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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

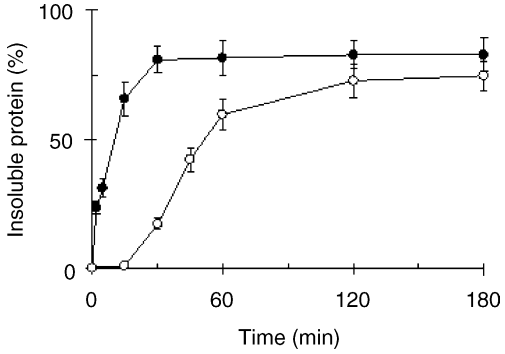

Fig. 1. Time-dependent insolubilization of rat eye lens proteins induced by hydroxyl radicals generated by Fenton reaction or peroxyl radicals generated by the azoinitiator AAPH in vitro. Soluble eye lens proteins (0.8 mg/ml) in 10 mM phosphate buffer (pH 7.4) were incubated in the presence: (filled circles) Fe^{2+} (0.8 mM), EDTA (0.96 mM), H_2O_2 (0.2%), ascorbate (4 mM) at 37 °C or (open circles) 10 mM AAPH at 50 °C. Results are mean values ± S.D. from three incubations.

buffer (20 mM, pH 7.4) saturated with nitrogen. The total homogenate was used for further analyses.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Pelleted protein samples (0.5 mg) were dissolved in a sample buffer solution of 5% SDS in boiling water bath, under either presence or absence of 5% 2-mercaptoethanol. Electrophoresis was conducted according to Laemmli [33] with stacking (4%) and separating (10%) polyacrylamide gels. The gels were stained in 0.1% Coomassie blue, and destained in 10% acetic acid in 25% methanol. The dried gels were scanned and the relative abundance of protein bands was evaluated by UN-SCAN-IT (Silk Scientific, Orem, UT, USA) software.

3. Results and discussion

The first part of the paper presents time-dependent molecular mass changes of rat eye lens proteins structurally modified by hydroxyl or peroxyl radicals under in vitro conditions, as detected by SDS-PAGE under reducing and non-reducing conditions. In the second part mass profiles of the eye lens proteins subjected to the free radical attack under in vitro conditions are compared with electrophoretic patterns of eye lens proteins isolated from diabetic rats with advanced stage of cataract.

As shown in Fig. 1, incubation of the water-soluble fraction of eye lens proteins in the presence of free radical generating systems resulted in a time-dependent accumulation of non-soluble protein products. About 80% of the total protein precipitated in the reaction system of Fenton after 30 min, while in the presence of the azoinitiator AAPH the proportion of the precipitated protein reached approximately 75% after 120 min. Once precipitated, the eye lens proteins showed only limited solubility in concentrated guanidine, suggesting that true covalent cross-linking of the protein had occurred.

The molecular masses of the native soluble eye lens proteins penetrating the stacking gel were in the range from M_r 18 000 to 94 000 (Figs. 2 and 3, lane 1). The high molecular mass (HMM) fraction (>M_r 108 000) was retained at the upper most portion of the resolving gel or failed to penetrate to the separating gel and was retained in the stacking gel. Under the reducing conditions in the presence of 2-mercaptoethanol, a profound increase in the intensity of low molecular mass proteins (LMM, M_r 18 000–32 000), corresponding to monomeric crystallins, was observed to the expense of higher molecular mass bands, reflecting the presence of disulfide cross-links in native eye lens proteins—a situation well described by other authors [10,13,34,35].

In the oxidatively modified samples, the intensity of staining of LMM protein bands (M_r 18 000–32 000) was time

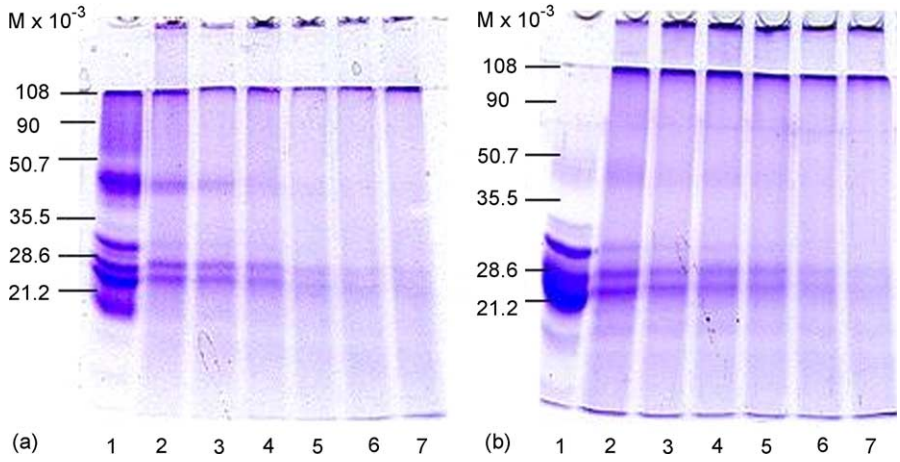

Fig. 2. Typical SDS-PAGE profiles of rat eye lens proteins oxidatively modified by hydroxyl radicals generated by Fenton reaction in vitro. Time dependence. Soluble eye lens proteins (0.8 mg/ml) in 10 mM phosphate buffer (pH 7.4) were incubated in the presence of Fe^{2+} (0.8 mM), EDTA (0.96 mM), H_2O_2 (0.2%), ascorbate (4 mM) at 37 °C. (a) Lane 1: t_0 control, lane 2: time of incubation 2 min, lane 3: t = 5 min, lane 4: t = 15 min, lane 5: t = 30 min, lane 6: t = 60 min, lane 7: t = 120 min; (b) the same as in (a) but in the presence of 2-mercaptoethanol.

