslinking in isolated bovine crystalline proteins when exposed to ultraviolet A (UVA)-visible light. These experiments showed evidence supporting the generation of a glucose-derived chromophore (GDC). The ability of this chromophore to induce oxidative damage in lens protein and bovine lens epithelial cells (BLEC) was further assessed. The analysis of dityrosine and carbonyl levels in lens proteins irradiated at 5 and 20% O_2 indicates the occurrence of mixed type I/type II photosensitizing mechanisms. When BLEC were exposed to photosensitized reactions induced by GDC a decrease in cellular viability and intracellular reduced (GSH) and oxidized (GSSG) glutathione ratio was observed, as well as an increase in the amount of intracellular reactive oxygen species. Our data suggest a major effect of type I photosensitizing mechanism in both lens proteins photo-oxidation and oxidative stress induced in BLEC.

Keywords: cataract disease, photosensitizing reactions, oxidative stress, bovine crystalline proteins, bovine lens cell model

Introduction

Cataract is one of the main causes of blindness worldwide and currently it has been proposed to play a role in systemic health through disruption of the circadian biological clock.[1,2] Sunlight has been established as a risk factor for nuclear and cortical cataract diseases.[3-5] In the same sense, the effect of the cumulative light-exposure from the modern and widely used white light-emitting diodes (LED) as a source of potential oxidative risk to the lens epithelium has recently been matter of study.[6,7] Hyperglycemia also constitutes a risk factor for cataract development.[8,9] This fact has been normally associated with the high prevalence of this disease in type 2 diabetes subjects,[8,9] but more recently, it has been determined that even dietary patterns associated with the intake of high glycemic index foods are also a risk factor for cataract disease.[10] The synergism between two risk factors for cataract development, such as hyperglycemia and ultraviolet A (UVA)-visible light, has not been previously assessed but could take place through the generation of chromophores with photosensitizing capacity produced as a consequence of chronic hyperglycemia states.

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Whereas nuclear cataract involves the accumulation of highly damaged proteins in the lens nucleus, cortical cataract has been mainly associated with impairment of lens epithelial cells. Considering that UVA light in sunlight is nearly a thousand times more intense than ultraviolet B (UVB), the study of the actual effects of UVA-Visible light in eye lens components is particularly relevant to understand the mechanisms involved in the photo-aging of this tissue. Taking into account the transparency of eye lens, the occurrence of UVA-Visible-mediated photochemical processes makes necessary the presence of photosensitizers that absorb in this region of the electromagnetic spectrum.[11-13] The human eye lens contains high concentrations of glycating agents, such as ascorbate and glucose.[14] These compounds can give rise to chromophores by means of the reaction with proteins and the consequent generation of advanced glycation end products (AGEs), but also by degradation reactions. We have recently reported that glucose degradation under simulated eye lens physiological conditions can generate a single chromophore with photosensitizing capacity.[15,16] This chromophore possesses identical chromatographic and spectral properties to one identified in the water soluble and non-protein fraction of human cataractous eye lenses.[15] The photosensitizing capacity of this chromophore is poorly understood and could constitute a pathway of oxidative stress generation in the eye lens. The induction of oxidative stress by photosensitized reactions can proceed by two mechanisms: a type I photosensitizing mechanism, which produces free radicals and hydrogen peroxide; and a type II photosensitizing mechanism, producing singlet oxygen as the main reactive species. The generation of reactive oxygen species (ROS) produced through photosensitized reactions in the human eye lens is strongly affected by the low oxygen concentration present in this tissue, the type I mechanism being favored, which does not involve the participation of oxygen in the inactivation of the triplet excited state of the photosensitizer.[17]

The induction of oxidative stress to crystalline proteins and lens epithelial cells are particularly relevant because both are key systems in maintaining lens functionality and are also impaired during nuclear and cortical cataract generation, respectively.[18-21] However, the photosensitizing effect mediated by the major chromophore arising from glucose degradation has not been assessed in these systems.

In this work we have studied the generation of chromophores from glucose and the photosensitizing capacity of the major chromophore arising from its degradation, in terms of the effects of the photosensitizing mechanism in oxidative chemical modifications in bovine lens proteins and epithelial cells.

Experimental

Reagents

D(+)-Glucose (100% pure) was obtained from Fluka Chemie GmbH (Buchs, Switzerland). Na_2HPO_4, NaH_2PO_4 and $CuSO_4$ were obtained from Merck (Darmstad, Germany). The OxyBlotTM protein oxidation detection kit was obtained from Chemicon International (Temecula, USA). Ethylenediaminetetraacetic acid (EDTA) was purchased from BDH Chemica (Poole, Dorset, England). Ultra-pure water from a Milli-Q system (Billerica, USA) was used for the preparation of all solutions, which were filtered through a 0.22 μm sterile nitrocellulose filter.

Isolation of water soluble bovine lens proteins

Bovine lenses (approximately 18 months old) were obtained from a slaughter-house. Twenty bovine lenses were decapsulated and stirred in a 50 mmol L^{-1} Tris-HCl buffer pH 7.4 containing 0.2 mmol L^{-1} KCl, 1 mmol L^{-1} EDTA, 10 mmol L^{-1} β-mercaptoethanol and 0.05% NaN_3. The suspension was homogenized and centrifuged at 15,000 rpm for 30 min at 4 ºC (Sorvall superspeed RC2B, Thermo Fisher Scientific, Waltham, USA). The supernatant was extensively dialyzed against deionized water at 4 ºC. Potassium phosphate buffer was added in order to achieve a protein concentration of 50 mg mL^{-1} and 0.5 mol L^{-1} phosphate buffer pH 7.4.

