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Original Contribution

Age-associated oxidative damage leads to absence of γ-cystathionase in over 50% of rat lenses: Relevance in cataractogenesis☆

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Abstract

Oxidative damage to lens proteins and glutathione depletion play a major role in the development of senile cataract. We previously found that a deficiency in γ-cystathionase activity may be responsible for glutathione depletion in old lenses. The aims of this study were: (1) to investigate the mechanism that causes the age-related deficiency in γ-cystathionase activity in the eye lens, and (2) to determine the role of γ-cystathionase deficiency in cataractogenesis. Two populations of old rats were found, one (56%) whose lenses lacked γ-cystathionase activity and the rest that exhibited detectable enzyme activity. γ-Cystathionase protein was absent in lenses from old rats without γ-cystathionase activity. Oxidative stress targeted γ-cystathionase in the eye lens upon aging, since the enzyme contained more carbonyl groups in old lenses than in young ones. γ-Cystathionase mRNA was also markedly reduced in old lenses, thus contributing to the age-associated deficiency in γ-cystathionase. Inhibition of γ-cystathionase activity caused glutathione depletion in lenses and led to cataractogenesis in vitro. In conclusion, the lack of γ-cystathionase activity in over 50% of old lenses is due to decreased gene expression and proteolytic degradation of the oxidized enzyme. This results in a high risk for the development of senile cataract.

Keywords: Glutathione; Oxidative stress; Carbonyls; Proteolysis; Senile cataract

Introduction

The eye lens is mainly formed by fiber cells packed with long-lived proteins. No measurable protein synthesis occurs in the center of the lens, but mRNA and protein synthesis takes place in the lens epithelium and in the cortex. Differentiated fiber cells do not contain nuclei or DNA, nor do they undergo mitosis [1].

Over 95% of the dry mass of the eye lens consists of specialized proteins called crystallins. Aging of the eye lens may lead to cataract formation when damage, cross-linking, and precipitation of crystallins cause a loss of lens clarity [2]. Damage to lens crystallins appears to be largely due to reactive oxygen species and UV radiation. The rate of crystallin damage increases as the antioxidant capacity in the eye lens declines with age. Oxidatively denatured crystallins contribute to cross-linking reactions that produce insoluble aggregates, resistant to protease digestion [2].

It is well known that oxidative damage to proteins is not a random process: some enzymes are much more sensitive than others. Thus, mitochondrial aconitase, liver malic enzyme, and carbonic anhydrase II have been reported as targets of oxidative damage with age [3–5]. Oxidative damage to enzymes involved in the antioxidant defense is of special importance, because it creates a vicious circle that may increase oxidative damage to cell components.

Abbreviations: AIDS, acquired immunodeficiency syndrome; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; GSH, reduced glutathione; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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γ-Cystathionase activity is essential to synthesize cysteine from methionine through the trans-sulfuration pathway. This enzyme is rate limiting in this pathway and regulates the availability of cysteine for glutathione synthesis. A deficiency in γ-cystathionase activity is, at least in part, responsible for glutathione depletion in several physiological and pathological situations, such as fetal life [6,7], surgical stress [8], cancer [9], and AIDS [10]. The present study started as a result of a previous observation in which we found that aging causes a decrease in γ-cystathionase activity in rat lenses [11]. Our aims were: (1) to investigate the mechanism responsible for the age-related deficiency in γ-cystathionase activity in the eye lens, and (2) to determine the role of γ-cystathionase deficiency in cataractogenesis.

Materials and methods

Animals

Young (5- to 6-month-old) and old (24- to 26-month-old) Wistar rats were used for studies on γ-cystathionase in the eye lens. Eye lenses were examined with the help of a magnifying glass to ensure that they were free of opacity. γ-Cystathionase was purified from 8- to 12-month-old rats. New Zealand rabbits were used to obtain antibodies against γ-cystathionase. This work was performed in agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Assay for γ-cystathionase activity in rat lenses

In eye lenses from rats, γ-cystathionase activity was measured as the rate of cysteine formation from cystathionine, as described by Sturman et al. [6] A pool of 2–3 lenses was used for each measurement. Lenses were homogenized in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM EDTA, 1 mM dithiothreitol, and 20 μM pyridoxal phosphate. The homogenate was spun at 10,000*g* for 30 min at 4°C, and the supernatant was used to determine γ-cystathionase activity.

Purification of γ-cystathionase

This was based on the method described by Bikel et al. [12] γ-Cystathionase was purified approximately 1000 times from rat liver as follows: Livers (50–100 g) were excised from male Wistar rats (8 to 12 months old), quickly cut in small pieces, washed in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM EDTA,[1] and homogenized in the same buffer (1 g/3 ml) containing 1 mM dithiothreitol, 20 μM pyridoxal phosphate, and 50 μM E-64. The homogenate was spun at 15,000*g* for 2 h at 4°C, and the supernatant was used for precipitation with ammonium sulfate. Two precipitations with ammonium sulfate were performed: the first at 55% saturation and the second at 85% saturation. Pellets were resuspended in 10 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol, and then precipitated with ethanol (50% final concentration) by spinning at 15,000*g* for 1 h 30 min at −6°C. The pellet was resuspended in 10 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol. This solution was further purified by ionic exchange chromatography using DEAE cellulose, and by gel filtration using FPLC (Pharmacia LKB) and the Superdex 200 HR 10/30 column (Pharmacia Biotechnology). Finally, the fraction of the eluate from the Superdex column corresponding to 160,000 g/mol contained the purified protein. This was confirmed by electrophoresis in a SDS-polyacrylamide gel.

In this purification process, we determined γ-cystathionase activity by using a continuous spectrophotometric method similar to that described by Flavin and Slaughter [13]. γ-Cystathionase activity was assayed at 37°C in 1 ml (final volume) of the following medium: 0.25 M Tris HCl, pH 8.1, 20 μM pyridoxal phosphate, 0.25 mM NADH, containing 30 IU of lactate dehydrogenase and using 30 mM cystathionine, as substrate. The rate for the decrease in absorbance at 340 nm was measured before and after the addition of cystathionine. γ-Cystathionase activity was calculated as the rate for NADH consumption. This continuous method could not be used to determine γ-cystathionase activity in lenses because they contain a high NADH oxidase activity.

Antibodies against γ-cystathionase

Polyclonal antibodies against γ-cystathionase were obtained from rabbits following the procedure described by Harlow and Lane [14]. Rabbits received three sc injections: the first with 20 μg of purified enzyme together with the complete Freund coadjuvant (Sigma Chemical Co., Spain); the second and third with 35 and 65 μg of purified γ-cystathionase, respectively, together with the incomplete Freund coadjuvant. Rabbit serum was obtained three weeks after the last sc injection. IgG was purified by precipitation with ammonium sulfate (25 and 50%) and by incubation with DEAE cellulose. An electrophoresis with SDS-polyacrylamide was performed to confirm the purification of the 25 and 55-kDa bands for IgG. A Western blotting was performed to confirm that these antibodies specifically detected the 40-kDa band which corresponds to the purified γ-cystathionase.

Western blotting of γ-cystathionase and protein oxidation

γ-Cystathionase was detected by Western blotting using the Protoblot Western Blot AP System (Bio-Rad, Spain) and the specific rabbit antibodies obtained in our laboratory as previously indicated. Oxidative modification