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Advanced Glycation Endproducts Induce Photocrosslinking and Oxidation of Bovine Lens Proteins Through Type-I Mechanism

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ABSTRACT

Advanced glycation endproducts (AGEs) have been suggested as photosensitizers that are capable of mediating eye lens photodamage during aging. In the present work, we investigate the photo-crosslinking and oxidation of bovine lens proteins sensitized by AGEs, with special regard to low oxygen conditions. A mechanistic study was conducted using different oxygen concentrations and specific additives with the aim either to scavenge or enhance Type-I or Type-II photoprocesses. Quantum yields for Trp decomposition were determined at 5%, 20% and 100% O_2, in the presence of ferricyanide and D_2O to elucidate the mechanism of action of AGEs. Type-I mechanism proved to be the most efficient pathway for AGE-sensitized Trp decomposition at low oxygen concentration. Photocrosslinking of lens proteins and crystallin fractions due to Type-I interaction was observed. The influence of the oxygen concentration and additives was also studied. The results show that both Type-I mechanism and oxygen-mediated reactions contribute to protein crosslinking. Carbonyl group formation due to protein photo-oxidation was detected with Oxyblot technique. The generation of high levels of hydrogen peroxide during the irradiations was detected and attributed mainly to Type-I reactions. The results support that AGEs act preferentially as Type-I sensitizers at the low oxygen concentration found in the lens and are capable of inducing protein crosslinking, oxidation and peroxide formation.

INTRODUCTION

Advanced glycation endproducts (AGEs) have been a subject of permanent interest through the last decade as they are related to many chronic diseases (1–5). Along with the multiple consequences of AGEs formation *in vivo* is their photosensitizing activity. This is of special relevance in tissues that are exposed to light, particularly the eye lens, where they are thought to be related to cataract development (6–8). Research concerning the presence of UVA-absorbing chromophores in the lens has attracted increasing interest (9–13). Within the lens, AGEs act as endogenous sensitizers generating reactive oxygen species (ROS) upon irradiation with UVA light, as has been demonstrated *in vitro* (14–17). Under aerobic conditions, singlet oxygen is the predominant species formed (18), overcoming the generation of superoxide radical anion (19) and hydrogen peroxide (20). Nonetheless, the actual potential of damaging lens proteins has not been addressed properly. Moreover, the real conditions of the lens, which is a poorly oxygenated tissue, have not been considered and the occurrence of Type-I mechanism has been overlooked.

We have previously demonstrated that glycation products arising from the reaction of lysine with either glucose or ascorbic acid are capable of photo-decomposing Trp residues within model proteins at the low oxygen concentration found in the lens (21,22). As the initial interaction between the sensitizer and Trp would produce radical intermediates, the possibility of a radical-mediated crosslinking and oxidation of lens proteins due to AGEs photosensitizing activity was examined, giving special emphasis to low oxygen conditions. Mechanistic aspects of the photoprocesses are discussed.

MATERIALS AND METHODS

Chemicals. *N*α-acetyl-L-lysine (*N*-Ac-Lys), *N*α-acetyl-L-arginine (*N*-Ac-Arg), L-tryptophan, xylenol orange tetrasodium salt (XO) and deuterium oxide were obtained from Sigma (St. Louis, MO). D-glucose (Glc) was purchased from Fluka (Seelze, Germany). NaH_2PO_4, Na_2HPO_4, $(NH_4)_2Fe(SO_4)_2 \times 6H_2O$ and $K_3Fe(CN)_6$ were obtained from Merck (Darmstad, Germany). OxyBlot™ protein oxidation detection kit was obtained from Chemicon International (Temecula, CA). Ultra pure water from a Milli-Q system (Billerica, MA) was used for the preparation of all solutions.

Preparation of lens proteins. Bovine lenses (approximately 18 months old) were obtained from the abattoir. Twenty calf lenses were decapsulated and stirred in a 50 mM Tris–HCl buffer pH 7.4 containing 0.2 mM KCl, 1 mM EDTA, 10 mM β-mercaptoethanol and 0.05% NaN_3. The suspension was homogenized and centrifuged at 12 320 ***g*** for 30 min at 4°C (Sorvall Superspeed RC2-B). The supernatant was extensively dialyzed against deionized water at 4°C. Subsequently, the protein was lyophilized and reconstituted in 100 mM sodium phosphate buffer pH 7.4.

Separation of α-, β_H- and β_L-crystallins. The isolation of crystallin fractions from lens proteins was performed by size exclusion chromatography on a Sephadex G-200 column equilibrated with Tris–HCl buffer at 4°C. Three major peaks were separated and collected, the first one containing alpha-crystallins in high proportion, and the second and third containing beta-H and beta-L crystallins, respectively. The collected samples were pooled, lyophilized and reconstituted in 100 mM sodium phosphate buffer pH 7.4.

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Preparation of AGEs. A solution containing 5 mM *N*-Ac-Lys, 5 mM *N*-Ac-Arg and 50 mM Glc was incubated in a sterile plastic centrifuge tube at 60°C for 5 days. At the end of this period a dark brown mixture of glycation products was obtained. After dilution, absorption and fluorescence spectra were recorded on a Hewlett Packard 8453 spectrophotometer (Colorado Springs, CO) and PerkinElmer LS55 luminescence spectrometer (Beaconsfield, UK), respectively. AGEs were aliquoted and kept frozen at −20°C for several weeks.

Irradiation conditions. Solutions were prepared by adding 1 mL of 9 mg mL^{-1} lens proteins, 1 mL of AGEs with an absorbance of 0.6 at 365 nm and 1 mL of either phosphate buffer or 3 mM potassium ferricyanide (FeCy). All solutions were prepared in 100 mM phosphate buffer pH 7.4. Final concentrations were 3 mg mL^{-1} lens proteins, AGEs with a final absorbance of 0.2 at 365 nm and 1 mM FeCy. Experiments in heavy water were performed by adding small aliquots of concentrated lens proteins and AGEs solutions to 3 mL of 100 mM phosphate buffer prepared in D_2O (pD = 7.4). For the experiments with α-, β_H- and β_L-crystallins, concentrated AGEs solution was added directly to 3 mL solutions of the crystallins (3 mg mL^{-1}) so as to reach a final absorbance of 0.2 at 365 nm.