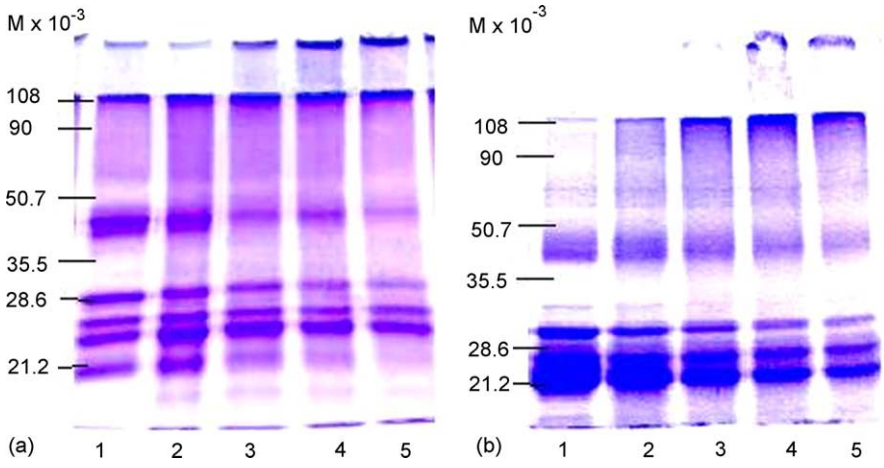

Fig. 3. Typical SDS-PAGE profiles of rat eye lens proteins oxidatively modified by peroxyl radical generated by azoinitiator AAPH in vitro. Time dependence. Soluble eye lens proteins (0.8 mg/ml) in 10 mM phosphate buffer (pH 7.4) were incubated in the presence of 10 mM AAPH at 50 °C. (a) Lane 1: t_0 control, lane 2: time of incubation 30 min, lane 3: $t = 45$ min, lane 4: $t = 60$ min, lane 5: $t = 120$ min; (b) the same as in (a) but in the presence of 2-mercaptoethanol.

dependently decreased compared to the respective bands of t_0 controls (Figs. 2 and 3 and Table 1). In the oxidatively modified samples, substantial amounts of the HMM fractions still resided in the stacking gel or in the start position of the separating gel even under the reducing conditions. The concomitant increase in the intensity of LMM bands to the expense of higher molecular ones (>M_r 50 000) was also much fainter when compared with the effect of reduction on the native protein. These results indicate that hydroxyl or peroxyl radical-mediated protein oxidation results in the accumulation of higher-molecular-mass cross-links (most likely dimers, trimers and higher unknown oligomers of eye lens crystallins) that are only partially reducible by 2-mercaptoethanol. As shown in Table 1, substantial amounts of the HMM fraction (>M_r 108 000) of the hydroxyl and peroxyl radical modified eye lens proteins remained unaffected by the reducing condition: 60 and 87%, respectively. Protein fragments with molecular masses below M_r 18 000 appeared in the oxidatively modified samples, with the relative proportion not exceeding 7.4% in mean under non-reducing conditions.

The reducibility of HMM aggregates by 2-mercaptoethanol indicates the presence of disulfide covalent cross-links. The aforementioned results showed that participation of disulfide bonding in free radical-mediated covalent HMM aggregation of soluble eye lens proteins under in vitro conditions reached about 40% [100(1 − 33.7/56.2)] for hydroxyl radical and approximately 13% [100(1 − 25.9/29.9)] for peroxyl radical treatment (Table 1, Fig. 4a). These findings are in agreement with those of other authors who reported predominance, yet without any quantitative estimate, of non-disulfide covalent cross-linking of eye lens proteins under similar conditions in vitro [18–21]. The type of covalent linkage in the remaining aggregates seen after reduction is not known.