Protein crosslinking in the presence and absence of UVA-Visible light

Water soluble lens proteins (10 mg mL^{-1}) were incubated with 30 mmol L^{-1} D(+)-glucose in a 0.1 mol L^{-1} phosphate buffer (pH 7.4) or a carbonate-bicarbonate buffer 0.1 mol L^{-1}, in the case of incubations performed at pH 9.4. Samples were incubated in the presence of 5 μmol L^{-1} $CuSO_4$ as a catalyst. Controls of glucose (30 mmol L^{-1}) and lens proteins (10 mg mL^{-1}) in the presence of $CuSO_4$ 5 μmol L^{-1} were prepared.

A volume of 5 mL of each reaction mixture was sterile-filtered through a 0.22 μm nylon syringe filter into a sterile glass test tube. The samples were bubbled with a nitrogen-oxygen gas mixture (5% O_2) and each test tube was covered by precision SealTM rubber septa (Sigma-Aldrich, St. Louis, USA) in order to keep the low oxygen concentration.

The samples were divided in two groups: one was wrapped in aluminum foil and the other was continuously exposed to light, 24 h *per* day, for 3, 6 and 9 days. Both

groups, the wrapped and unwrapped tubes, were incubated at 37 ºC in a water bath under exposure to UVA-Visible light. The light source was a high-pressure mercury lamp (400 W Philips E-40, Philips, Amsterdam, The Netherlands) and an ordinary glass was used to filter the radiation below 330 nm. Light intensity measurements were performed with a Suga RAX34C radiometer (Tokyo, Japan). Incident intensity on the tubes was 18 W m^{-2}.

Irradiation conditions for bovine lens proteins

Solutions of bovine eye lens proteins at 20 mg mL^{-1} were prepared with the glucose-derived product at an absorbance of 0.2 at 365 nm, diluting the proteins and the chromophore with phosphate buffer, pH 7.4. During the experiments, solutions were bubbled with 5 or 20% O_2. All experiments were performed in a 1 cm light-path quartz cuvette at 25 ºC. Irradiations were performed with an OSRAM HBO 500 W high-pressure mercury lamp (Munich, Germany) filtered with a 5% (m/v) $CuSO_4$ solution in a 2.5 cm optical glass cell. Light intensity measurements were performed with a YSI Kettering 65A radiometer (Yellow Spring, USA). Incident intensity on the cuvette was 1500 W m^{-2}. Samples were lyophilized and kept frozen at −20°C until different analyses were performed.

SDS-PAGE analysis

Protein crosslinking was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis under reducing conditions. Samples (22.5 µg) were boiled for 5 min in Laemmli sample buffer and loaded onto 12% (m/v) SDS-PAGE gels.[22] Electrophoresis was performed at 100 V for 1-2 h. Gels were stained with 0.1% (m/v) Coomassie Brilliant Blue and destained in a solution containing 10% (v/v) ethanol and 0.75% (v/v) acetic acid up to 24 h. Gels were scanned with an Amersham Bioscience image scanner (Piscataway, USA) and the quantification of the crosslinked proteins was performed by densitometry analysis using ImageJ software.

Immunodetection of derivatized protein carbonyl groups

The determination of protein oxidation was performed by immunoblotting, using the OxyBlot™ Protein Oxidation Detection Kit (Chemicon International, Temecula, USA), according to manufacturer instructions, as it was previously described.[23] Scanning of the films was performed with Amersham Bioscience image scanner (Piscataway, USA). Signal intensities were quantified with ImageJ software.

Immunodetection of dityrosine modifications

Proteins were separated by SDS-PAGE according to the procedure described in previously and electrotransferred onto a Hybond nitrocellulose membrane (GE Healthcare, Piscataway, USA) that was then blocked with phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) and 0.1% tween 20. Membranes were incubated with an anti-dityrosine monoclonal primary antibodies (Cosmo Bio Corp, Tokyo, Japan), at a dilution of 5/1,000 and incubated overnight under agitation at 4 °C. Posteriorly, membranes were washed with PBS, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Membranes were revealed with enhanced chemiluminescence (ECL) plus chemiluminescent detection system (GE Healthcare, Piscataway, USA). Scanning of the films was performed with Amersham Bioscience image scanner (Piscataway, USA). Signal intensities were quantified with ImageJ software.

In vitro generation and purification of the major chromophore arising from glucose degradation

A solution of glucose (30 mmol L^{-1}) in phosphate buffer 100 mmol L^{-1}, pH 7.4 was bubbled with oxygen at 5% during 30 min and then sealed in order to keep the low oxygen concentration. All this procedure was made in sterile conditions. The solution was incubated during 30 days at 37 ºC in the dark. After this period, glucose concentration was determined by photometric measurements by means of glucose-dye-oxidoreductase, with color indicator. At the end of this period, the absorption spectra were recorded on a Hewlett Packard 8453 spectrophotometer (Colorado Spring, USA). The samples were distributed in aliquots and kept frozen at −20 ºC, until purification. Thawed samples were purified by exclusion chromatography on a Sephadex G-15, considering that it has been demonstrated to be efficient in the separation of low molecular mass molecules, such as tyrosine (Tyr) and dityrosine, among others.[24,25] Milli Q water was used as the mobile phase. The different fractions were recovered by means of a fraction collector LKB/Pharmacia 2070 (Uppsala, Sweden). The flow rate was adjusted to 0.2 mL min^{-1}, collecting in each tube a volume of 1 mL. The fractions were analyzed by means of absorption spectroscopy.

