Protein Oxidation and Lens Opacity in Humans

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PURPOSE. Oxidative damage to lens proteins is a major factor leading to cataract formation. It is of pathogenic importance to determine a threshold of protein oxidation over which opacification of the lens takes place.

METHODS. Sixty-two lenses extracted from patients affected by idiopathic senile, diabetic, or myopic cataract were studied. Clear lenses were obtained from subjects undergoing enucleation (n = 10) or vitrectomy for giant retinal tears (n = 9), and were age- and sex-matched to those with cataract. The content of carbonyls and sulfhydryls (P-SH) in proteins in the lens was assessed using spectrophotometric assay.

RESULTS. An age-associated inverse relation (P < 0.01) was noted in the content of P-SH, the concentrations of which were also inversely related (P < 0.05) to the content of protein carbonyls. These changes were more pronounced in cataracts than in clear lenses and in diabetic and myopic cataracts when compared with senile cataracts. The drop of P-SH concentration occurred earlier in diabetic and in myopic cataracts than in senile cataracts. The accumulation of protein carbonyls >2 nmol/mg protein and the decrease of P-SH below 12 to 10 nmol/mg protein were always accompanied by lens opacification.

CONCLUSIONS. Idiopathic senile, diabetic, and myopic cataractogenesis appear to be dependent on oxidative damage to lens proteins. This damage occurs earlier in myopic and diabetic patients. Values of P-SH below and protein carbonyls above their specific threshold were found to be predictive for the presence of cataract. Because increased oxidation was observed in clear lenses removed from myopic and diabetic subjects, oxidation may be involved in the pathogenesis of these forms of human cataract. (Invest Ophthalmol Vis Sci. 2000;41:2461–2465)

The light-scattering process is the primary factor responsible for the turbidity and wave front distortion by the cataractous lens. The aggregation of lens protein into randomly distributed high molecular weight clusters are thought to produce sufficient fluctuation in protein density to account for the opacification. In fact, protein aggregation results in the development of very high molecular weight aggregates of sufficient size to directly scatter the light and in the creation of protein-rich and protein-poor phases causing local changes in refractive index and thus increased light scattering.

Protein aggregation increases with age. The crystallins, which constitute approximately 90% of the total protein content of the lens, accumulate and show many age-related oxidative changes. These include formation of disulfide and other inter- and intramolecular cross-links and methionine oxidation, all of which result in the aggregation of high molecular weight molecules. Therefore, the protein redox status seems to be fundamental to maintain the lens function and transparency. It may be possible that local or systemic conditions affecting the protein redox status, such as myopia and diabetes, influence this process.

Several authors have tried to identify a threshold for lens protein aggregation in view of the causal relationship of these molecular changes with the increased lens turbidity. Light-scattering measurements have shown a threshold of turbidity, six to eight times that of a newborn lens, at which point clinically significant opacification is found. A threshold of green fluorescence, perhaps caused by protein oxidation, in both nuclear and cortical cataractous soluble fractions has been suggested as a marker for cataractogenesis. Recently, it was hypothesized that a threshold of lipid oxidation might exist above which the opacification takes place and that this could be surpassed earlier in some subjects predisposed to cataract formation. It may be of pathogenic and therapeutic importance to determine a threshold of protein oxidation over which the opacification of the lens becomes clinically evident. General strategies for the inhibition of cataract and other molecular condensation diseases are being developed. A method to measure the efficacy of reagents that inhibit lens protein oxidation and aggregation could rely on the assessment of a threshold. It may also be relevant to understand whether local or systemic conditions, such as myopia and diabetes, may affect this threshold.

The assessment of carbonyl and sulfhydryl proteins has been suggested as being a valuable index of the protein redox status in the lens. In fact, the level of carbonyl proteins, derived from amino acids during metal-catalyzed oxidation of proteins in vitro and in vivo, represents a direct measure of the oxidative injury to these molecules. The sulfhydryl proteins,
known to have structural and functional role in the crystallin lens, contain an elevated number of thiol groups and, therefore, are reduced as a result of oxidation. For these reasons, the level of these compounds is an indirect measure of protein oxidation leading to protein aggregation. A linear relationship between subject age and the amount of protein carbonyl groups has been found in the human eye lens cortex.11 It has been already shown that during senile cataract development a progressive decrease in SH content of the crystallins occurs.12–14 However, a threshold of these protein oxidation products has never been accurately identified. Therefore, we determined whether an age-related threshold of carbonyl and sulfhydryl proteins concentration, over which the opacification of the lens becomes clinically evident, exists and if myopia and diabetes affect this threshold.

