Temporal Relationship between Lens Protein Oxidation and Cataract Development in Streptozotocin-Induced Diabetic Rats

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Summary

We compared the progression of lens opacification with the time course of oxidation of lens proteins under conditions of streptozotocin-induced experimental diabetes in rats. By the end of the 17th week, approx. 50% of the diabetic animals developed mature cataracts. During the following month 95% of the eyes in the diabetic group became cataractous. In the course of lens opacification we observed a time-dependent increase in the content of protein carbonyls and decrease in the concentration of protein sulfhydryls in the lenses of diabetic animals. Significantly higher protein carbonyl ($p<0.01$) and lower protein sulfhydryl ($p<0.001$) content was found in lenses with the advanced stage of cataract when compared with the diabetic lenses still transparent. We showed that the values of protein carbonyls exceeding 1.2 nmol/mg protein and of sulfhydryls falling below 60 nmol/mg protein corresponded to an about 50% incidence of mature cataract development. At the end of the 34th week, when all lenses of diabetic rats became cataractous, the corresponding values of protein carbonyls and sulfhydryls were 2.5 nmol/mg protein and 27 nmol/mg protein, respectively. The main finding of this study is the disclosure of a quantitative relationship between the degree of protein oxidation and the rate of advanced cataract development in the widely used model of streptozotocin-induced experimental diabetes in rats.

Key words: *C*ataract – STZ-diabetes – Protein oxidation – Protein sulfhydryls – Protein carbonyls

Running Title: *Lens protein oxidation and diabetic cataract*

Introduction

Current evidence supports the view that cataractogenesis is a multifarious process, in which combination of more closely linked events induces subtle post-translational modifications in the lens structural proteins, enhancing their aggregation, fragmentation, and precipitation, resulting eventually in lens opacification (Brownlee 1996, Bron *et al.* 1998, Baynes and Thorpe 1999, Stitt 2001, Hockwin *et al.* 2002). Chronic elevation of blood glucose in diabetes presents a severe risk factor for development of cataract as one of the earliest secondary complications of diabetes mellitus. Free radical production is increased in the diabetic lens and natural antioxidant defenses are compromised, which results in increased oxidative stress. Human studies, as well as *in vitro* and *in vivo* animal experiments strongly suggest that there is an association between increased oxidative stress and the development of cataract (Bhuyan and Bhuyan 1984, Altomare *et al.* 1996, Altomare *et al.* 1997, Kubo *et al.* 1999, Boscia *et al.* 2000). Antioxidant supplementation was found to inhibit the development of cataract in experimentally induced diabetes in rats (Ross *et al.* 1982, Ansari and Srivastava 1990, Linklater *et al.* 1990, Ohta *et al.* 1999, Agardh *et al.* 2000, Packer *et al.* 2001). In their study on human cataractous lenses, Boscia *et al.* (2000) identified a threshold of protein oxidation above which clinically significant cataracts developed.

The aim of this study was to determine the temporal relationship between oxidation of the lens proteins and the course of lens opacification in hyperglycemic rats during a period of 34 weeks following induction of experimental diabetes by streptozotocin. Two distinctive parameters of lens protein oxidation were measured: protein carbonyls and free sulfhydryls. Accumulation of protein free carbonyls has been closely related to the development of both senile and diabetic cataracts (Altomare *et al.* 1996, Vendemiale *et al.* 1999, Boscia *et al.* 2000, Chevion *et al.* 2000). Sulfhydryl oxidation is also thought to be one of the pathological events leading, through disulfide cross-linking and molecular aggregates, to protein precipitation and lens opacification (Truscott and Augusteyn 1977, Monnier *et al.* 1979, Hum and Augusteyn 1987, Perry *et al.* 1987, Swamy and Abraham 1987, Duhaiman 1995, Vendemiale *et al.* 1996, Takemoto 1996, 1997). To characterize the role of disulfide cross-links in protein aggregation, changes in molecular weight of lens proteins determined by sodium dodecyl sulfate polyacryl amide gel electrophoresis were compared under reducing and non-reducing conditions.