Isolation of bovine lens epithelial cells and *in vitro* culture

Cells from bovine lens were prepared according to the method described by Kurosaka and Nagamoto.[26]

Briefly, bovine eyes were washed with physiological saline containing 50 μg mL^{-1} gentamicin. The lenses were excised under aseptic conditions in a laminar flow hood; the epithelial cells were gently scraped off, aspirated and added on to 5 mL of complete culture medium, composed by Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, USA) supplemented with 20% fetal bovine serum (FBS, Hyclone, St. Louis, USA), and 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin (Invitrogen, Carlsbad, USA). The cell suspension was centrifuged at 4,000 rpm during 5 min at 4 °C, the supernatant was discarded and the pellet was re-suspended in 2 mL of the same culture medium. Cells were seeded into 75 cm^2 tissue culture flasks and grown in complete medium under a humidified atmosphere at 37 °C and 10% CO_2. After reaching confluence, cells were harvested using trypsin/EDTA (Invitrogen, Carlsbad, USA) and subcultured at a ratio of 1:5. Only cells from passages 1 and 2 were stored frozen in liquid nitrogen, since their morphology assessed under light phase-contrast microscopy maintains their epithelial features. Within 1-3 days after becoming confluent, the cells were harvested with trypsin/EDTA and plated on Costar cell culture inserts at a density of 10^6 cells *per* insert. Cells reached confluence in 5-7 days and then, they were fed every 2-3 days. The epithelial morphology of the lens cells was assessed under fluorescence microscopy, using a mouse anti-cytokeratin monoclonal antibody (clone 8.60 of IgG1 isotype, Sigma-Aldrich, St. Louis, USA).[27] All experiments were performed with epithelial cells between passages 2-8, to avoid cellular differentiation. For experiments, lens cells were grown at 37 °C in a humidified atmosphere (10% CO_2 in air) in Dulbecco's medium supplemented with 10% FBS, penicillin-streptomycin mix, L-glutamine 0.04 mmol L^{-1} and fungizone 5 μL mL^{-1} (Invitrogen, Carlsbad, USA).

UVA-Visible light treatment in BLEC

Bovine lens epithelial cells (BLEC) were platted at 200.000 cells mL^{-1} and grown to 80% confluence on a 100 mm plastic culture dishes (Corning Costar, Cambridge, USA). Cells were irradiated using Hanks medium, pH 7.4. The absorbance of glucose-derived chromophore (GDC) was adjusted to 0.2 (at 365 nm) with Hanks medium and the irradiations were carried out for 2 and 4 hours, under low oxygen concentration (5%). Cells were exposed to sub lethal doses, using 12 black light lamps Philips model T5/G5/8W/BLB (UVA radiation at 365 nm = 1.2 W). Light intensity measurements were performed with a YSI Kettering 65A radiometer (Yellow Spring, USA). Incident intensity on the system was 10 W m^{-2}. After irradiation, the solution was completely removed and the BLEC were immediately analyzed or incubated in medium at 37 °C and 5% CO_2 for 24 h. Two types of controls were used, cells irradiated in absence of GDC and cells incubated at dark in the presence of GDC (A_{365} = 0.2).

MTT assay for cell viability

Cells were grown in a 96 well plate (1.5×10^4 cells *per* plate) and irradiated with 100 μL of a solution of GDC prepared in Hank medium, previously adjusted to an absorbance of 0.2 at 365 nm. Cell viability was evaluated 24 h after UV irradiation, by means of the reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay, Sigma Aldrich, St. Louis, USA), according to the manufacturer instructions.[28] The absorbance was measured at 570 nm and against a reference wavelength (690 nm) with an enzyme-linked immunosorbent assay (ELISA) reader (Cambridge Technology, Inc., Bedford, USA). All experiments were performed three times in triplicate. The reported data were expressed as percentages, relatives to the control value (medium alone, assigned as 100%).

Analysis of intracellular ROS levels

The BLECs were seeded at 10,000 cells *per* well in 96-well plates. Twelve hours after seeding, non-adherent BLECs were washed with culture medium and 100 μL of a GDC solution (previously prepared adjusting to an absorbance of 0.2 at 365 nm) were added. The cells were exposed to light as it was previously described, in the presence of 20 μmol L^{-1} of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Invitrogen, Carlsbad, USA) in PBS for 30 min. Thereafter, the medium was discarded and the cells were washed with PBS. The fluorescence intensity was determined using a spectrofluorometer Perkin Elmer LS 55 (Waltham, USA), at excitation and emission wavelengths of 485 nm and 535 nm, respectively. Changes in peroxide levels were expressed in relative fluorescence unit (RFU) mg^{-1} of protein. All experiments were performed three times in triplicate.

GSH/GSSG intracellular levels

Cellular reduced glutathione (GSH) levels were analyzed using the glutathione assay kit (Sigma-Aldrich, St. Louis, USA). At least 10^8 cells were washed and suspended with PBS and then centrifuged at 600 × g to obtain a packed cell pellet and the quantification of GSH/oxidized glutathione (GSSG) ratio (GSH/GSSG) levels were carried out according to the manufacturer instructions.

Protein concentration was assessed using BCA protein assay kit (Thermo Fisher Scientific, Waltham, USA). Results were normalized to the protein content of each culture well. All experiments were performed three times in triplicate.

Statistical analyses

The results of the experiments are expressed as the mean ± standard deviation (SD). Comparisons between groups were made using two-way analysis of variance (ANOVA). Graphics and statistical analysis were performed using Graph Prism 5 software (La Jolla, USA).

Results and Discussion

UVA-Visible light induces additional cross-linking in lens proteins incubated in the presence of glucose

We have previously reported that models of AGEs derived from incubations of lysine (Lys) with glucose are approximately 25% more effective than AGEs produced from incubations of Lys with ascorbate to induce photosensitized degradation of tryptophan (Trp).[29] For this reason we conducted experiments aimed to determining the contribution of photosensitized reactions mediated by AGEs derived from glucose, in a model where bovine eye lens proteins were incubated with this monosaccharide in the presence and absence of permanent exposure to UVA-visible light.

For these purposes, bovine lens proteins were incubated at low oxygen concentration for 10, 20 and 30 days in the presence of 30 mmol L^{-1} glucose and 5 μmol L^{-1} $CuSO_4$, at pH 7.4 and 9.4. The aim of increasing pH was to enhance the nucleophilicity of amino acids that possess basic side chains (mainly Lys and arginine), improving the reactivity towards the carbonyl group of the sugar and, consequently, generating AGEs in a faster way, compared to that observed at physiological pH.

