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INCREASED SUSCEPTIBILITY TO TRANSGLUTAMINASE OF EYE LENS PROTEINS EXPOSED TO ACTIVATED OXYGEN SPECIES PRODUCED IN THE GLUCOSE-GLUCOSE OXIDASE REACTION

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In order to test whether a mild oxidative stress could promote the transglutaminase damaging effect on eye lens proteins, total lens soluble proteins and purified β_L-crystallin have been exposed to H_2O_2 slowly produced by the glucose-glucose oxidase reaction. Soon after the pretreatment, the substrate capacity of the lens proteins for an exogenous transglutaminase has been evaluated. Exposure to the oxidative stress increased the susceptibility of the lens proteins to transglutaminase. When ferrous ions were added to the preincubation medium, in order to convert the H_2O_2 into the hydroxyl radical, the increase was more evident.

KEY WORDS: Oxy-radicals, crystallins, transglutaminase, aging.

INTRODUCTION

The major structural proteins of the eye lens, the crystallins, are the longest-lived proteins of the vertebrate body, some of them having been synthesized before birth. Thus, the lens is a very useful model system for studying age-related structural modifications of proteins.[1] The most studied modifications of lens proteins are those induced by oxidative stress and transglutaminase-mediated reactions. The eye lens is exposed to high levels of activated species of oxygen that may be generated by metabolic of photochemical processes. Several groups of workers (see [2] for rev.) have reported the presence of substances in the excited state and free radical species in the human lens. Singlet oxygen may be generated by photosensitizing chromophores present in the lens.[3] The aqueous humor that bathes the lens and the lens itself contain levels of H_2O_2 in the 20–50 μM range.[4] Oxidative processes are known to generate many of the changes occurring in the aged lens and in cataracts, namely browning and insolubilization of proteins, formation of disulfide and nondisulfide covalent cros-slinks between crystallin polypeptides, partial degradation, decreased susceptibility to proteolytic enzymes, formation of large protein aggregates.[5-9]

Some age-related modifications of lens proteins are dependent on transglutaminase activity. Transglutaminases (TGase, EC 2.3.2.13) are a family of calcium-dependent

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enzymes that catalyse the crosslinking of proteins by γ-glutamyl-ε-lysine bridges and the covalent binding of primary amines to γ-glutaminyl sites of proteins.[10] TGases are expressed in many cell types, but normally present in an inactive form, owing to the low level of intracellular calcium. The observation that calcium can induce aggregation of bovine lens α-crystallin [11] and produce cataract in cultured rabbit lenses,[12] the findings of TGase activity in the lens and of significant amounts of γ-glutamyl-ε-lysine isopeptides in protein polymers from cataractous lenses,[13] strongly point out that TGase-mediated changes are important in aging of the lens. TGase activity may be regulated at the enzyme level, or at the substrate level.[10] The first type of control is exerted by the concentration of calcium ions. Intracellular TGases show an apparent calcium requirement for half-maximal activation in the 0.1–0.5 mM range, that may be only occasionally reached in most normal tissues. The eye lens, however, is peculiar, because intracellular calcium levels in it vary between 0.5 and 2 mM,[14] being therefore sufficient to activate TGase to an appreciable extent. The substrate-level control of TGase activity may be exercised through changes in the amount of enzyme-reactive γ-glutaminyl acceptor sites. The latter may increase after conformational changes induced by post-translational modifications. We sought to explore the possibility that in the eye lens such modifications are consequent to oxidative stress. In previous studies [15] bovine lens proteins were exposed to acute oxidant injury by gamma irradiation or by initiating a Fenton reaction. These treatments increased the substrate capacity for an exogenous TGase of lens proteins. A strong oxidative injury, however, reduced it, probably by inducing protein degradation. In the present work we have studied the effect of a mild oxidative stress on the substrate capacity for TGase of total lens soluble proteins or purified β_L-crystallin. Proteins have been exposed to H_2O_2 slowly produced in the glucose-glucose oxidase reaction; in some experiments ferrous ion was added to convert H_2O_2 in the hydroxyl radical.

MATERIALS AND METHODS

Bovine lens β_L-crystallin, glucose oxidase (Type VII-S), catalase, N, N-dimethylcasein and guinea pig liver TGase were purchased from Sigma Chemicals Co. (St.Louis,-MO.USA). [1,4-^{14}C] putrescine was purchased from Amersham International plc. BTS-450 tissue solubilizer and Ready-Solv NA counting cocktail were purchased from Beckman Analytical SpA (Milano, Italy).