Material and Methods

Disease model

The investigation conforms with the Guide for the Care and Use of Laboratory Animals. Male Wistar rats, 8 - 9 weeks old, weighing 200 - 230 g, were used. The animals were of monitored conventional quality and came from the Breeding Facility of the Institute of Experimental Pharmacology Dobra Voda (Slovak Republic). Experimental diabetes was induced by a single *i.v.* dose of streptozotocin (STZ, 55 mg/kg). STZ was dissolved in 0.1 mol/l 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 mmol/l (n = 40) were considered diabetic and were included in the study. Control animals (n = 24) received 0.1 mol/l citrate buffer. During the experiment the animals were housed in groups of two in cages of the type T4 Velaz (Prague, Czech Republic) with bedding composed of wood shaving (exchanged daily). Tap water and pelleted standard diet KKZ-P-M (Dobra Voda, Slovak Republic) were available *ad libitum*. The animal room was air-conditioned and the environment was continuously monitored for the temperature of 23±1 ^{o}C and relative humidity of 40 - 70%. For biochemical analyses the animals were killed at the 8th, 17th and 34th week with the respective number of animals: control group, 8, 8, 8; diabetic group, 12, 16, 12.

Blood measurements

Plasma glucose levels were measured in two-month intervals using the commercial Glucose (Trinder) kit (Sigma, St. Louis, MO, U.S.A.). Glycated hemoglobin was determined by standard kit GHB 100 obtained from Bio-La-Test (Czech Republic).

Observation of cataract

The progress of cataract was monitored weekly by an individual without prior knowledge of affiliation of an animal to an experimental group. Eyes were inspected by hand-held slit-lamp, preceded by topical administration of 1% mydriacyl drops. Cataract formation was scored on the basis of 0 for absence of advanced cataract (clear lenses and cataractous lenses still transparent) and 1 for presence of advanced cataract (opaque lens). No attempt was made to grade the cataract, only a binomial paradigm was used: present or absent. Cataract formation

was considered complete when the red fundus reflex was no longer visible through any part of the lens (Follansbee *et al.* 1997, Agardh *et al.* 2000).

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 teflon pestle in 1.2 ml of ice-cold phosphate buffer (20 mmol/l, pH 7.4) saturated with nitrogen. The total homogenate was used for further analyses.

Protein determination

Total protein concentration in the lens homogenate was determined by Lowry's method (Lowry *et al.* 1951) using bovine serum albumin as a standard.

Carbonyl determination

Content of free carbonyls in the total lens proteins was determined by the procedure of Levine *et al.* (1990) using the 2,4-dinitrophenylhydrazine (DNPH) reagent. Two aliquots of lens homogenate of approximately 3 mg of proteins were precipitated with equal volume of 10% trichloroacetic acid (TCA), and after centrifugation, the pellets were treated with 0.5 ml of 10 mmol/l DNPH dissolved in 2 mol/l HCl as a sample or with 0.5 ml of 2 mol/l HCl as a control blank. The reaction mixtures were allowed to stand for 1 h at room temperature with stirring at 10-min intervals. Next, 0.5 ml of ice-cold 20% TCA was added and left on ice for 15 min. The precipitated proteins were subsequently washed three times with 1 ml of ethanol-ethyl acetate (1:1). The washed pellets were dissolved overnight in 1.8 ml of 6 mol/l guanidine. Any insoluble material was removed by centrifugation at 3000 rpm for 15 min. The difference spectrum of the DNPH derivatives vs. HCl controls was scanned at 322-370 nm on Hewlett Packard 8452A Diode Array Spectrophotometer. Carbonyl content was calculated from the absorbance readings, using 22 000 $l.mol^{-1}cm^{-1}$ as the molar absorption coefficient. The final values were normalized to actual protein amount determined on the basis of absorbance readings at 280 nm of parallel HCl-treated control blank samples.

Sulfhydryl determination

Content of –SH groups in the lens proteins was determined using the Ellman´s procedure modified by Altomare *et al.* (1997). Aliquots of total lens homogenate of approximately 3 mg

of proteins were precipitated with equal volume of 4% sulfosalicylic acid (SSA); the pellets obtained after centrifugation were washed with 1 ml of 2% SSA to remove free thiols. The washed pellets were taken up to 0.2 ml of 6 mol/l guanidine (pH 7.4) and read spectrophotometrically at 412 nm and 530 nm on Labsystems Multiscan RC Spectrophotometer, before and after 30 min incubation in the dark with 50 µl of 10 mmol/l 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Content of protein sulfhydryls was calculated using a calibration curve prepared with reduced glutathione.