Figure 1a shows the SDS-PAGE profile under reducing condition of eye lens proteins incubated in the presence of glucose at the two mentioned pH values. An increase in protein crosslinking with the incubation time was observed in both pH values (7.4 and 9.4), particularly in the top region of the resolving portion of the gel, where fractions with molecular mass higher than 37 kDa are found. However, the samples incubated at the highest pH values were characterized by a more extensive crosslinking, which is in accordance with the previous hypothesis. This fact is in agreement with previous studies, where it has been demonstrated that the formation of Amadori compounds increases at high pH values.[30] In this study, the formation of the Schiff base complex was proposed to be the rate limiting step in Maillard reaction.[30]

The contribution of UVA-Visible light to the generation of glucose-induced protein crosslinking was assessed by incubating bovine lens proteins at low oxygen concentration with glucose (30 mmol L^{-1}) at pH 9.4 and 37 °C for 3, 6 and 9 days, under permanent exposure to UVA-Visible light. Control experiments were also carried out using the same conditions, but incubating in the absence of UVA-Visible light. When bovine lens proteins were incubated during the same time period in the absence of glucose and exposed to UVA-Visible light, no significant modifications in the SDS-PAGEs profiles were observed (data not shown).

Figure 1b shows that UVA-Visible light induces additional protein crosslinking in the region between 28 and 195 kDa of the resolving portion of the gel, to that produced as a consequence of AGE generation (samples incubated in the dark). This complementary photochemical contribution was more intense than those observed in an experiment with a similar design but performing the incubations in the presence of ascorbic acid (3 mmol L^{-1}), at pH 7.4.[23] In those experiments it was determined that the additional protein crosslinking was originated from photosensitized reactions mediated by a colored product arising from ascorbate degradation.[23] For this reason and with the aim to identify the presence of the chromophore generated during decomposition of glucose, the spectral changes that experiment glucose solutions incubated during 10, 20 and 30 days at pH 9.4 were assessed. Figure 1c shows the spectral modifications that occur in the UV-Visible region during the glucose degradation. The presence of absorption bands at 210, 270, 345 (shoulder) and 365 nm is clearly appreciated. These spectral properties can also be observed when incubations were performed at pH 7.4, suggesting that both degradation compounds are the same,[15] which was confirmed by high-performance liquid chromatography (HPLC) analyses (see Supplementary Information). In a previous report, the chemical nature of this chromophore was analyzed by means of HPLC mass spectrometry and was found to be composed of furan subunits.[15] The pH value also plays a role in the extension of glucose degradation. Thus, 7.3, 11.5 and 19.5 μmol of glucose were decomposed when a 30 mmol L^{-1} of glucose solution was incubated at pH 7.4 for 10, 20 and 30 days, respectively. These values increase to 10.5, 16.9 and 28.6 μmol, respectively, of decomposed glucose when the experiments were made at pH 9.4.

The contribution of photosensitized reactions mediated by the major chromophore derived from glucose degradation to protein crosslinking was assessed irradiating bovine lens proteins with a purified fraction of this compound. Figure 1d