All experiments were performed in a 1 cm light-path quartz cuvette at 25°C. During the experiments, 3 mL solutions were bubbled with 5%, 20% or 100% O_2. Irradiations were performed with an OSRAM HBO 500 W high-pressure mercury lamp filtered through a 5% $CuSO_4$ solution in a 2.5 cm glass cell. The glass of the cell has the property to filter out all the light below 320 nm, as confirmed spectrophotometrically. Light intensity measurements were performed with a YSI Kettering 65A radiometer (Yellow Spring, OH). Incident intensity on the cuvette was 0.15 W cm^{-2} in the UVA-visible range of the spectrum. Small aliquots were withdrawn at defined time intervals for analysis. A dark control was performed containing the protein and the AGEs. A sensitizer control was performed by irradiating a 3 mg mL^{-1} lens proteins solution in the absence of any sensitizer.

Tryptophan photodecomposition quantum yields. Freshly prepared solutions of 50 μM Trp were irradiated in the presence of AGEs set at an absorbance of 0.2 at 365 nm, with monochromatic light at 367 nm (Schott band pass filter, I_0 = 7 mW cm^{-2}) at different oxygen concentrations (5%, 20% and 100% O_2) at the same conditions described above. In some experiments, FeCy was added at a final concentration of 0.1 mM, or 100 mM phosphate buffer prepared in deuterium oxide was used. Trp decay was analyzed by the second-derivative procedure, as described in a previous paper (21). All measurements were performed in triplicate.

SDS-PAGE analysis. Samples were boiled for 5 min in 62.5 mM Tris buffer pH 6.8 containing 2% SDS, 10% glycerol and 100 mM β-mercaptoethanol as reducing agent, with traces of bromophenol blue as a tracking dye. Three percent acrylamide stacking gel, 12% acrylamide resolving gel and a running buffer of 25 mM Tris, 400 mM Gly and 0.1% SDS pH 8.3 were used. Electrophoresis was performed at 100 V for 1–2 h. Gels were stained with 0.1% Coomassie Brilliant Blue and destained in a solution of methanol and acetic acid up to 24 h.

Gels were scanned with Amersham Bioscience Image Scanner (Uppsala, Sweden) and the quantification of the crosslinked protein was performed with Image Master 1D software.

Oxyblotting. The determination of protein oxidation was performed by immunoblotting using the Oxyblot assay. Samples containing 15 μg of proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH) to the corresponding 2,4-dinitrophenylhydrazone (DNP). DNPH-derivatized protein samples were separated in a 12% polyacrylamide gel by electrophoresis and transferred to a nitrocellulose membrane (Pierce, Rockford, IL) for 90 min at 100 V. The membranes were incubated with blocking buffer (PBS-Tween 0.05% containing 5% BSA) for 2 h at room temperature, and then treated with a rabbit polyclonal antibody specific to the DNP moiety of the proteins, diluted in blocking buffer (1:150) and incubated overnight at 4°C. Native lens proteins (100-fold greater in weight than the applied protein) were added to the primary antibody solution to compete with the proteins in the membrane, to avoid any nonspecific binding of the antibody. After several washings in PBS-Tween 0.05%, the membranes were incubated with a goat horseradish peroxidase-conjugated antirabbit IgG (1:300 in blocking buffer) directed against the primary antibody for 2 h at room temperature. After several washings and later incubation with a chemiluminescent reagent (Western Lightning chemiluminescence reagent; Perkin Elmer-Life Sciences, Boston, MA), the membranes were exposed to autoradiography films (Kodak BioMax XAR Film, Rochester, NY) and revealed according to the manufacturer's recommendations. Lens protein samples without DNPH-derivatization or treated only with the secondary antibody were used as negative controls.

Carbonyl group determination. Quantification of protein carbonyl content was performed by the DNPH spectrophotometric assay according to Levine *et al*. (23) with minor modifications. Briefly, 300 μL of the samples were incubated with 1.5 mL of 0.2% DNPH in 2 N HCl for 30 min. Protein was precipitated with the same volume of 20% ice-cold TCA and centrifuged at 1970 ***g*** for 10 min. The pellet was washed three times with 1:1 ethanol–ethyl acetate mixture. The protein was solubilized in 6 M of guanidine for 10 min at 37°C and the absorbance was measured at 370 nm using an extinction coefficient of 22 000 M^{-1} cm^{-1}.

Peroxide generation throughout the irradiation. Modified FOX-2 method was used for the determination of hydrogen peroxide and protein hydroperoxides (24,25). During the irradiations, 200 μL aliquots were withdrawn every 10 min and kept on ice during the irradiation period. Total peroxides were determined by adding to the samples 20 μL of water and 2 mL of FOX reagent containing 250 μM of ammonium ferrous sulfate and 125 μM of XO in 25 mM H_2SO_4. An additional 15 min preincubation step with 20 μL of catalase in a concentration of 715 U mL^{-1} instead of water was performed so as to eliminate H_2O_2 and measure only protein hydroperoxides. The mixtures were vortexed thoroughly and left to react for 30 min in the dark at room temperature. The formation of Fe(III)-XO complex was quantified at 560 nm and compared to a standard curve made with H_2O_2. To eliminate the interference of phosphate buffer, hydrogen peroxide standards were prepared in 100 mM phosphate buffer and a good correlation was found.

RESULTS AND DISCUSSION

Advanced glycation endproducts are well described as photosensitizers being capable of generating ROS when they are irradiated with UVA light. This fact has been proposed as one of the causes of cataract development (6–22). Nonetheless, their actual potential for damaging eye lens proteins is poorly understood. Specific damage to some reactive amino acids within lens proteins as a consequence of singlet oxygen generation has been measured (18), regardless of the low oxygen concentration found in the lens, which is unable to sustain a significant singlet oxygen formation (14).

In a previous work, we have demonstrated that UVA-visible irradiation of lysine-derived AGEs at low oxygen concentration can induce the photodecomposition of free Trp and Trp-residues in human serum albumin (21). In the present paper, we extend the study to eye lens proteins, using simple models to elucidate the sensitizing mechanism that leads to photo-crosslinking and oxidation of the proteins.