Bovine eye lenses were immediately cooled with ice after removal and stored for not more than 1 month at −80°C. Frozen lenses were homogenized in 3 volumes (w/v) of 50 mM Tris-HCl buffer, pH 7.5. The homogenate was centrifuged at 78,000 × g for 30 min, to obtain the lens soluble fraction. TGase was purified from rat liver by QAE-Sephadex ion-exchange and hydroxylapatite adsorption, according to the method described by Brookhart *et al.*[16] for purification of guinea pig liver transglutaminase; its activity was 0.57–0.62 units per mg protein when compared with Sigma guinea pig liver TGase. The enzyme was diluted to 0.4 unit/ml with 50 mM Tris-HCL buffer, pH 7.5, containing 2 mM dithiothreitol, and stored in small aliquots at −80°C.

Oxidative stress

Lens soluble fraction, or β_L-crystallin solutions, were diluted to 2 mg protein per ml

with 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM glucose and different amounts of glucose oxidase. In some experiments the mixture contained EDTA-chelated iron as reported by Zigler *et al.*:[17] $FeSO_4$, pre-mixed with EDTA, was added to give final concentrations in the reaction mixture of 0.1 mM iron and 0.3 mM EDTA. All operations were conducted rapidly at temperature below 5°C. Aliquots of 0.5 ml of the solutions were placed into flat bottom, 18 mm diameter, glass tubes and incubated for different times at 37°C in a vigorously shaken water bath, to ensure a good oxygenation of the reaction mixture. The oxidative stress was terminated by addition of 100 U of catalase. In some control samples catalase was added before starting the incubation.

Transglutaminase-directed putrescine incorporation

The substrate capactiy for TGase of the lens soluble proteins and β_L-crystallin was evaluated 30 min after catalase addition by determining the TGase-directed, Ca^{2+}-dependent, incorporation of ^{14}C-putrescine into trichloroacetic acid-insoluble material.[18] The assay reaction mixture (0.2 ml) contained 50 mM Tris-HCl buffer, pH 7.5, 5 mM $CaCl_2$, 0.2 mg of substrate protein and 0.01 units of TGase. Triplicate samples and blank samples containing 5 mM EDTA instead of $CaCl_2$ were prepared. The reaction was started by the addition of 0.5μCi of [1,4 – ^{14}C] putrescine to a final concentration of 1 mM. Samples were incubated at 37°C for 30 min, then the reaction was stopped with 10% trichloroacetic acid. The precipitates were collected by suction on Sartorius 25 S glassfibre filters and washed five times with 5% trichloroacetic acid. Filters were dried and transferred into scintillation vials; precipitates were dissolved with 0.5 ml of BTS-450 solubilizer. Radioactivity was determined with a liquid

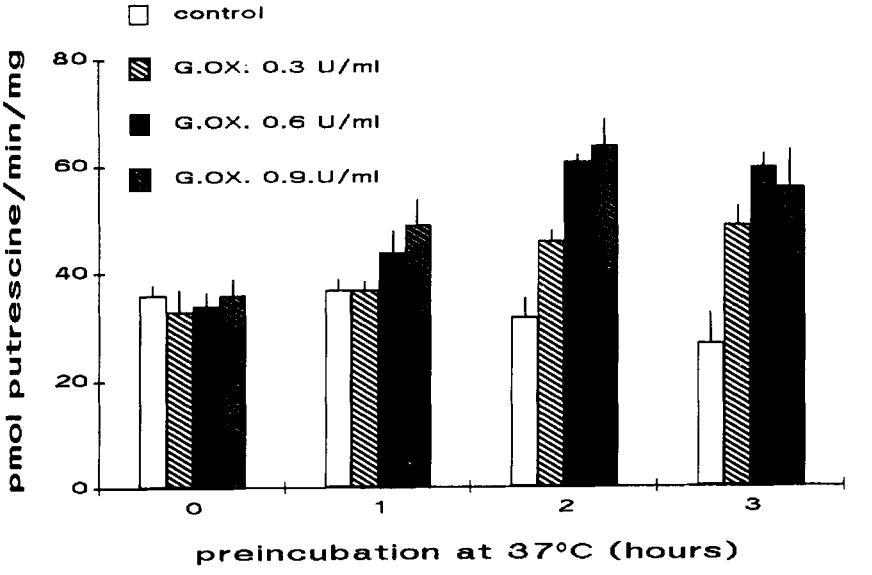

FIGURE 1 Substrate acceptor capacity for TGase (pmol of putrescine incorporated/min/mg of protein) of eye lens soluble proteins after preincubation at 37°C for different times with 10 mM glucose and 0.3, or 0.6, or 0.9 U/ml of glucose oxidase (G.OX.). Columns represent the means of 3 triplicate experiments and vertical bars show the SD.

scintillation counter after addition of 5 ml of Ready-Solv NA. The radioactivity of the calcium-free blank samples was less than 10% of the total radioactivity; its was subtracted from the total radioactivity to yield the specific TGase-dependent putrescine incorporation. The latter is reported as pmol of putrescine incorporated/min/mg of lens protein of β_L-crystallin.