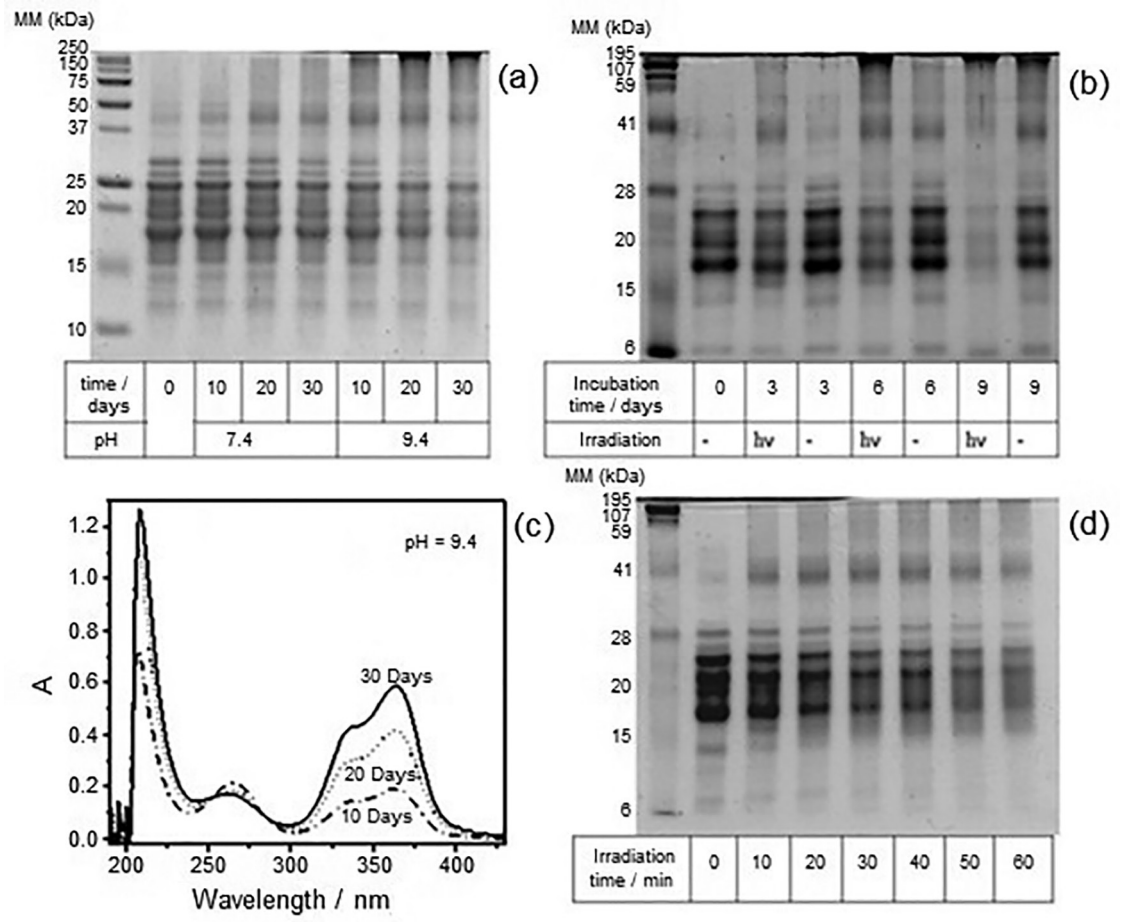

Figure 1. Eye lens proteins incubated with glucose, induce protein crosslinking in a pH-dependent manner and through ultraviolet A (UVA) visible light-induced photosensitized reactions mediated by a side product of the glucose degradation. (a) SDS-PAGE analysis of bovine lens proteins (10 mg mL^{-1}) incubated with glucose 30 mmol L^{-1} for 10, 20 and 30 days at pH 7.4 or 9.4; (b) SDS-PAGE analysis for lens proteins (10 mg mL^{-1}) incubated with glucose in the absence and presence of UVA-visible light; (c) absorption spectra of glucose (30 mmol L^{-1}) incubated for 10, 20 and 30 days in sterile conditions at 37 ºC, pH 9.4; (d) SDS-PAGE analysis of lens proteins (20 mg mL^{-1}) irradiated with UVA-Visible light (at 5% O_2) and the presence of purified glucose degradation chromophore.

shows the effect of the irradiation with UVA-Visible light in terms of the generation of protein crosslinking. A high efficiency in the production of intermolecular crosslinking for the glucose-derived chromophore (GDC) can be observed even at short times (10 min). Protein crosslinking (molecular mass region higher than 28 kDa) was observed to increase substantially after 10 min of irradiation and after the crosslinking degree was kept constant with irradiation time in the range of molecular mass until 195 kDa. However, a continuous decrease in the amount of proteins with molecular mass between 15-28 kDa can be noted, suggesting the generation of highly crosslinked proteins, which are not able to enter into the separating gel and, consequently, these products are not observed in the SDS-PAGEs profile.

When colored glycated lens proteins, obtained after 30 days of incubation in the presence of glucose and posterior dialysis to eliminate low molecular weight compounds, were exposed to UVA-Visible light and analyzed by SDS-PAGE, no modification in the electrophoretic pattern was observed (data not shown). This behavior was also observed when irradiations were performed with ascorbate-glycated proteins. These results were explained because AGEs-chromophores covalently bound to the proteins restrict their photosensitizing effect within the vicinity of their location and do not have the capacity to induce photochemical crosslinking.[31] Numerous studies have shown that the chemical environment of a photosensitizer plays a major role in its behavior when binding to proteins.[32-34] Protein-self-quenching of photo-induced transient species in the interaction between human serum albumin and Rose bengal has been reported.[32,33] In addition, it has been shown that partial denaturation of alpha crystalline alters the excited state properties of the photosensitizer oxyblepharismin when it is bound to the hydrophobic pocket of alpha crystallin.[34] The interaction between GDC and crystalline proteins deserves additional studies and will be assessed in the future.

Taking into account that the evidence found in this work, which suggest a simultaneous generation of glucose derived chromophores together with the generation of AGEs, the mechanisms of photo-oxidation mediated by this chromophore were assessed.

GDC photosensitized oxidative modifications in lens proteins are induced by a mixed type I/type II photosensitizing mechanism

The chemical nature of oxidative modifications introduced in proteins by photosensitized reactions depends on the photosensitizing mechanism, which can be modulated varying the oxygen concentration. In particular, the prevalence of the type II photosensitizing mechanism is favored when the oxygen concentration is increased. At low oxygen concentration, the type I mechanism is favored, where product arising from radical intermediaries should prevail. Therefore, to study the effect of the photosensitizing mechanism in the generation of oxidative modifications in lens proteins, the irradiations were performed at 5 and 20% of oxygen.

Figures 2a and 2b show representative oxyblots, where a continuous increase in protein carbonylation along the irradiation time can be observed, this increase was higher when the experiments were performed at 20% oxygen, which indicates the effectiveness of photosensitized reactions mediated by GDC to induce oxidative modifications in lens proteins. The presence of carbonylated proteins with molecular mass higher than 37 kDa indicates the occurrence of oxidative processes together with the generation of intermolecular crosslinking.