Advanced glycation endproducts were prepared considering the important role of lysine and arginine in the generation of known glycation products. For this purpose, equimolar amounts of *N*-Ac-Lys and *N*-Ac-Arg were incubated with a high concentration of Glc. The absorption and emission properties of the sensitizer used in this work are shown in Fig. 1. A major fluorophore was detected at Ex330/Em390 nm along with a minor fluorophore at Ex380/Em440 nm. These data are consistent with the generation of known Lys–Arg and Lys–Lys crosslinks (26,27). This mixture of glycation products was used directly as a sensitizer to study the photochemical behavior of AGEs.

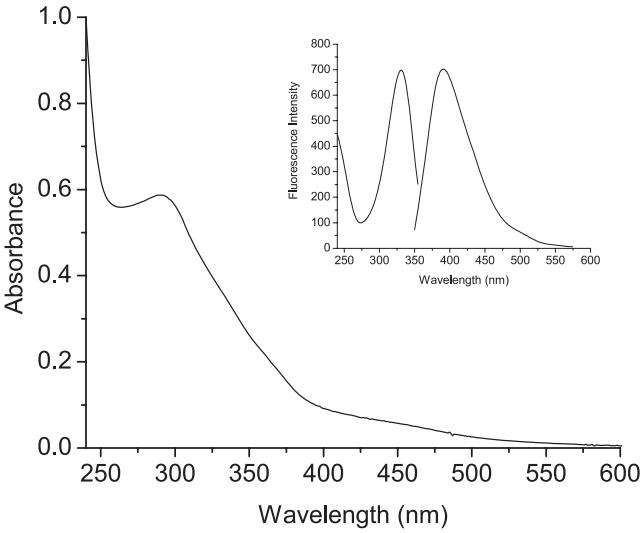

Figure 1. Absorption spectra of advanced glycation endproducts obtained through the incubation of a mixture of *N*-Ac-Lys (5 mM), *N*-Ac-Arg (5 mM) and Glc (50 mM) at 60°C during 5 days. The concentrated dark brown mixture of glycation products was diluted to an absorbance of 0.2 at 365 nm. Inset: fluorescence excitation and emission spectra of the mixture at the same concentration.

AGE-sensitized tryptophan photodecomposition quantum yields

To investigate the photosensitizing mechanism of AGEs, Trp was used as a model target and the photodecomposition quantum yields at 367 nm of this amino acid were determined at different oxygen concentrations. FeCy, a known electron-scavenger (28), was used with the aim to interfere with electron-transfer mechanism, whereas deuterium oxide buffer was used so as to enhance singlet oxygen-mediated photodamage.

The results shown in Table 1 support a combined Type-I–Type-II mechanism for all oxygen concentrations, being Type I and Type II favored at low and high oxygen concentrations, respectively. The fraction of Type-I ($f_{type\ I}$) and Type-II ($f_{type\ II}$) mechanisms that contributes to Trp photodecomposition depends on both of the following: Trp and oxygen concentration, and the rate constants of the reactions between triplet AGEs and Trp or O_2, as expressed by the equations below:

$$f_{type\ I} = k_{et}[Trp]/(k_{ic} + k_P + k_{et}[Trp] + k_q[O_2]) \quad (1)$$

$$f_{type\ II} = k_q[O_2]/(k_{ic} + k_P + k_{et}[Trp] + k_q[O_2]) \quad (2)$$

where k_{et}, k_{ic}, k_P and k_q correspond to the rate constants of the electron-transfer between triplet AGEs and Trp, internal conversion, phosphorescence emission and quenching of triplet AGEs by oxygen, respectively.

Table 1. Quantum yields for AGE-sensitized Trp decomposition at 367 nm under different oxygen pressures.

Trp photodecomposition quantum yields ($\phi_{367} \times 10^3$)			
O_2 (%)	Control	Added FeCy*	D_2O buffer†
5	0.55	0.28	1.44
20	0.35	0.29	1.74
100	0.49	0.38	2.42

Initial concentration of Trp was 50 μM and AGEs were set at an absorbance of 0.2 at 365 nm; *FeCy was added at a final concentration of 0.1 mM; †Phosphate buffer 100 mM was prepared in D_2O (pD = 7.4).

Given that Trp concentration was the same in all the experiments, the contribution of Type-I and Type-II photoprocesses depends solely on the oxygen concentration and the values for the rate constants of the processes.

At 5% O_2, Type-I photosensitizing mechanism is predominant because the presence of FeCy considerably drops the quantum yield to 51% with respect to the control. A similar result was reported by our group for the predominantly Type-I sensitizer, riboflavin (29). Under the same conditions, D_2O increases the damage 2.6 times, which is low if we consider that deuterium oxide prolongs the lifetime of singlet oxygen about 10–17 times compared to water (30). This result indicates a small contribution of singlet oxygen at low oxygen concentration.

At 20% O_2, the quantum yield drops to 64% with respect to that at 5% O_2. This result is consistent with AGEs acting preferentially as Type-I sensitizers, and therefore, the four-fold increase in the oxygen concentration disfavors the interaction between the triplet sensitizer and tryptophan, and this is reflected in a lower damage to the target. The presence of FeCy does not inhibit the process to a great extent (down to 80% of the control), which agrees with the fact that at 20% oxygen, Type-I mechanism is disfavored. A high oxygen concentration favors the quenching of the triplet state of the sensitizer by ground state oxygen in a Type-II mechanism that generates singlet oxygen, and this is observed as a five-fold increase in the quantum yield when the reaction is performed in deuterium oxide buffer.

At 100% O_2, the quantum yield rises again to 89% of the value observed at 5% O_2. This result was surprising because for a Type-I sensitizer, an even greater decrease in the quantum yield was expected compared to the value at 20% O_2. A plausible explanation for this behavior is that at 100% O_2, the increased amount of singlet oxygen generated in this condition compensates the loss in Type-I mechanism, balancing the total efficiency of the process. The influence of FeCy and D_2O in the quantum yield shows the same behavior as that observed at 20% O_2, being consistent with the occurrence of Type-II mechanism at high oxygen concentrations.

Notwithstanding the fact that an enhancement of the photodamage in the presence of deuterium oxide is observed for all oxygen concentrations, supporting the participation of Type-II mechanism, the increase is small compared to the expected enhancement of the lifetime of singlet oxygen. The ratio between the quantum yields in D_2O and in H_2O is 2.62 at 5% O_2, which is low compared to that for known Type-II sensitizers such as methylene blue, where a ratio of 8.29 in oxygen-saturated solutions is observed (31). In the same work, a ratio of 4.99 is obtained for riboflavin in aerated solutions, which agrees well with the values of 4.97 and 4.94 observed for 20% and 100% oxygen concentrations found for AGEs in our conditions. These data indicate that Type-I photoprocesses are the principal mechanism by which AGEs induce photodamage under low oxygen conditions.