Validation of the biochemical analyses

To validate the aforementioned assays of carbonyl and sulfhydryl groups, total lens proteins obtained from control healthy rats were repeatedly analyzed. Assay reproducibility characterized by S.D. and obtained in repeated determinations (n=6) was as follows: 0.58 ± 0.09 nmol/mg protein for carbonyls, and 92.0 ± 14.7 nmol/mg protein for sulfhydryls.

Sodium dodecyl sulfate polyacryl amide electrophoresis

An aliquot of total lens homogenate equivalent to 0.5 mg of protein was precipitated with equal volume of 10% TCA and the washed pellets were treated with 5% SDS under either reducing conditions in the presence of 5% 2-mercaptoethanol or non-reducing conditions. Electrophoresis was conducted according to Laemmli (1970) with stacking and separating gels containing 4% and 10% acryl amide, respectively. 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 software.

Reagents

Streptozotocin (STZ), 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and electrophoresis grade chemicals were obtained from Sigma. Other chemicals were purchased from local commercial sources and were of analytical grade quality.

Statistical analysis

Statistical analysis of the data was done using unpaired Student's t-test and Fischer's exact test. Least square procedure was used to correlate the parameters.

Results

Body weight and blood glucose

The weights of diabetic rats were significantly lower as compared with those in the control group: diabetic animals weighed 239 ± 31 g (mean ± S.D., n = 12) at the end of experiment, while control animals gained weight from 234 ± 29 g (mean ± S.D, n = 24) at the beginning of experiment to 501 ± 54 g (mean ± S.D., n = 8) at the end of the 34th week.

Persistent hyperglycemia, on average over 20 mmol/l, was observed in diabetic animals throughout the whole experiment. The average blood glucose of control rats during the 34 week experiment was 6.7 ± 2.5 mmol/l (mean ± S.D., n = 8). At the end of the 24th week, the values of glycated hemoglobin were as follows: 4.1 ± 0.7 and 7.6 ± 0.7% (mean ± S.D., n=6) for control and diabetic animals, respectively.

Cataract formation

As shown in Fig. 1a, the appearance of advanced cataract in diabetic rats became apparent after 10 weeks. By the end of the 17th week approximately 50% of the animals in the diabetic group developed mature cataracts and with the week 22 more than 95% of all eyes became opaque in the diabetic group ($p<0.001$, diabetic vs. control rats, Fischer's exact test). None of the control rats developed lens opacity.

Changes in free carbonyls and sulfhydryls of lens proteins

The diabetic state led to an increase in free carbonyl groups of lens proteins as measured by absorbance of DNPH bound to total lens proteins (Fig. 1b). In the 17th and 34th week of the experiment, significantly higher values of free carbonyls were observed in the lenses of diabetic animals compared to healthy controls ($p<0.05$ and $p<0.001$, respectively). The time dependent change in DNPH-reactive carbonyls was accompanied by a steady decrease in protein free sulfhydryl groups titratable by DTNB (Fig. 1c). At the sampling time points in the 17th and 34th weeks significantly lower values of absolute content of lens sulfhydryls were observed in diabetic rats compared to age matched controls ($p<0.001$). At the end of the 17th week when lenses of diabetic rats were classified according to the formation of cataract, significantly higher carbonyl content ($p<0.01$) and significantly lower free sulfhydryls ($p<0.001$) were observed in the group of opaque lenses in comparison with the diabetic lenses that were still transparent (Fig. 2).

As shown in Fig. 3, an inverse correlation was found in STZ-diabetic animals between the eye lens protein content of free carbonyls and sulfydryls ($R^2 = 0.862$).

Sodium dodecyl sulfate polyacryl amide electrophoresis

At the end of the experiment, SDS-PAGE was used to detect qualitative and quantitative changes in lens protein composition on the basis of molecular size. The molecular weights of the proteins penetrating the stacking gel were in the range from 20 to 94 kDa (Fig. 4 and Table 1). The high molecular weight (HMW) fraction (>101 kDa) 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. In the diabetic group, the intensity of staining of all the protein bands apparent in the separating gel (18 – 94 kDa) was significantly decreased compared to the respective bands of controls (Table 1). Under the reducing conditions in the presence of 2-mercaptoethanol, profound increase in intensity of low molecular weight proteins (18 – 32 kDa) was observed both in the control and diabetic groups to the expense of HMW bands.