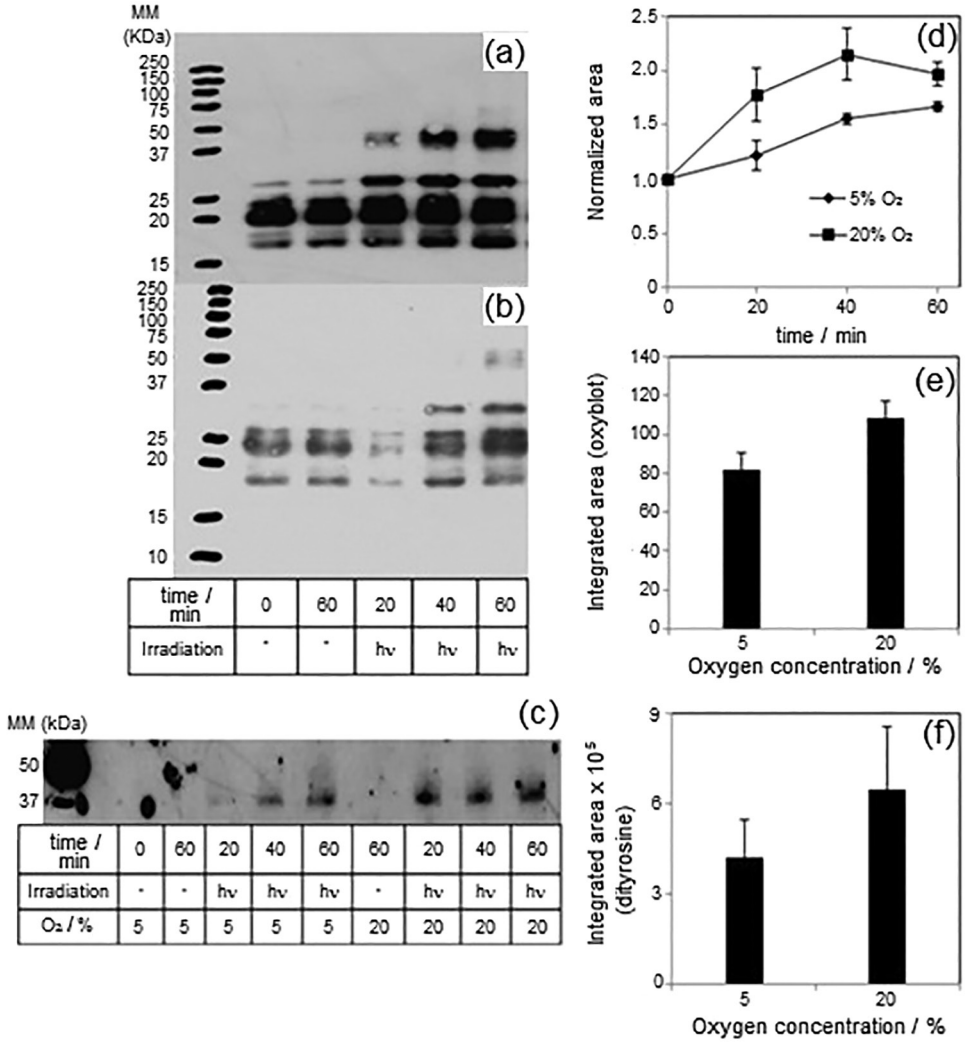

Figure 2. Effect of oxygen in oxidative modifications induced by photosensitized reactions mediated by the major chromophore arising from glucose-derived in eye lens proteins. (a) and (b) show oxyblot analyses when lens proteins were irradiated in the presence of the glucose degradation chromophore (GDC) under constant bubbling with 5 and 20% O_2; (c) shows immunochemical detection of dityrosine produced when lens proteins were irradiated in the presence of GDC at 5 and 20% O_2; (d) shows the densitometric analyses of oxyblots performed at 5 and 20% O_2; (e) and (f) show the integrated area of densitometric analyses of oxyblots and dityrosine blots performed at 5 and 20% O_2.

To determine the behavior of protein carbonylation by photosensitized reactions mediated by GDC, densitometric analyses were performed quantifying the peak intensity. Figure 2d shows the behavior of normalized area of carbonylated proteins during the irradiation time. The area under the curve of each plot was calculated in order to compare the effect of different oxygen concentrations. Figure 2e shows an increase in protein carbonylation when increasing oxygen concentration. These results indicate that singlet oxygen can also induce oxidative processes that result in increased protein carbonylation. However, the ratio (protein carbonyls, 20% O_2)/(protein carbonyls, 5% O_2) is only 1.3, although the oxygen concentration increases 4 times, which indicates the simultaneous and competitive occurrence of mixed type I/type II oxidizing mechanisms.

Dityrosine is an oxidative modification that can contribute to protein crosslinking. The role of photosensitized reactions mediated by GDC in the generation of dityrosine was assessed performing the irradiations at 5 and 20% O_2. The generation of dityrosine by photosensitized reactions was carried out by means of immunochemical detection using monoclonal antibodies that recognize specifically dityrosine adducts. Figure 2c shows the behavior of dityrosine generation when bovine lens proteins were irradiated for 60 min. It can be observed that dityrosine is generated during the irradiation of lens proteins and their levels increase with time, at both 5 and 20% oxygen concentration. Figure 2f shows a densitometric quantification of the integrated area of dityrosine formation with time, where irradiations performed at 20% O_2 can be observed to possess slightly higher levels of this modification. The ratio (protein dityr, 20% O_2)/(protein dityr, 5% O_2) is 1.5, which is also indicating the simultaneous and competitive occurrence of mixed type I/type II oxidizing mechanisms.

The occurrence of protein carbonyls and dityrosine formation induced by both type I and type II photosensitizing mechanisms has been previously established.[17,35-37] In particular, amino acids susceptible to being involved in photosensitizing reactions induced by a type I mechanism are mainly Trp, Tyr and histidine.[38,39] Formation of Trp-Trp and Tyr-Tyr crosslinks through radical mechanisms has been observed.[40-42] These processes are characterized by very low efficiencies in anaerobic conditions because oxygen is required to promote the photosensitizer regeneration.[43] This fact is in agreement with the results presented in this work.

BLEC exposed to UVA-Visible light in the presence of GDC show a reduction in their viability

Taking into consideration the results obtained *in vitro* that pointed to a possible *in vivo* role of GDC in the genesis of the protein modifications observed during aging and in patients with cataract,[44] it seemed opportune to study the effect of GDC on BLEC, when exposed to UVA-Visible light under a low oxygen concentration. For this purpose, a primary bovine lens epithelial cells culture was employed, which was extracted from fresh bovine eye lenses. Their epithelial nature was confirmed by immunofluorescent staining of cytokeratin (data not shown).