Proteins were determined by a biuret method.[19]

RESULTS AND DISCUSSION

Data reported in Figures 1 and 2 show that the TGase-directed putrescine incorporation was much higher in purified β_L-crystallin (about 90 pmol/min/mg) than in total lens soluble proteins (about 35 pmol/min/mg). This is in agreement with the finding that β-crystallin acts selectively as substrate for lens TGase.[20] The lens soluble proteins (Figure 1) and β_L-crystallin (Figure 2), after incubation at 37°C for 1 to 3 hours with glucose and different amounts of glucose oxidase, showed changes in their putrescine binding capacity. In both kinds of proteins incubations up to 2 hours at enzyme concentrations of 0.3 and 0.6 U/ml induced a time and dose-dependent increase of the putrescine binding capacity. This change was surely caused by the H_2O_2 produced, since it did not occur when catalase was present in the incubating medium (not shown). A higher glucose oxidase concentration (0.9 U/ml) and/or a longer incubation time (3 hours) elicited different effects in the two kinds of lens protein. In β_L-crystallin 0.9 U/ml of glucose oxidase were less effective than 0.6 U/ml in increasing the putrescine binding capacity; moreover, at all enzyme concentrations, the

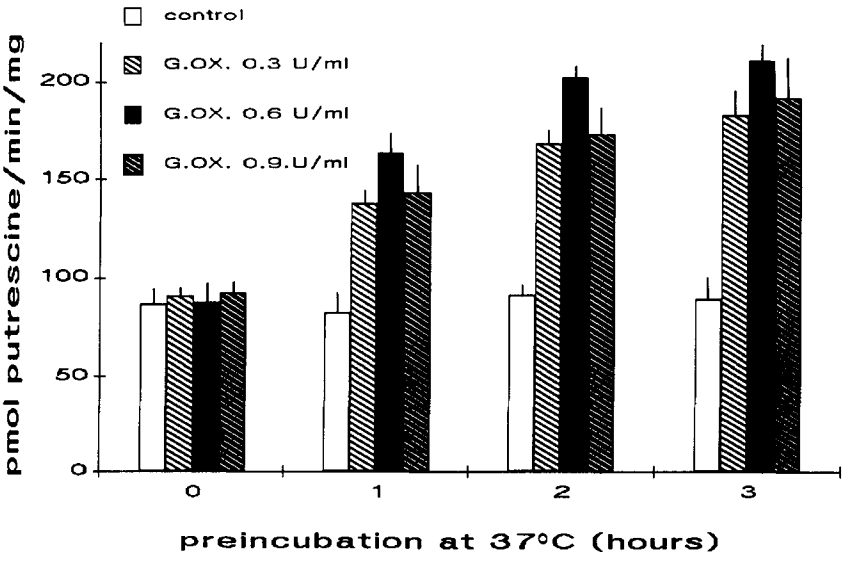

FIGURE 2 Substrate acceptor capacity for TGase (pmol of putrescine incorporated/min/mg of protein) of β_L-crystallin, after preincubation at 37°C for different times with 10 mM glucose and 0.3, or 0.6, or 0.9 U/ml of glucose oxidase (G.OX.). Columns represent the means of 3 triplicate experiments and vertical bars show the SD.

longer incubation did not significantly increase the same capacity. The lens soluble fraction displayed an initial resistance to the oxidant action of the glucose–glucose oxidase reaction, probably because it contains antioxidant factors, including glutathione, glutathione peroxidase and catalase.[21] In the total lens soluble proteins, indeed, the lower glucose oxidase concentration increased the putrescine binding capacity only after 2 hours and 0.9 U/ml of the enzyme were more effective than 0.6 U/ml in inducing the same change in the first 2 hours of incubation. A prolonged incubation at 37°C, however, induced a significant decrease of the putrescine binding activity of the control samples. Possibly this was a consequence of degradative processes occurring in the not purified lens fraction.

Subsequent experiments were conducted on the more stable purified β_L-crystallin. Figure 3 shows the calcium requirement for the TGase-directed putrescine incorporation into samples subjected to the oxidative stress induced by 2 hours of incubation with glucose and 0.6 U/ml of glucose oxidase and into appropriate controls. In all samples putrescine incorporation at 0.4 mM $CaCl_2$ was about 70% of the maximal activity measured at 5 mM $CaCl_2$. The samples exposed to oxidative stress showed a higher putrescine incorporation at all calcium concentrations; the increase however, as compared to controls, was higher at low calcium concentration, appreciable amount of putrescine being incorporated at 0.2 mM $CaCl_2$. This suggests that in the eye lens an oxidative stress may induce a loss of the TGase activity regulation exerted by calcium levels.