Discussion

In this study we compared the time course of lens opacification with the time-related oxidation of lens proteins under conditions of STZ-induced experimental diabetes in rats. Free carbonyls and sulfhydryls were used as markers of oxidative modifications of lens proteins.

As examined by a slit-lamp ophthalmoscope, by the end of the 17^{th} week approx. 50% of the diabetic animals developed mature cataracts. During the following month more than 95% of the eyes in the diabetic group became cataractous. In this type of experimental cataract, a similar progression of lens opacification was observed (Follansbee *et al.* 1997, Agardh *et al.* 2000).

In the course of cataract development we observed a time-dependent increase in the content of protein carbonyls ($p<0.05$ for 17^{th} week vs. 8^{th} week; $p<0.01$ for 34^{th} week vs. 17^{th} week) and decrease in the concentration of protein sulfhydryls ($p<0.001$ for 17^{th} week vs. 8^{th} week; $p<0.001$ for 34^{th} week vs. 17^{th} week) in the lenses of diabetic animals. As measured at the end of the 17^{th} week in the diabetic group, a significantly higher protein carbonyl content ($p<0.01$) and lower protein sulfhydryls ($p<0.001$) were found in lenses with the advanced stage of cataract when compared with the lenses still transparent. Analogously, in their human studies, Altomare *et al.* (1997) and Boscia *et al.* (2000) found higher protein carbonyl content and

lower protein sulfhydryls in cataractous lenses from diabetic patients compared to clear lenses from non-diabetic subjects. Similar changes were observed under *in vitro* conditions in lens proteins incubated in the presence of sugars or their metabolites (Beswick and Harding 1987, Argirova and Breipohl 2002). All these findings are in line with the generally accepted notion that oxidative events are involved in the onset of diabetic eye complications (Baynes 1991, Varma 1991, Jahngen-Hodge *et al.* 1994, Bron *et al.* 1998, Vendemiale *et al.* 1999, Chevion *et al.* 2000, Spector 2000).

In their study on human cataractous lenses, Boscia *et al.* (2000) identified a threshold of protein oxidation above which clinically significant cataracts developed. They found protein sulfhydryls below and protein carbonyls above their specific thresholds to be predictive for the presence of cataract. In this study on STZ model of cataract in rats, at the end of the 17^{th} week when the incidence of development of advanced cataract reached approximately 50%, the corresponding values of protein carbonyls and sulfhydryls were about 1.2 nmol/mg protein and 60 nmol/mg protein, respectively. Increase of free carbonyls and decrease of free sulfhydryls beyond the above mentioned values resulted in further steep increase in the rate of advanced cataract development.

Lysine, arginine, proline, histidine and tryptophan are the amino acid residues most likely to form carbonyl derivatives as a result of direct metal-catalyzed oxidation (Stadtman and Berlett 1997). The conventional assay of protein carbonyls measures spectrophotometrically the binding of 2,4-dinitrophenylhydrazine (DNPH) (Levine *et al.* 1990, Reznick and Packer 1994). In protein glycation, however, the appearance of carbonyl groups is not specific for oxidative modification. Early glycation products and some of the final advanced glycation endproducts (AGEs) contain carbonyls derived from a protein-bound sugar component, and they may contribute to the total pool of DNPH-reactive carbonyls. Recently, however, in their *in vitro* glycation experiments, Liggins and Furth (1997) and Stefek *et al.* (1999) came to the conclusion that the early glycation products do not contribute to the DNPH reaction. The DNPH assay was found selective enough to discriminate between protein-bound carbonyls produced by metal-catalyzed oxidations and those formed in the early glycation steps. Despite the fact that the contribution of the final AGEs to the total DNPH-reactivity of glycation-altered protein remains unclear, the aforementioned findings stress involvement of oxidative steps in the formation of the majority of DNPH-reactive carbonyl compounds generated by glycoxidation. In the light of the above findings, the presence of the increased levels of free carbonyls in proteins of the cataractous diabetic eye lens can be interpreted as a result of oxidative insult initiated by hyperglycemia.