The interest on use of epithelial cells as a case study lies in the implication that has the epithelium at the origin of the cataract. Several studies suggest that the single layer of epithelial cells might be the first site of damage and, in fact, lens opacification frequently begins in this region,[45] as in the specific case of diabetic patients with cataract.[46]

BLEC cultures were exposed to UVA-Visible light during 2 and 4 h in the presence of GDC, which initial concentration was adjusted until an absorbance value of 0.2 at 365 nm. This concentration of GDC did not produce any interference with the MTT cell viability assay. In the first experiment, cell viability was determined immediately after the exposure of cells to light (Figure 3, 0 h) with the aim of determining the direct deleterious effect of light on cells and, in a second experiment, the previously irradiated cell cultures were incubated for subsequent 24 h in the dark, previous to the viability determination (Figure 3, 24 h). Control cultures not exposed to UVA-Visible radiation and incubated during 24 hours did not show toxicity in

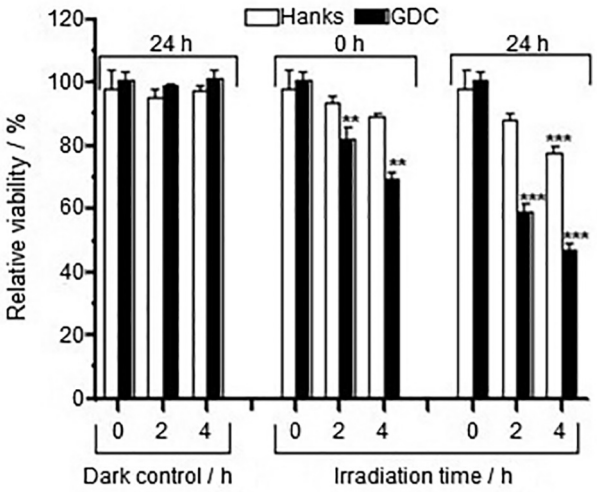

Figure 3. Effect of photosensitized reactions mediated by glucose derived chromphore on the viability of bovine lens epithelial cells. The cell viability was determined by means of the reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay), of bovine lens epithelial cells (BLEC) culture irradiated with ultraviolet A (UVA) visible light during 2 and 4 h in the presence of GDC. Non irradiated samples that were incubated during the same periods of time were used as dark controls. The cell viability was determined immediately after the incubation (0 h) and 24 h after the end of the experiment. The white and black bars correspond to samples incubated in the absence and presence of GDC, respectively. Results are expressed as means ± standard deviations (n = 3). $^{**}p < 0.01$, $^{***}p < 0.0001$ compared to their respective controls.

absence or presence of GDC (Figure 3, dark control). A decrease in cell viability was observed when the BLECs were irradiated with UVA-Visible light in the Hanks culture medium in the absence of GDC. This behavior was observed both in cells harvested immediately after the irradiation (Figure 3, 0 h) and also when they were analyzed 24 h later (Figure 3, 24 h). This fact can be explained by the presence of endogenous chromophores, such as riboflavin or nicotinamide adenine dinucleotide reduced (NADH), which can induce photosensitizing reactions in the intracellular milieu.[47] However, when the cells were irradiated in the presence of GDC, there was a considerable decrease of cell viability measured both, immediately after the irradiation (18 and 31% decrease at 2 and 4 h, respectively), and 24 h later of the irradiation periods (41 and 53% decrease at 2 and 4 h, respectively), showing a time-dependent decrease in cell viability.

During hyperglycemia, extracellular glucose diffuse into the lens, which can lead to post-translational modifications, including glycation and glycoxidation, among other processes, together with glucose autoxidation. In this respect, it seems important to underline that the presence of a compound with identical spectral and chromatographic properties to GDC, was observed in the water-soluble fraction of cataractous human eye lenses.[15]

The exposure of BELC cells to UVA-Visible light and GDC decrease GSH with a corresponding increase in GSSG levels

The photosensitizing effect of GDC on the levels of GSH and GSSG in UVA-Visible light exposed BLEC cells was studied. BLEC cell cultures were exposed for 2 and 4 h to UVA-Visible light in the presence or absence of GDC. Control cells incubated for 2 and 4 h in the dark, with or without GDC presented levels of GSH of approximately 80 μmol mg^{-1} of protein and only residual amounts of GSSG were detected (Figure 4a). The exposure of the cell cultures to light in absence of GDC produced a decrease of 15 and 24% in the normal level of GSH and a corresponding increase in the concentration of GSSG. When the same experiments were conducted in the presence of GDC, it was observed a 44 and 57% of loss in GSH level, which was also concomitant with an equivalent increase in GSSG.

Reduced glutathione in the lens epithelium has different functions. One of them is to protect the thiol groups of lens proteins, which are relevant for the normal activity of lens epithelium, i.e., enzymes Na-K-ATPase, thus influencing cell permeability.[48] A decrease in the relationship GSH/GSSG, which is normally high in