In vitro systems of isolated eye lens crystallins treated with reactive oxygen species generated in solutions are often used as experimental models of cataract [18–26]. In this work we focused on the role of disulfides in HMM protein aggregation in relevance to the diabetic eye lens. Under the diabetic state, hyperglycemia-induced oxidative stress is considered the most likely cause of structural changes of lens proteins during diabetic cataractogenesis [1]. A progressive decrease of protein sulfhydryls has been observed during development of diabetic and senile cataracts [10,12,14,16]. Sulfhydryl oxidation is thought to be one of the main pathological events leading, through disulfide cross-linking, to protein precipitation and lens opacification [5,7,11,13,15,17,36]. Indeed, in

Table 1
SDS-PAGE separation of eye lens proteins modified by hydroxyl and peroxyl radicals in vitro

Molecular mass ($M_r \times 10^{-3}$)	<18	18–32	50–70	88–94	>108
Without 2-mercaptoethanol					
Control: t_0	4.9 ± 1.8	48.2 ± 7.2	13.9 ± 1.8	20.5 ± 8.0	12.5 ± 3.2
Fenton: $t_{30\,min}$	6.8 ± 2.7	19.9 ± 3.0	5.2 ± 1.3	11.9 ± 2.5	56.2 ± 11.3
AAPH: $t_{120\,min}$	7.4 ± 1.8	32.0 ± 5.7	9.1 ± 1.0	21.6 ± 1.3	29.9 ± 7.2
With 2-mercaptoethanol					
Control: t_0	10.1 ± 0.8	72.4 ± 10.1	5.2 ± 1.4	8.2 ± 4.6	4.1 ± 2.7
Fenton: $t_{30\,min}$	9.8 ± 0.5	30.3 ± 5.2	6.9 ± 1.2	19.4 ± 3.4	33.7 ± 7.1
AAPH: $t_{120\,min}$	9.8 ± 2.2	33.8 ± 5.8	8.7 ± 1.0	21.8 ± 7.0	25.9 ± 4.7

Values, relative percentage of total protein on the lane, are mean ± S.D. from three incubations.

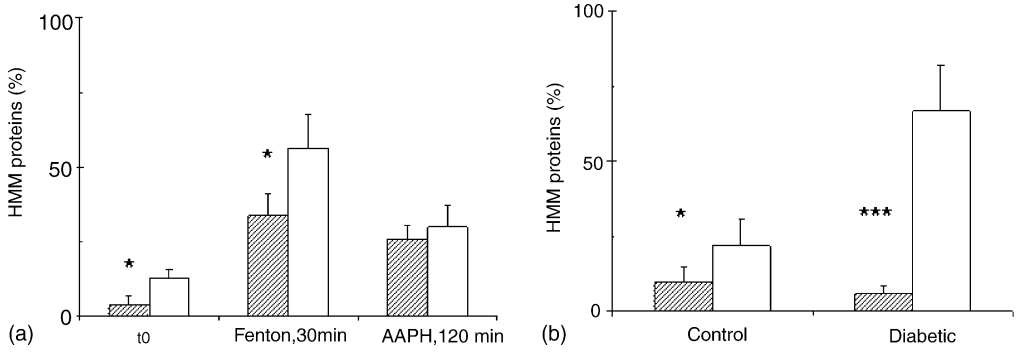

Fig. 4. HMM cross-links (>M_r 108 000) as determined by SDS-PAGE in the absence (blank columns) or presence (dashed columns) of 2-mercaptoethanol: (a) in soluble rat eye lens proteins exposed to hydroxyl radical (Fenton system) or peroxyl radical (thermal azoinitiator AAPH) in vitro and (b) in total eye lens proteins of control and STZ-diabetic rats after 34 weeks of diabetes. (a) In vitro experimental conditions as described in Figs 2 and 3. Results are mean values ± S.D. from three incubations; (b) in vivo experimental conditions as described in Section 2.3.1. Results are mean values ± S.D. (n = 8 and 12 for control and diabetic animals); $^{*}p < 0.05$, $^{***}p < 0.001$ non-reducing vs. reducing conditions (Student's t-test).

cataractous lenses of streptozotocin-diabetic rats, a substantial increase in HMM aggregates was observed [5,7,9,10,37]. These aggregates, often not determined as to size, were mostly linked by disulfide bonds since reducing agents decreased dramatically the amount of HMM proteins compared with samples not reduced. For example, in streptozotocin-diabetic rats, Abraham et al. [8] found after 150 days of diabetes almost 95% of the HMM aggregates of eye lens proteins to be disulfides reducible by 2-mercaptoethanol. In our recent work [10], in cataractous lenses of streptozotocin-diabetic rats exposed to 34 weeks of diabetes more than 90% of HMM protein cross-links (>M_r 108 000) were fully dissociated by 2-mercaptoethanol (for comparison see Fig. 4b).

On balance then, 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. Similarly, in both systems subjected to SDS-PAGE analysis, increase of densities in high molecular mass protein bands was observed to the expense of low molecular mass ones. On considering diabetic cataract conditions in vivo, there was a noticeable contribution of disulfide bridges to protein cross-linking. In contrast, under model 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 HMM products was markedly lower. These in vivo – in vitro differences indicate that the generally accepted role of reactive oxygen species (e.g. hydroxyl or peroxyl radicals) in diabetic cataractogenesis may be overestimated in connection with the processes of protein cross-linking. Our results thus point to a significant contribution of mechanisms, at least partially independent of hydroxyl and peroxyl radicals, involved in the formation and accumulation of HMM protein cross-links in the diabetic eye, which eventually may contribute to lens opacification. There is also obvious need to reconsider the adequacy of in vitro free radical models of experimental cataract for studying molecular mechanisms involved in the etiology of diabetic cataract.

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