Photocrosslinking of bovine lens proteins sensitized by AGEs

As the results described above show the presence of Type-I photosensitizing mechanism, and this would lead to the

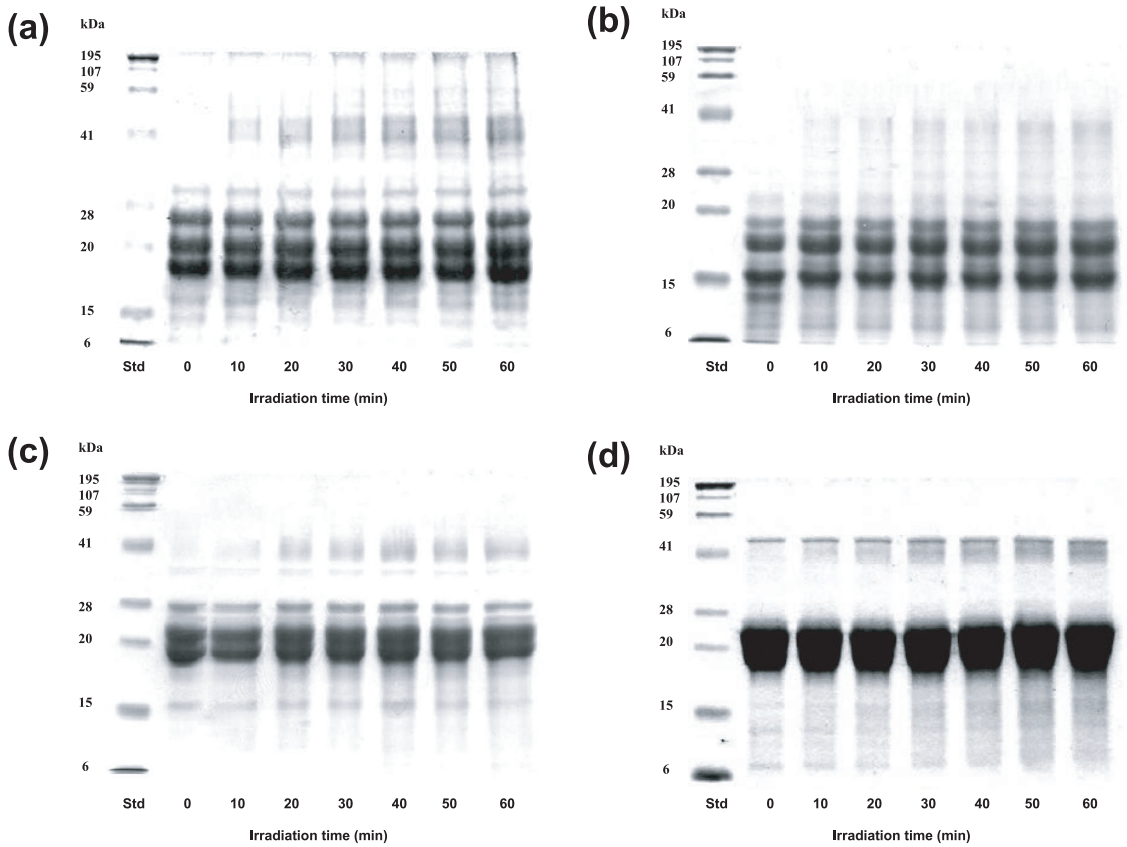

Figure 2. SDS-PAGE analysis of (a) bovine lens proteins, (b) α-, (c) β_H- and (d) β_L-crystallins, irradiated during 10, 20, 30, 40, 50 and 60 min with UVA-visible light in the presence of AGEs (absorbance of 0.2 at 365 nm) in 100 mM phosphate buffer pH 7.4 under a 5% oxygen atmosphere. The main crosslinking band represents protein dimers with an average molecular weight of 41 kDa.

formation of radical intermediates, we investigated the possibility of a radical-mediated photo-crosslinking of lens proteins. Figure 2a shows the time-dependent formation of crosslinking when the total water-soluble bovine eye lens proteins are irradiated with UVA-visible light in the presence of AGEs at 5% O_2 concentration.

At initial time, eye lens proteins contain a negligible amount of crosslinking generated *in vivo*. When these proteins are incubated in the dark in the presence of AGEs (dark control) or irradiated during 1 h in the absence of the chromophores (sensitizer control), the electrophoretic pattern of these samples does not show any modifications (data not shown). SDS-PAGE analyses were performed in the presence of a reducing agent so as to exclude disulfide bonds and leave only the irreversible covalent crosslinking. The main band centered at 41 kDa represents the formation of dimers with molecular weights in the range 36–47 kDa, arising from the crosslinking of the crystallin subunits with an average molecular weight of about 20 kDa (32). The photochemical aggregation of lens crystallins due to UV irradiation has been described before, although in these studies the nature of the crosslinking was probably the direct photoexcitation of Trp residues or its oxidation products (33–35). Lee *et al.* described the crosslinking of lens proteins as a result of the sensitizing activity of the chromophores extracted from brunescent cataractous lenses in the presence of air (36); however, no further investigations were performed in this area.

Crystallins represent 95% of the total lens proteins, of which the major components are α- and β-crystallins (40% each). To observe what happened with each one of the fractions, a separation through size exclusion chromatography was conducted. Isolated α-, β_H- and β_L-crystallins were irradiated under the same conditions as those for the whole lens proteins homogenate, as described in Materials and Methods. Crosslinking of all the fractions was observed. Alpha- and beta-crystallins showed different grades of crosslinking, decreasing in the order α- < β_H- < β_L-crystallins (Fig. 2b–d, respectively). These results are consistent with an early study from our group using riboflavin as sensitizer for lens crystallins, where a good correlation between the decomposition of Trp residues and protein aggregation was found (37). These data point to Trp as a key target in the photoprocesses that lead to the observed crosslinking, as the number of Trp residues within the fractions are 2.6- and 2.7-fold higher for the beta fractions (β_H- and β_L-crystallins, respectively) than the alpha fraction (37), which is consistent with the experimental observations. Nevertheless, the participation of other amino acids cannot be discarded.