Hyperglycemia-induced oxidative stress is considered as the most likely cause of changes in the sulfhydryl status of lens proteins during diabetic cataractogenesis. A progressive decrease of protein sulfhydryls has been observed generally during development of diabetic and senile cataracts (Hum and Augusteyn 1987, Duhaiman 1995, Boscia *et al.* 2000). Sulfhydryl oxidation is thought to be one of the main pathological events leading, through disulfide cross-linking and molecular aggregation, to protein precipitation and lens opacification (Truscott and Augusteyn 1977, Monnier *et al.* 1979, Perry *et al.* 1987, Swamy and Abraham 1987, Takemoto 1996, 1997). Indeed, in diabetic cataractous lenses, we observed an increase in HMW aggregates. These HMW proteins were present in a small amount also in controls, but lenses in long-term diabetic animals showed a profound increase in the HMW protein level. These aggregates of unknown size are mostly linked by disulfide bonds since the reducing agent 2-mercaptoethanol decreased dramatically the amount of HMW proteins compared with samples not reduced with 2-ME (see Table 1). Disulfide-linked HMW aggregates of a size ranging from 40 to 176 million daltons were observed in cataractous lenses of diabetic rats by Monnier *et al.* (1979).

In conclusion, the main finding of this study is the disclosure of a quantitative relationship between the degree of protein oxidation and the rate of advanced cataract development in the widely used model of experimental diabetes induced in rats by streptozotocin corroborating thus the role of oxidative mechanisms in the development of diabetic cataract.

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TABLE 1. SDS-PAGE separation of lens proteins quantified after Coomassie blue staining at the end of week 34

Mol. weight (kDa)	18 – 32	50 – 70	88 – 94	> 101
Without 2-mercaptoethanol				
Control	43.8 +/- 8.2	28.9 +/- 7.2	5.6 +/- 2.3	21.6 +/- 9.1
Diabetic	14.7 +/- 5.8 ***	14.1 +/- 5.1 ***	3.5 +/- 1.8 *	66.6 +/- 15.6 ***
With 2-mercaptoethanol				
Control	60.3 +/- 18.9	25.7 +/- 9.4	4.2 +/- 2.3	9.8 +/- 5.1
Diabetic	68.0 +/- 19.5	23.10 +/- 8.9	3.5 +/-1.9	5.6 +/- 2.6

Values, relative percentage of total protein on the path, are means +/- S.D. (n=8 and 12 for control and diabetic animals, respectively); * $p < 0.05$; ***$p < 0.001$ Diabetic group vs. Control group (Student's t-test).