Activated oxygen species, namely single oxygen [21] and hydroxyl radical, [22] are known to induce nondisulfide crosslinks formation in α- and β-crystallins, in the absence of TGase. A recent work [17] has shown that exposure of lens crystallins to the hydroxyl radical generated by H_2O_2 and EDTA-chelated iron leads to crosslinking of

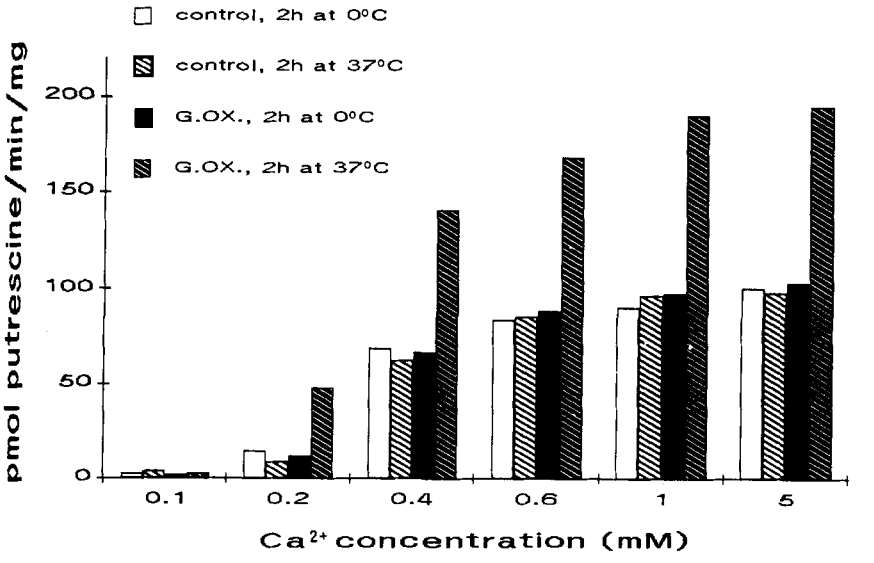

FIGURE 3 TGase-directed putrescine incorporation (pmol of putrescine/min/mg of protein) into β_L-crystallin, at different $CaCl_2$ concentrations. β_L-crystallin has been previously subjected to the treatments reported in the legend. Columns represent the means of 2 triplicate experiments.

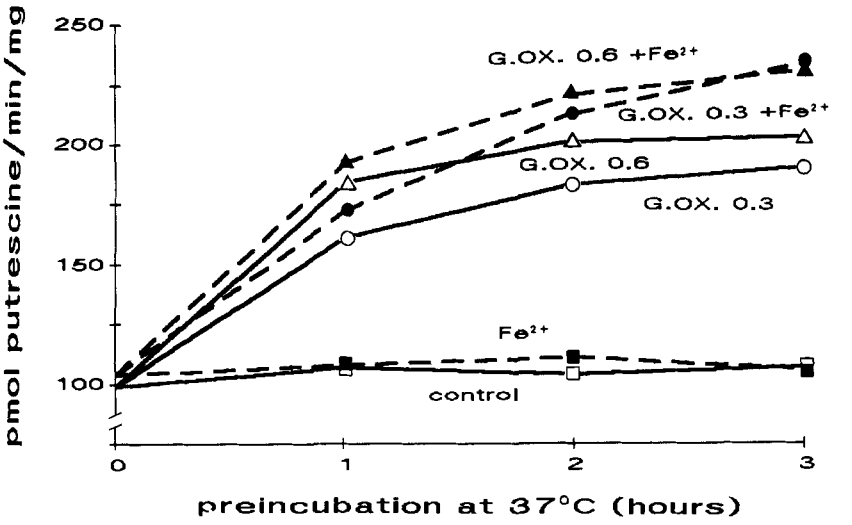

FIGURE 4 Substrate acceptor capacity for TGase (pmol of putrescine incorporated/min/mg of protein) of β_L-crystallin after preincubation at 37°C for different times with 10 mM glucose and 0.3, or 0.6 U/ml of glucose oxidase, in the presence, or in the absence, of $FeSO_4$/EDTA (0.1/0.3 mM) complex. Data are means of 2 triplicate experiments.

crystallin polypeptides, H_2O_2 alone being ineffective. Some experiments reported in Figure 4 were performed to test the effect of the hydroxyl radical on the putrescine binding capacity of β_L-crystallin. EDTA-chelated iron, in the form and concentration found to generate high levels of hydroxyl radical in solutions of H_2O_2 [17] was added to the crystallin-glucose-glucose oxidase mixture. Figure 4 clearly shows that the ferrous ion significantly increased the effect of the H_2O_2-generating system on the putrescine binding capacity of β_L-crystallin. Thus, the hydroxyl radical seems to be more effective than H_2O_2 in increasing the susceptibility of β_2-crystallin to TGase. Other studies are now in progress to explore the role of specific oxy-radicals on TGase-mediated changes of eye lens proteins.

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