Photocrosslinking mechanism of bovine lens proteins

To further investigate the mechanism of AGE-sensitized protein crosslinking, a series of experiments were performed by irradiating 3 mg mL^{-1} lens proteins solutions in the

presence of AGEs adjusted to an absorbance of 0.2 at 365 nm under increasing oxygen concentrations (5%, 20% and 100%). Figure 3 shows the SDS-PAGE analysis of the samples irradiated during 60 min under different oxygen pressures. A rise in the crosslinking degree with the increase in oxygen concentration can be observed, indicating that Type-II mechanism and/or secondary reactions mediated by oxygen begin to be important in the aggregation process.

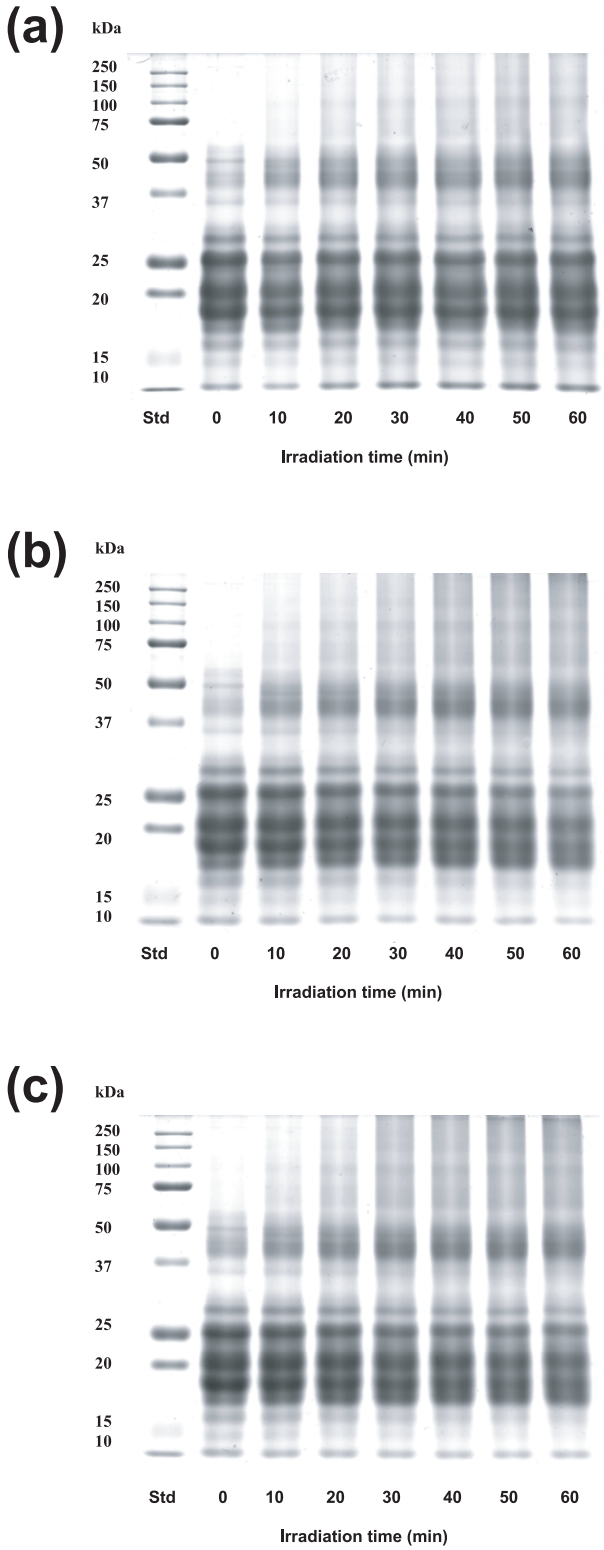

Figure 3. SDS-PAGE analysis of bovine lens proteins irradiated during 10, 20, 30, 40, 50 and 60 min with UVA-visible light in the presence of AGEs (absorbance of 0.2 at 365 nm) in 100 mM phosphate buffer pH 7.4 under (a) 5%, (b) 20% and (c) 100% oxygen concentrations.

Subsequently, the influence of FeCy and D_2O on lens proteins photo-crosslinking was investigated for every oxygen concentration. Figure 4a shows the effect of the additives at 5% O_2. A great decrease in the crosslinking was observed in the presence of FeCy, indicating the occurrence of Type-I interaction between AGE-sensitizers and the proteins. In the presence of D_2O buffer, only a slight increase in the crosslinking was observed, giving evidence for a small participation of singlet oxygen in the crosslinking at low oxygen concentration, as expected due to the predominance of Type-I mechanism.

Based on the above results, it is plausible to propose that at low oxygen concentration, an initial Type-I interaction between triplet AGEs and reactive amino acids on the protein takes place, generating charged radicals and then neutral radicals after deprotonation. The sequence of reactions is indicated below

$$\text{AGEs} + h\nu \rightarrow {}^1\text{AGEs} \rightarrow {}^3\text{AGEs} \quad (3)$$

$${}^3\text{AGEs} + \text{Protein} \rightarrow \text{AGEs}^{\bullet -} + \text{Protein}^{\bullet +} \quad (4)$$

$$\text{AGEs}^{\bullet -} + \text{Protein}^{\bullet +} \rightarrow \text{AGEs}(+\text{H})^{\bullet} + \text{Protein}(-\text{H})^{\bullet} \quad (5)$$

Carbon-centered radicals produced on the protein are prone to react with oxygen, generating reactive species that can further enhance the production of neutral radicals (38). The reactions are the following:

$$\text{Protein}(-\text{H})^{\bullet} + \text{O}_2 \rightarrow \text{Protein-OO}^{\bullet} \quad (6)$$

$$\text{Protein-OO}^{\bullet} + \text{Protein} \rightarrow \text{Protein-OOH} + \text{Protein}(-\text{H})^{\bullet} \quad (7)$$

It is important to note that the oxygen concentration present in the reaction medium can modulate both, the occurrence of this oxygen-mediated radical mechanisms and the prevalence of Type-I or Type-II mechanisms.

