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Electrophoretic analysis of oxidatively modified eye lens proteins in vitro: implications for diabetic cataract

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Abstract

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of eye lens proteins showed that both progression of diabetic cataract in rats in vivo and precipitation of soluble eye lens proteins stressed by free radicals in vitro were accompanied by significant protein cross-linking. There was a noticeable contribution of disulfide bridges to protein cross-linking in diabetic eye lens in vivo. In contrast, under conditions in vitro, when eye lens proteins were exposed to hydroxyl or peroxyl radicals, we showed that the participation of reducible disulfide linkages in the formation of high molecular mass products was markedly lower. These in vivo – in vitro differences indicate that the generally accepted role of reactive oxygen species in diabetic cataractogenesis may be overestimated in connection with the processes of protein cross-linking.

Keywords: Eye lens proteins; Diabetic cataract; Protein cross-linking; Protein sulfhydryls; Peroxyl radical; Hydroxyl radical; SDS-PAGE

1. Introduction

The mammalian lens has an excessively high protein content. The proteins are organized in a sophisticated manner to maintain lens transparency. There is virtually no protein turnover in the lens, which provides great opportunities for post-translational modifications to occur. It is generally accepted that oxidative free-radical damage is an initiating or very early event in the overall sequence that leads to diabetic cataract [1]. In diabetic individuals, oxidative stress stemming from auto-oxidation of free or bound glucose may cause direct modification of the inner lens proteins, such as cross-linking, aggregation and precipitation. Indeed, a substantial increase in the occurrence of high molecular mass (HMM) aggregates was observed in cataractous lenses [2–10]. These aggregates, often not determined as to size, were mostly linked by disulfide bonds. Correspondingly, a progressive decrease of protein sulfhydryls was observed during development of diabetic and senile cataracts [5,7,10–17].

Isolated eye lens crystallins treated with hydrogen peroxide, hydroxyl or peroxyl radicals, generated in the solution by different chemical or physical methods, are often used as experimental models of cataract [18–26] on studying structural changes of eye lens proteins in aging or diabetic eye lenses. A general concern is the relevance of such models to the authentic situation in vivo. Unlike the situation in vivo [3–5,7,8,10,27], processes of non-disulfide covalent cross-linking were found to dominate in these in vitro models of cataract [18–21].

The aim of the present study was to analyze mass profiles of rat eye lens proteins structurally modified by hydroxyl or peroxyl radicals under in vitro conditions and to compare them with electrophoretic patterns of lens proteins isolated from diabetic rats with advanced stage of cataract. By using the standard separation method sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), special attention was paid to the processes of covalent cross-linking leading to high-molecular-mass protein aggregates.

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To characterize the role of disulfides in protein cross-linking, we inspected the samples treated under reducing and non-reducing conditions.

2. Experimental

2.1. Chemicals

Electrophoresis grade chemicals, ascorbic acid, deferoxamine mesylate, 2-mercaptoethanol, and streptozotocin (STZ) were obtained from Sigma (St. Louis, MO, USA). 2,2′-Azobis-(amidinopropane)-dihydrochloride (AAPH) was from Fluka (Buchs, Switzerland). Prestained SDS-PAGE standard (M_r 21 200–108 000) was from Bio-Rad Labs (Hercules, CA, USA). Other chemicals were purchased from local commercial sources and were of analytical grade quality.

2.2. In vitro experiments

2.2.1. Eye lens proteins

Male Wistar rats, 8–9 weeks old, weighing 200–230 g, were used as eye lens donors for preparation of soluble eye lens proteins. The animals were killed by cervical dislocation (anesthetized with thiopental 65 mg/kg i.p.) and the eye globes were excised. The lenses were dissected, rinsed with ice-cold saline. The pool of lenses was homogenized in a glass homogenizer with a PTFE pestle in ice-cold phosphate buffer (20 mM, pH 7.4, 1.2 ml/each pair) saturated with nitrogen. The total homogenate was sonified for 1 min and subsequently centrifuged for 30 min at 4 °C and 9000 × *g*. The supernatant was dialyzed for 20 h at 4 °C against 100-time-excess of 50 mM phosphate buffer, pH 7.4. The protein containing solution was analyzed for protein [28] and stored deep frozen (below −20 °C) up to the time of processing (not longer than 2 months).