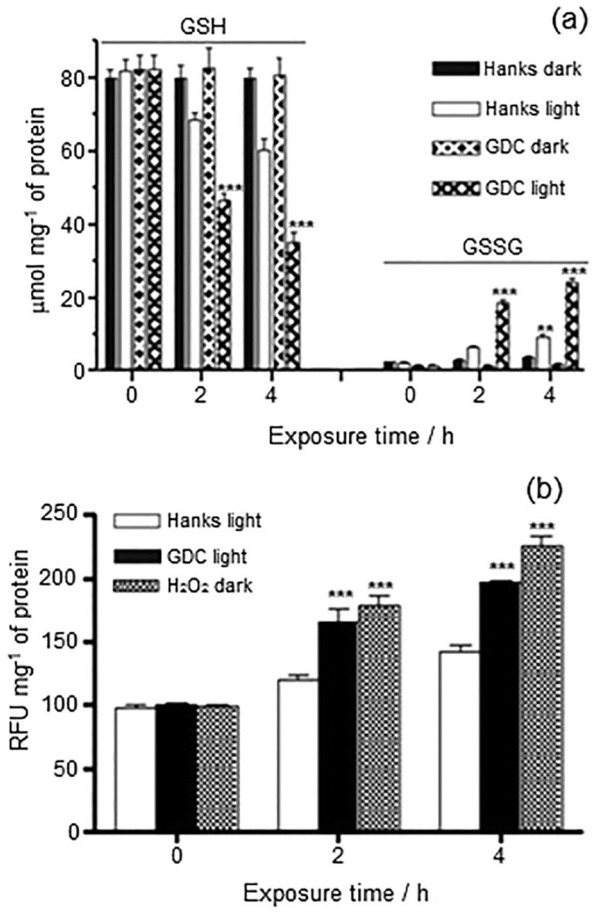

Figure 4. Effect of photosensitized reactions mediated by GDC on the redox status of bovine lens epithelial cells (BLEC). (a) Effect of ultraviolet A (UVA) visible irradiation in the presence of GDC on cytosolic levels of reduced glutathione (GSH) and oxidized glutathione (GSSG). Results are expressed as means ± standard deviations (n = 3), $^{**}p < 0.01$, $^{***}p < 0.0001$ compared to their respective controls (Hanks dark); (b) reactive oxygen species in BLEC after UV exposure in presence of GDC. The intensity of fluorescence caused by intracellular 2,7-dichlorofluorescein increased with UV exposure in a dose-dependent manner with time exposure. The measurement of reactive oxygen species (ROS) in the untreated cells was taken as 100%. As positive control, cells were incubated in the presence of H_2O_2 100 μmol L^{-1} for the same period of the experiment. Results are expressed as means ± standard deviations (n = 3), $^{***}p < 0.0001$ compared to their respective controls (time 0).

the normal lens, indicates a sign of weakness in the antioxidant response of the lens epithelium and this behavior has been observed during the development of senile cataract. The disappearance of GSH in the epithelium cell affect the glutathione redox cycle, which is responsible for the reduction of dehydroascorbic acid (DHA).[49] The reconversion of DHA in ascorbic acid in the lens is important because of glycating and photosensitizing properties of the degradation products of ascorbate.[23,29,50-53] The quantity of GSH is also determinant for glutathione peroxidase and glutathione S-transferase functioning, and the activity of these enzyme has been found significantly decreased in mature cataract.

BLEC exposed to UVA-Visible light in presence of GDC evidenced formation of reactive oxygen species

The generation of oxygen reactive species as a consequence of the UVA-Visible light exposure of the BLEC cultures in the presence of GDC was assessed using dichloro-dihydro-fluorescein diacetate (DCFH-DA) as a fluorescent probe. The non-fluorescent lipophilic DCFH-DA easily crosses the cell membrane and passes into the cytosol, where it is rapidly cleaved by unspecific cellular esterases, giving rise to the non-fluorescent alcohol DCFH.[54] The oxidation of this molecule to the fluorochrome DCF has been considered as an indicator of the intracellular presence of ROS. Figure 4b shows the increase of DCF fluorescence after 2 and 4 h of UVA-Visible light exposure of BLEC in the presence of GDC under a 5% oxygen atmosphere. It has been demonstrated that neither superoxide, nor hydrogen peroxide (H_2O_2), directly oxidized DCFH, and in consequence, this process is dependent either on Fenton-type reactions or of an unspecific enzymatic oxidation mediated by cytochrome c.[55] As positive and negative controls, BLEC cultures were also incubated in the dark during 2 and 4 h in the presence and absence of 100 μmol L^{-1} H_2O_2. BLEC incubated in a Hank medium in the dark, without H_2O_2, and in the presence or absence of GDC, exhibited a basal fluorescence corresponding to the oxidation of DCFH mediated by *in vivo* ROS generated in the cells. ROS levels, measured indirectly through the oxidation of DCFH, begin to rise after two hours of irradiation of BLEC exposed to UVA-Visible light in the presence of GDC, and at 4 h of irradiation there is a larger increase in ROS production, corresponding to 101% compared to the dark controls. A significant increase in the intracellular amount of ROS was also found in the BLEC culture incubated in a medium with 100 μmol L^{-1} H_2O_2. In this sense, the results obtained in this work are indicating the participation of redox-active transition metals and H_2O_2 and/or oxidative processes mediated by cytochrome c. Furthermore, the slight effect seen in controls irradiated in the absence of GDC can be due to the presence of endogenous chromophores that act as photosensitizers. At low oxygen concentrations, a type I photosensitization mechanism is favored. In previous studies, Johar *et al.*[56] established a relationship between the oxidative defense mechanisms and the alteration caused in the cell density of the lens epithelium.

The BLEC exposed to UVA-Visible light during 2 and 4 h in the presence of GDC showed a significant decrease in free GSH levels that correlate with the concomitant increase of GSSG and increase in ROS levels. It has been previously reported in the literature that a decrease in GSH levels in human lens epithelial cells is normally accompanied by a decrease in cell viability, together with an increase in the amount of reactive oxygen species, which is in agreement with the results obtained in this work exposing the cells to UVA-Visible light in the presence of GDC.[57]

Conclusions

Taken together, our data shows that glucose, commonly associated with cataractogenesis in diabetic patients, gives rise to a colored compound with type I photosensitizing capacity, which is extremely important in a medium where the oxygen pressure is low and is permanently exposed to UVA-Visible light, like the eye lens.

It was proven that the incubation of eye lens proteins in the presence of glucose gives simultaneously rise to the generation of advanced glycation products, as well as to the production of a colored decomposition product of the sugar with photosensitizing properties. When the incubation was performed under the exposure of UVA-Visible light, under low oxygen concentration, an increase in protein carbonylation and protein crosslinking was observed.

The irradiation of bovine epithelial cells in culture with UVA-Visible light, at low oxygen pressure and in the presence of the colored glucose decomposition product, is accompanied by a decrease in cell viability. In these conditions, an increase in the oxygen reactive species together with a decrease in the concentration of reduced glutathione concomitantly with an augment in the concentration of oxidized glutathione at cell level was observed. All these data resemble fairly well to the biological and chemical changes observed during the development of cataract.

Supplementary Information

Supplementary Information is available free of charge at http://jbcs.sbq.org.br.

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