Notwithstanding the fact that the reaction of neutral radicals with oxygen is an efficient process, protein radical recombination could also make a contribution to protein crosslinking, especially at low O_2 concentration (Eq. 8).

$$\text{Protein}(-\text{H})^{\bullet} + \text{Protein}(-\text{H})^{\bullet} \rightarrow \text{Protein}(-\text{H})\text{-Protein}(-\text{H}) \quad (8)$$

Although this reaction does not require oxygen to proceed and hence could be favored in its absence, a reduced protein crosslinking was observed when the solutions were irradiated under a nitrogen atmosphere (data not shown). This result is consistent with earlier studies from our group that indicate that the efficiency of AGE-sensitized photoprocesses decreases dramatically under oxygen-free conditions due to the impossibility of recovering the sensitizer in its active form, limiting the extension of the reaction (22).

The improvement in the efficiency of this recombination process in the case of lens proteins can be explained by the conformational properties of the lens crystallins that possess a quaternary structure configuring multimeric complexes

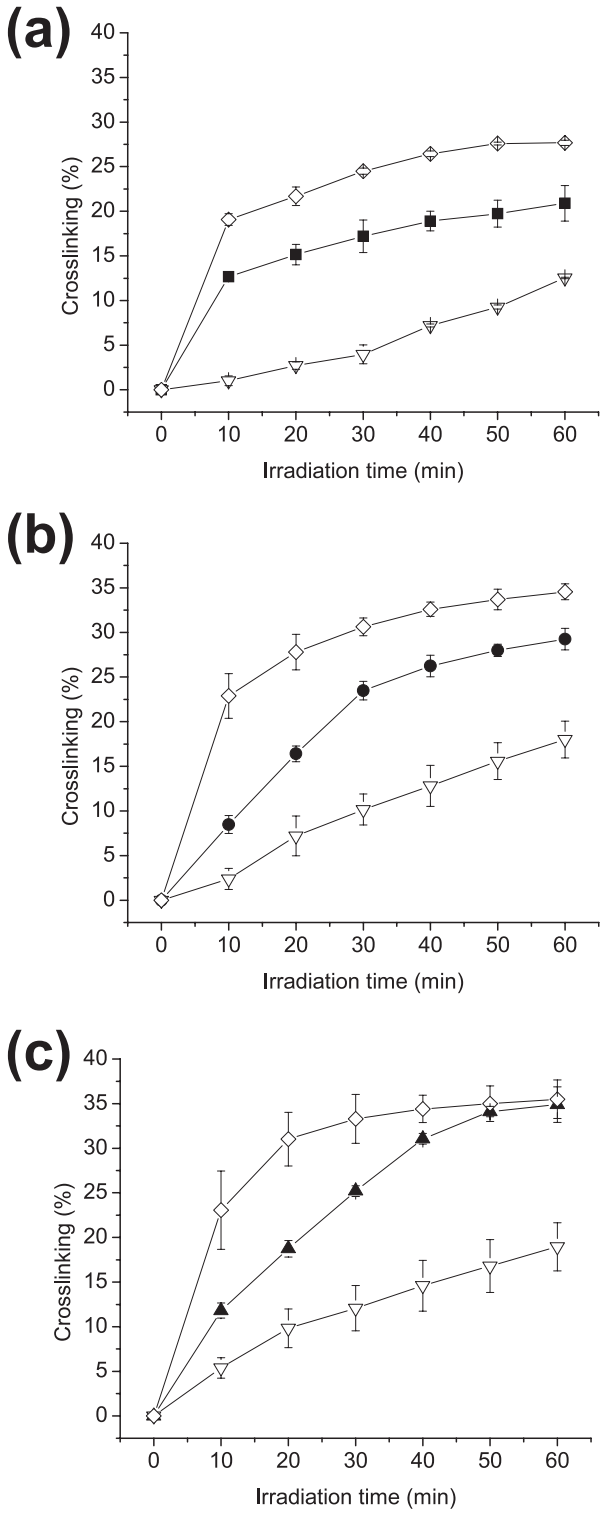

Figure 4. Quantification of the photoinduced crosslinking of bovine lens proteins when a 3 mg mL^{-1} solution is irradiated with UVA-visible light in the presence of AGEs (absorbance of 0.2 at 365 nm) under different oxygen pressures. Panels (a–c) show the effect of FeCy and D_2O at 5%, 20% and 100% O_2, respectively. Initial crosslinking was normalized to zero in all cases. Symbols: (■) 5% O_2, (●) 20% O_2, (▲) 100% O_2, (∇) Added FeCy and (◇) D_2O buffer. Data are the mean ± SD of three experiments. The percentage of crosslinking corresponds to the integrated area of the bands above 37 kDa referred to the area of the total protein.

(39–41) in which the subunits are in close vicinity, which could facilitate radical recombination. When a model protein such as glucose 6-phosphate dehydrogenase is irradiated in the presence of AGEs in the same conditions described in this paper, crosslinking is not observed despite the fact that an important photosensitized inactivation is clearly appreciated (data not shown). A similar behavior with respect to this Type-I–mediated crosslinking has been observed for riboflavin, whose photochemical efficiency on eye lens proteins has been compared with those of AGEs (9). The interaction between triplet riboflavin and crystallins together with the induction of protein crosslinking at low oxygen concentration has been demonstrated (37). The amino acids susceptible to Type-I interaction are mainly Trp, Tyr and His (42,43). Formation of Trp–Trp (44) and Tyr–Tyr (45,46) crosslinks through radical mechanisms has been observed. These processes are characterized by very low efficiencies in anaerobic conditions because oxygen is required to prevent photobleaching of the sensitizer and promote its regeneration (22). In spite of this, riboflavin-sensitized crosslinking of proteins other than crystallins had not been observed until recently (47), using an extremely high concentration of the target protein, suggesting that the radical recombination is highly dependent on the proximity of the participating groups and emphasizing that this condition should be especially favorable in crystallin multimeric complexes.

At 20% O_2 (Fig. 4b) and 100% O_2 (Fig. 4c), the influence of FeCy is smaller than that at 5% O_2 and this is consistent with a higher proportion of Type-II mechanism over Type I at higher oxygen concentrations. The effect of D_2O on the enhancement of the crosslinking is more marked than that at 5% O_2, giving evidence for the contribution of singlet oxygen to the observed crosslinking at high oxygen concentrations.