2.2.2. Protein treatment with hydroxyl radicals

Hydroxyl radicals were generated by a Fenton reaction system of Fe^{2+}-EDTA-H_2O_2-ascorbate [29,30]. The in vitro incubation mixtures of 2.5 ml total volume contained reagents added at the final concentrations in the sequence as follows: eye lens proteins (0.8 mg/ml), phosphate buffer, pH 7.4 (10 mM), EDTA (0–4.8 mM), $Fe(NH_4)_2(SO_4)_2$ (0–4 mM), ascorbate (4 mM) and H_2O_2 (0.2%). The reaction mixture was incubated for different time periods from 0 to 180 min at 37 °C. Reaction was terminated by adding deferoxamine mesylate (0.5 ml, 6 mM) and cooling on ice. After centrifugation at 1000 × *g* for 15 min at 4 °C, the supernatant was precipitated with ice-cold trichloroacetic acid (TCA, 1.5 ml, 30%), followed by centrifugation at 1000 × *g* for 10 min. The pellet thus obtained was washed with TCA (1 ml, 5%) and the precipitate was redissolved in 1 ml of Na_2CO_3 (10%) in NaOH (0.5 M). Water was added to the protein solution to obtain a final volume of 2.5 ml and an aliquot of the solution was taken for protein determination [28]. For SDS-PAGE analysis, the reaction mixture was terminated by deferoxamine mesylate (0.5 ml, 6 mM) and precipitated by ice-cold TCA (1.5 ml, 30%). The pellet, obtained after centrifugation (1000 × *g* for 15 min), was washed with TCA (1 ml, 5%) and dissolved in the sample buffer.

2.2.3. Protein treatment with peroxyl radicals

Peroxyl radicals were generated by thermal decomposition of AAPH at 50 °C [31,32]. The in vitro incubation mixtures of 2.5 ml total volume contained reagents added at the final concentrations in the sequence as follows: eye lens proteins (0.8 mg/ml), phosphate buffer, pH 7.4 (10 mM) and AAPH (10 mM). The reaction mixture was incubated for different time intervals from 0 to 180 min at 50 °C. Reaction was terminated by cooling on ice. After centrifugation at 1000 × *g* for 15 min at 4 °C, the supernatant was precipitated by TCA (1.25 ml, 30%). The pellets were washed with TCA (1 ml, 5%), the precipitate was redissolved in 1 ml of Na_2CO_3 (10%) in NaOH (0.5 M), water was added to the protein solution to obtain a final volume of 2.5 ml and an aliquot of the solution was taken for protein determination [28]. For SDS-PAGE analysis, the reaction mixture was terminated by cooling on ice and precipitated by ice-cold TCA (1.25 ml, 30%). The pellet, obtained after centrifugation (1000 × *g* for 15 min), was washed with TCA (1 ml, 5%) and dissolved in sample buffer.

2.3. In vivo experiments

2.3.1. Disease model

The investigation conforms with the Guide for the Care and Use of Laboratory Animals and was approved by the local ethics committee and performed in accordance with Principles of Laboratory Animals Care (NIH publication 83-25, revised 1985) and the Slovak law regulating animal experiments (Decree 289, part 139, 9 July 2003). Male Wistar rats, 8–9 weeks old, weighing 200–230 g, were used. Experimental diabetes was induced by a single i.v. dose of streptozotocin (STZ, 55 mg/kg). STZ was dissolved in 0.1 M citrate buffer, pH 4.5. The animals were fasted overnight prior to STZ administration. Water and food were available immediately after dosing. Ten days after STZ administration, all animals with plasma glucose level >15 mM were considered diabetic and were included in the study. Control animals received 0.1 M citrate buffer. For more detail see our report by Kyselova et al. [10].

2.3.2. Lens preparation

At the indicated time intervals, the rats were killed and the eye globes were excised. The lenses were then dissected, rinsed with ice-cold saline and preserved deep-frozen under saline. Each pair of lenses was homogenized in a glass homogenizer with a PTFE pestle in 1.2 ml of ice-cold phosphate