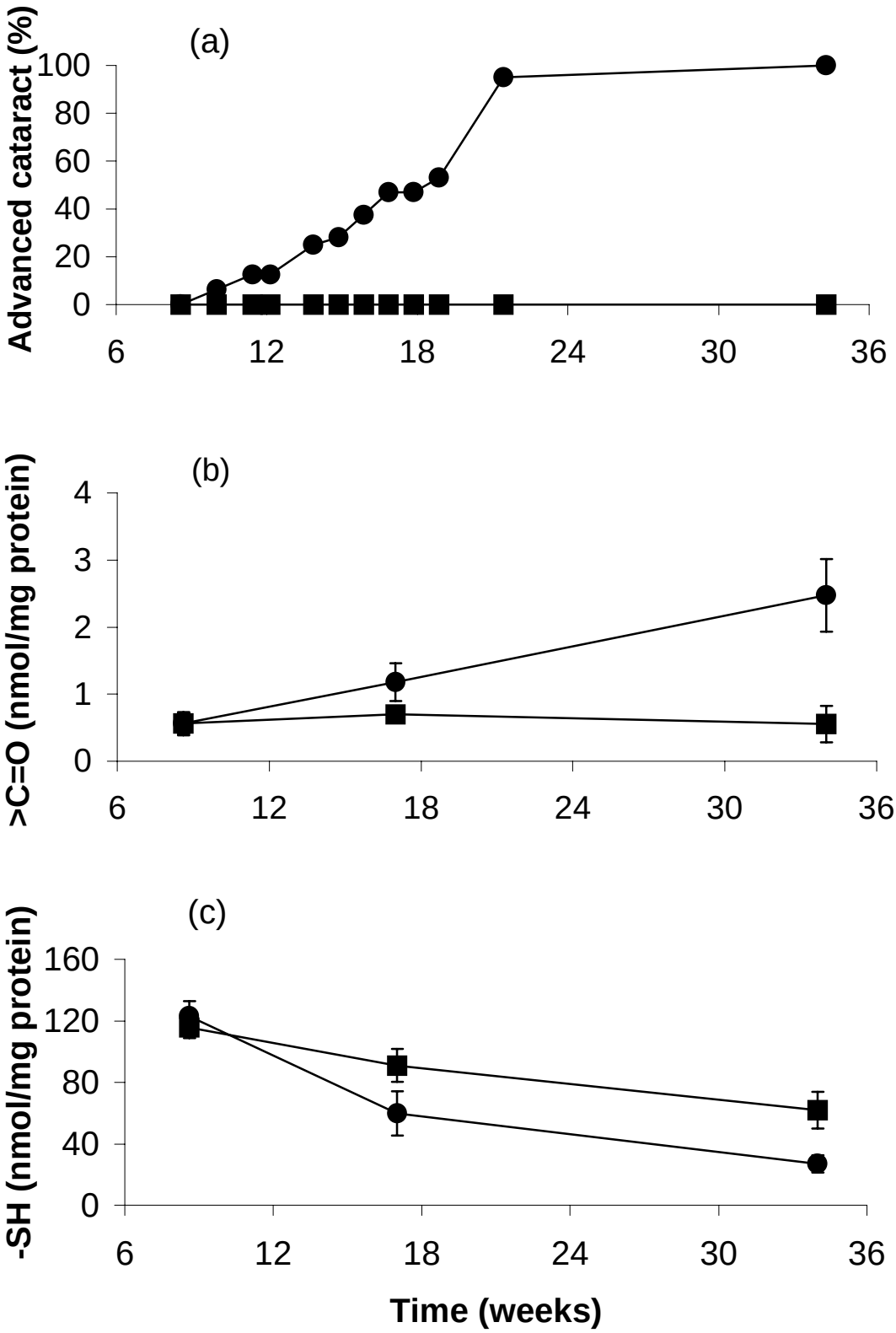
Figure legends

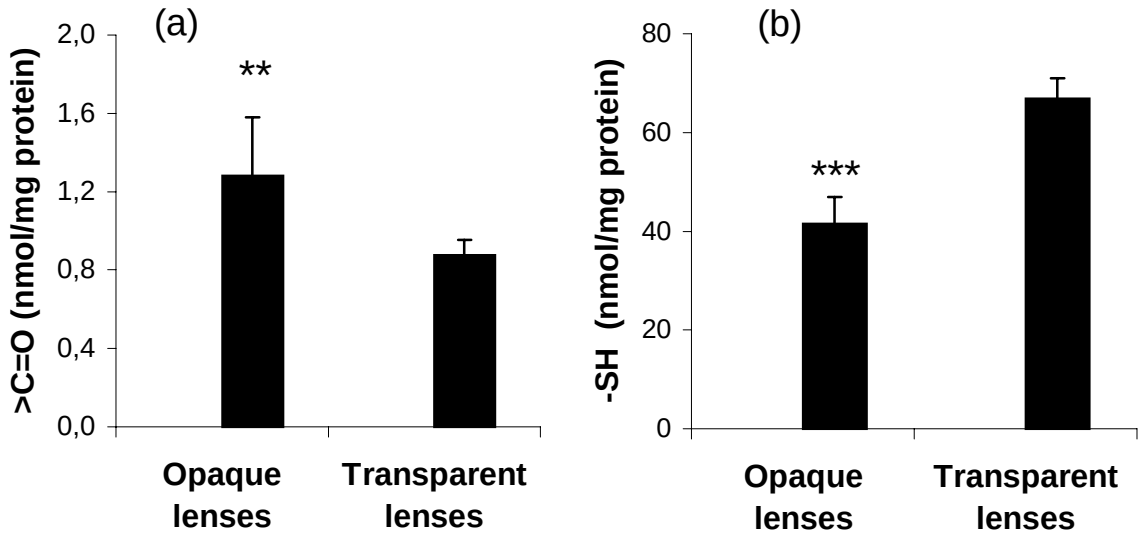
Fig. 1. *Progress of cataract formation and changes of free carbonyls and sulfhydryls in lens proteins of experimental animals. (■) Control group; (●) Diabetic group. Results are mean values ± S.D. (n = 8 – 40)*