The experiments performed at high oxygen concentration, in which the interactions between triplet AGEs and oxygen is favored, showed an efficient covalent protein aggregation. The involvement of $^1O_2$ in the photodynamic crosslinking of proteins has been reported previously (48–51). It has been proposed that photodynamically generated singlet oxygen interacts with photo-oxidizable amino acids residues such as His, Cys, Trp and Tyr in one protein molecule to generate reactive species. These in turn interact nonphotochemically with residues of these types or with free amino groups in another protein molecule to form a crosslink such as His–His or His–Lys (52–55). In the case of His–His singlet oxygen-mediated crosslinking, the first step is the 1,4-cycloaddition of singlet oxygen to the His-imidazole ring to give a stable endoperoxide. This then undergoes changes followed by nucleophilic addition of a second imidazole ring and the elimination of one molecule of water to give His–His crosslinks (52).

Photo-oxidation of lens proteins and peroxide formation sensitized by AGEs

Carbonyl groups can be introduced into proteins as a consequence of the sensitized photo-oxidation of amino acids such as Trp, His and Tyr in both Type-I and Type-II mechanisms (56,57). Therefore, the presence of carbonylated proteins due to AGEs photosensitizing activity at 5%, 20% and 100% O_2 was detected with the Oxyblot assay.

Lens proteins are known to bind nonspecifically to other proteins. To minimize nonspecific binding, the antibodies were incubated in the presence of native lens proteins to observe only DNP-specific signals. Clear blots were obtained for all oxygen concentrations, as depicted in Fig. 5. Negative controls, which included both irradiated and nonirradiated samples that were not derivatized with DNPH, showed no signal. On the other hand, derivatized samples, both irradiated and nonirradiated, which were only incubated with the secondary antibody, also failed to produce any signal. Figure 5 shows an increase in the carbonyl content throughout the irradiation period at all oxygen concentrations. The results show that the amount of carbonyl groups formed increases with the rise in oxygen concentration, which suggests an important role of oxygen-mediated reactions in the photo-oxidation of lens proteins. Both the secondary reactions of radical species formed in Type-I mechanism and the decomposition of singlet oxygen intermediates appear to be important. Additional experiments showed that the photo-oxidation of lens proteins is partially inhibited by FeCy and increased in the presence of D_2O, in the same manner as the effect observed for protein crosslinking (data not shown).

For a better accuracy in the interpretation of the results, carbonyl groups were quantified by the DNPH spectrophotometric method. At initial time, bovine lens proteins contain a small amount of carbonyl groups (1.87 nmol mg^{-1} of protein) and this quantity does not change significantly during a 1 h incubation period in the presence of AGEs (<1 nmol mg^{-1} protein). Carbonyl group formation was observed exclusively when the samples were irradiated in the presence of AGEs, with maximal values obtained at 60 min of irradiation and corresponding to 8.70, 10.49 and 12.35 nmol mg^{-1} of protein for 5%, 20% and 100% O_2, respectively. These results are in agreement with the corresponding Oxyblots shown in Fig. 5. It is noteworthy that carbonyl group introduction due to photo-oxidation is particularly low and may not represent a major end-result of AGE-sensitized photoprocesses.

Recent studies point out that the main products of Type-I and Type-II photosensitizing mechanisms could be the generation of reactive peroxides (24,58,59). Singlet oxygen, generated in Type-II mechanism, reacts mainly with Trp-, Tyr- and His-residues, with the consequent formation of hydroperoxides and/or endoperoxides (60,61). Type-I interaction can also generate hydroperoxides through the reactions of radical intermediates with oxygen (Eq. 7) and later hydrogen abstraction (Eq. 8) (38). The peroxides formed by either Type-I or Type-II mechanisms can further decompose thermally or undergo a catalytic breakdown in the presence of metal ions in a pseudo-Fenton reaction (62), promoting the formation of radicals that could eventually lead to the crosslinking of lens proteins.

To investigate the formation of peroxides in the process of photo-crosslinking of lens proteins, these species were determined by a modified FOX-2 method. The results shown in Fig. 6 show the generation of peroxides during the irradiation of lens proteins in the presence of AGEs at 5%, 20% and 100% oxygen concentrations. The amount of peroxides generated during the irradiations was higher for 5% O_2 concentration, followed by 100% O_2 and 20% O_2. The