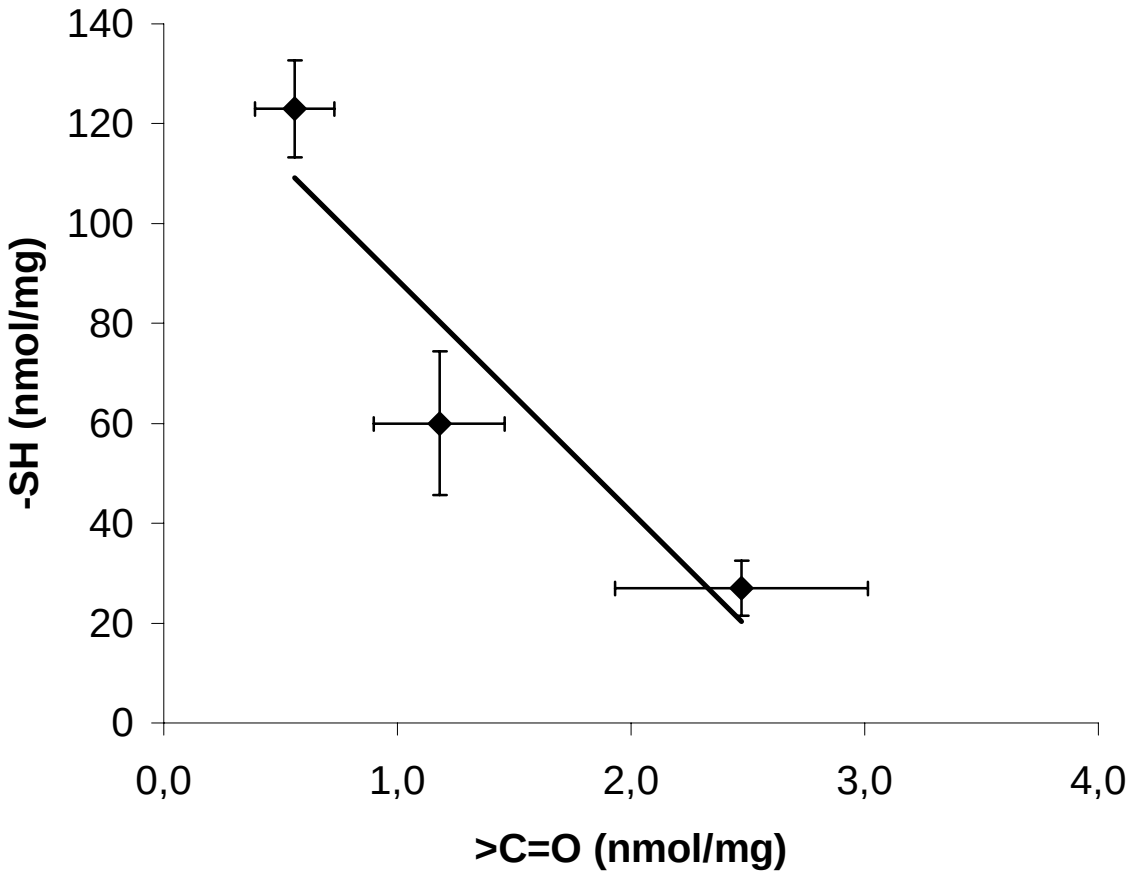
Fig. 2. *Free carbonyls and sulfhydryls in lens proteins of diabetic rats classified according to formation of cataract at the end of week 17. Results are mean values ± S.D. (n = 16). ** $p < 0.01$, *** $p < 0.00$, (Student's t-test). Opaque lenses (mark 1) vs. Transparent lenses (mark 0).*

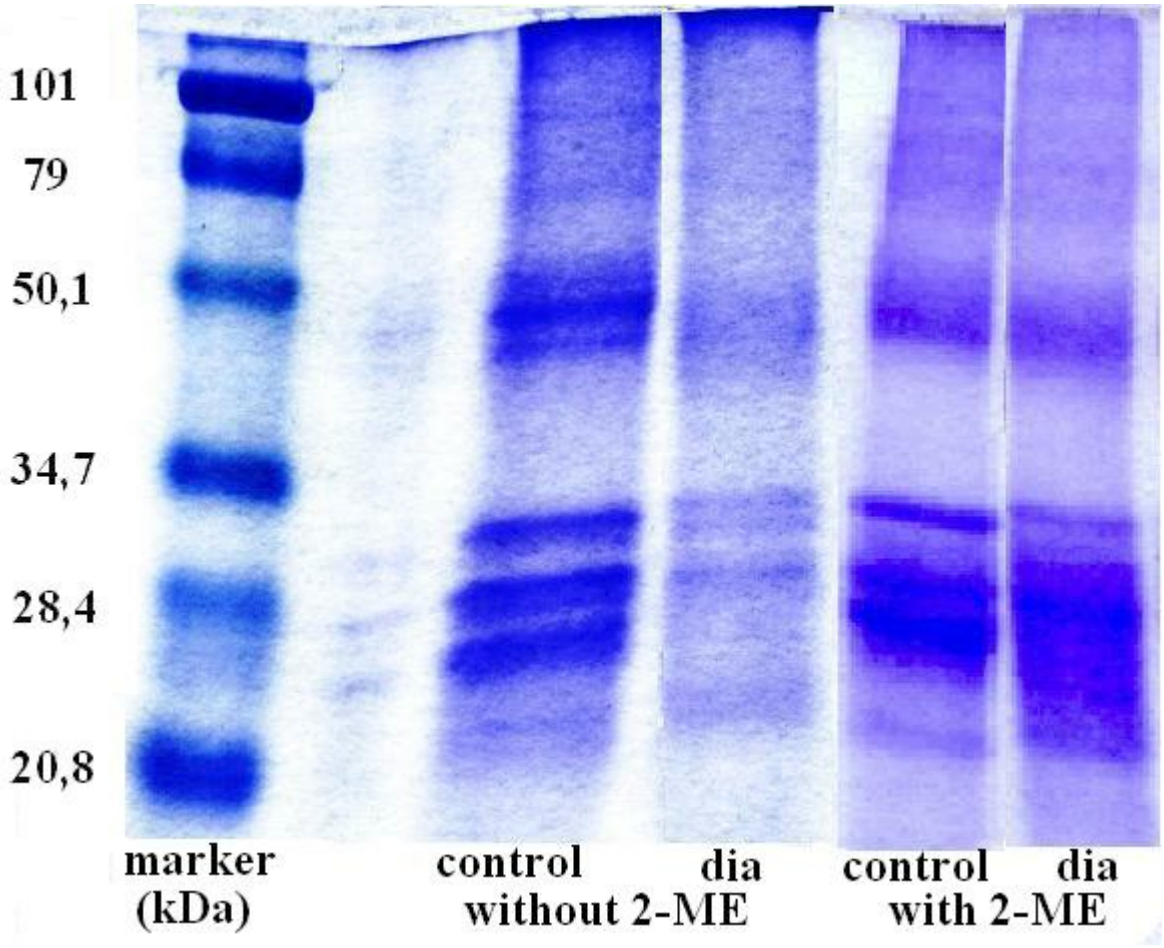
Fig. 3. *Correlation between the eye lens protein carbonyls and sulfhydryls in STZ-diabetic rats. $R^2 = 0.862$. Results are mean values ± S.D. (n = 8 – 16).*

Fig. 4. *Typical SDS-polyacrylamide gel electrophoretic pattern of lens proteins obtained at the end of week 34. 2-ME, 2-mercaptoethanol.*


(a)
Advanced cataract (%)
100
80
60
40
20
0
6
12
18
24
30
36
(b)
>C=O (nmol/mg protein)
4
3
2
1
0
6
12
18
24
30
36
(c)
-SH (nmol/mg protein)
160
120
80
40
0
6
12
18
24
30
36
Time (weeks)


(a)
>C=O (nmol/mg protein)
2,0
1,6
1,2
0,8
0,4
0,0
**
Opaque lenses
Transparent lenses
(b)
-SH (nmol/mg protein)
80
60
40
20
0
***
Opaque lenses
Transparent lenses


140
120
100
80
60
40
20
0
-SH (nmol/mg)
0,0
1,0
2,0
3,0
4,0
>C=O (nmol/mg)


101
79
50,1
34,7
28,4
20,8
marker (kDa)
control without 2-ME
dia
control with 2-ME
dia