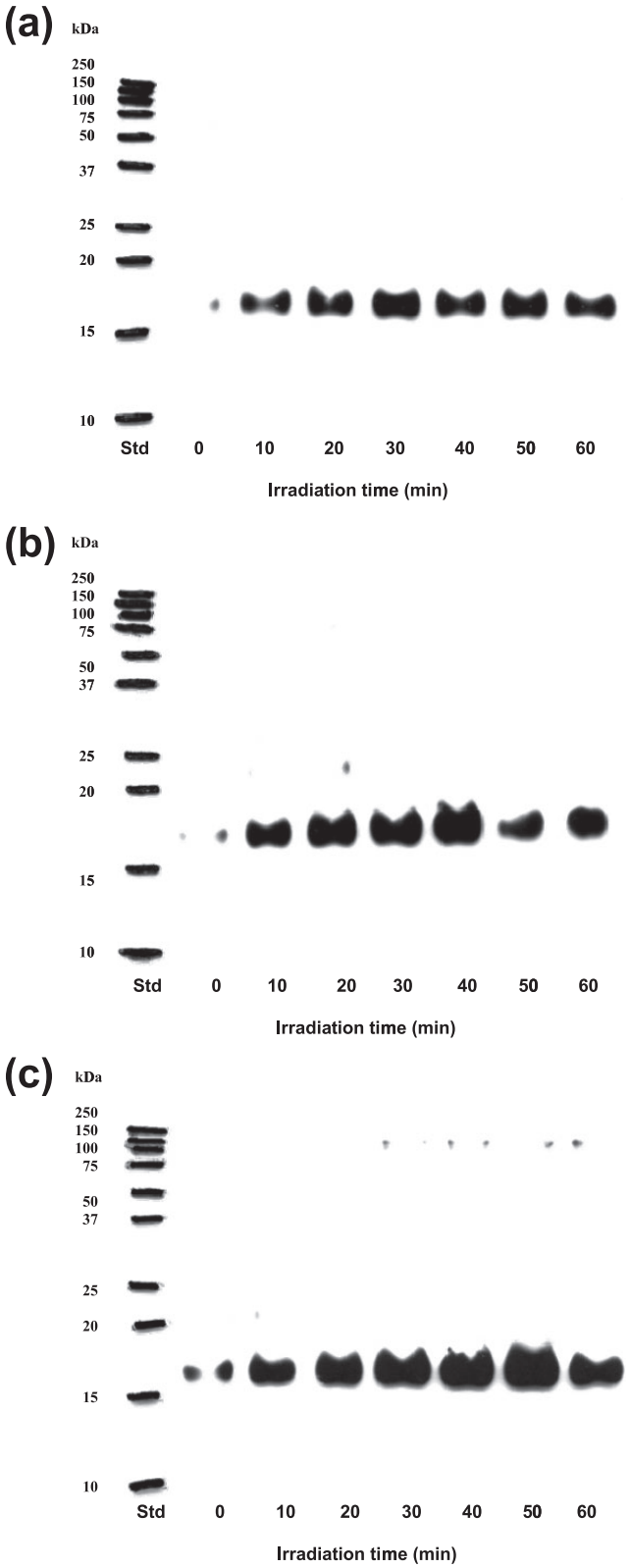

Figure 5. Oxyblot analysis of bovine lens proteins (3 mg mL^{-1}) irradiated with UVA-visible light during 60 min, in the presence of AGEs (absorbance of 0.2 at 365 nm) under different oxygen pressures. Panels (a), (b) and (c) show the increase in carbonyl content at 5% O_2, 20% O_2 and 100% O_2, respectively. These Oxyblots correspond to the SDS-PAGE shown in Fig. 3.

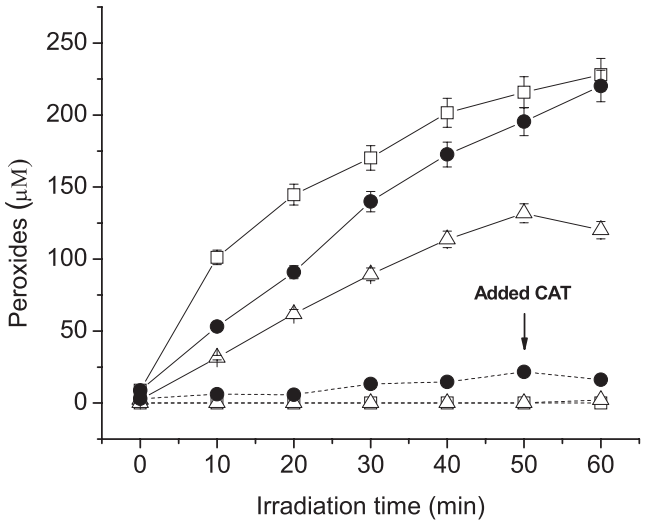

Figure 6. Generation of peroxides during the irradiation of bovine lens proteins (3 mg mL^{-1}) in the presence of AGEs (absorbance of 0.2 at 365 nm) under different oxygen pressures. The addition of CAT 65 U mL^{-1} after the irradiations eliminated most of the peroxides detected (dashed lines). Symbols: (□) 5% O_2, (△) 20% O_2 and (●) 100% O_2. Data are the mean ± SD of two experiments.

addition of catalase (65 U mL^{-1}) after the irradiations almost completely abolishes the peroxides detected, indicating the presence of H_2O_2 as the main peroxide generated in the reaction medium. Under our experimental conditions, hydrogen peroxide accounted for more than 98% of the total peroxides generated in the photoprocesses at 5% and 20% oxygen concentrations, while protein hydroperoxide formation was detected exclusively at 100% O_2, with values lesser than 7% of the total generated peroxides. Taking into account the experimental conditions of this study, a low yield of protein peroxides is expected as these species are particularly prone to decompose in the presence of metals and UV light (63–65), which were both present in our system. However, the decomposition of these intermediates gives rise to oxidation products and radical species that are consistent with the oxidation and crosslinking observed for lens proteins. Formation of H_2O_2 was not increased in the presence of 500 U mL^{-1}superoxide dismutase (data not shown) indicating that the main source of hydrogen peroxide is associated to the reaction between molecular oxygen and the reduced sensitizer (66,67), as proposed in the reactions below:

$$\mathrm{AGEs(+H)^{\bullet} + AGEs(+H)^{\bullet} \rightarrow AGEs + AGEsH_2} \quad (9)$$

$$\mathrm{AGEsH_2 + O_2 \rightarrow AGEs + H_2O_2} \quad (10)$$

The lack of increase in H_2O_2 production in the presence of superoxide dismutase suggests that superoxide radical anion generation by the reaction between AGEs radical anion and oxygen could not be significant in our system or that this specie is rapidly consumed in additional reactions with carbon-centered radicals.

Given the presence of hydrogen peroxide in the reaction medium together with the ubiquitous presence of metal traces, the generation of hydroxyl radicals is very probable. These species constitute a new source of radicals that lead to crosslinking and/or oxygen-mediated reactions, where the oxygen concentration modulates the prevalence of one or another process.

CONCLUSIONS

Previous studies have shown that AGEs have Type-II sensitizer properties under aerobic atmosphere, which is not the actual condition of the eye lens that possesses a very low oxygen concentration (68,69). The results obtained in this study support a predominant Type-I photosensitizing mechanism for AGEs at the low oxygen concentration found in the lens, in which the main process is the direct interaction between triplet AGEs and reactive amino acids within the proteins. This photo-process leads to the crosslinking of lens proteins mainly through radical reactions. At higher oxygen concentrations, Type-II mechanism begins to take place with a consequent increase in the singlet oxygen-mediated crosslinking together with the enhancement of secondary radical reactions that are modulated by oxygen. Simultaneously with lens proteins crosslinking, photo-oxidation occurs and this was evidenced by an increase in the carbonyl groups content dependent on irradiation time and oxygen concentration. Hydrogen peroxide was produced during the photoprocesses and this could be attributed to Type-I mechanism mostly by the re-oxidation of the fully reduced sensitizer by oxygen. On the contrary, hydroperoxides were generated as a consequence of Type-II mechanism in a very low yield.

As a final remark, this paper deals with eye lens photodamage and reports on the photo-crosslinking and oxidation of bovine lens proteins as a consequence of AGEs photosensitizing activity. The occurrence of Type-I photosensitizing mechanism is demonstrated, with a consequent formation of protein crosslinking, oxidation and ROS.

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