**MATERIALS AND METHODS**

This study was performed on clear and cataractous lenses coming from four groups of subjects: (1) clear lenses from healthy, nonmyopic, nondiabetic subjects; (2) cataractous lenses from nonmyopic, nondiabetic subjects older than 43 years, without posterior subcapsular opacities, previous eye trauma, or signs of significant ocular inflammation (senile cataract); (3) cataractous lenses from myopic subjects (axial length demonstrated by A-scan ultrasonography of 24.0 mm or greater); and (4) cataractous lenses from diabetic patients on treatment (diabetic cataract; Table 1). The first group included 19 subjects (age, 43–75 years; men 

| Table 1. Clear Lenses and Cataracts Included in the Study and Divided per Group |
|---------------------------------|-----------|-----------|-----------|-----------|
| Clear lenses from               | 43–50     | 51–60     | 61–70     | 71–75     |
| age (years)                     | Years     | Years     | Years     | Years     |
| healthy subjects                | 5         | 5         | 5         | 4         |
| Senile cataracts                |           |           |           |           |
| Diabetic cataracts              |           |           |           |           |
| Myopic cataracts                |           |           |           |           |
| Cataracts from                  |           |           |           |           |
| diabetic subjects               |           |           |           |           |
| Clear lenses from               |           |           |           |           |
| myopic subjects                 |           |           |           |           |

The study conformed to the tenets of the Declaration of Helsinki and was approved by the local Committee for Human Experimentation.

**Protein Carbonyl Measurement**

Equal aliquots of 2 mg of protein were precipitated with 10% trichloroacetic acid (TCA) and, after centrifugation, the pellet was treated with 1 ml of 2% (w/v) dinitrophenylhydrazine (DNPH) in 2N HCl or with 1 ml of 2N HCl as a control. Samples were incubated at room temperature and stirred for 5-minute intervals. Two hundred microliters of 50% TCA were then added, and the precipitated proteins were subsequently washed three times with 1:1 ethanol-ethylacetate and three times with 10% TCA. The final precipitate was dissolved in 6 M guanidine, and the difference spectrum of the DNPH derivatives versus HCl controls was followed spectrophotometrically at 340 to 370 nm with a scan program.15 The concentrations of carbonyl groups were calculated from the absorbance spectrum, using 21.5 mM/cm as the extinction coefficient for aliphatic hydrazones.

**Protein Sulphhydryl Measurement**

The content of protein sulphhydrils (P-SH) in the lens was assessed spectrophotometrically with a modification of the Ellman procedure.16 Proteins were precipitated by adding 4% (w/v) sulfosalicylic acid (SSA); the pellet, obtained after centrifugation, was washed twice with 2% SSA to remove free thiols, centrifuged at 3000g for 3 minutes and finally resuspended in 200 μl of 6 M guanidine, pH 6.0. Samples were read spectrophotometrically at 412 and 530 nm, before and after 30 minutes of incubation in the dark with 5,5-dithiobis 2-nitrobenzoic acid. P-SH concentrations were calculated using a standard curve prepared with reduced glutathione.

**Protein Measurement**

Protein concentration in lens homogenate and in guanidine-treated samples was assessed using a Bio-Rad kit for protein assay (Bio-Rad GmbH, Munchen, Germany). Bovine serum albumin, dissolved in guanidine, was used to prepare a standard curve.

**Chemicals**

All the reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) or represented the best commercial grades.

**Statistical Analysis**

Data were analyzed using the Student’s t-test for unpaired data and by the χ² analysis of contingency tables followed by Friedman repeated measures on ANOVA on ranks, when appropriate. The Pearson Product Correlation test was used to predict the presence of cataract.

**RESULTS**

As shown in Figures 1 and 2, an age-related decrease in the P-SH concentration and increase in the content of protein carbonyls was observed both in clear and cataractous lenses. An inverse correlation (P < 0.01) was noted between the P-SH concentrations and the age of the subjects. The significant (P < 0.01) relationship between the lens content in carbonyl proteins and the age of the subjects observed in clear lenses did
not reach the level of significance in cataractous lenses. These changes were significantly more pronounced in cataracts compared with clear lenses and in diabetic and myopic cataracts compared with senile cataracts.

Despite a fluctuation of values among the groups, the maximal percentage drop of P-SH concentrations in diabetic and myopic cataracts occurred earlier than in senile cataracts. In diabetic and myopic cataractous lenses, the maximal significant ($P < 0.05$) drop of P-SH ($\sim 40\%$ and $\sim 28\%$, respectively) was observed between the sixth and the seventh decade of life. In contrast, the fall of P-SH concentrations in senile cataracts was significant only between the seventh and the eighth decade of life ($\sim 24\%, P < 0.05$). This finding was not observed for protein carbonyls. However, there was an inverse correlation ($P < 0.03$) between the content of P-SH and the amount of carbonyl proteins both in clear and in cataractous lenses.

In all instances, when the concentration of P-SH fell below 12 to 10 nmol/mg protein (Fig. 1) and the accumulation of protein carbonyls increased to $> 2$ nmol/mg protein (Fig. 2) the probability of a cataract being present was increased ($P < 0.05$).

Eight additional clear lenses, extracted from diabetic or myopic patients, showed an impaired oxidative status of the proteins when compared with clear lenses obtained from nonmyopic, nondiabetic subjects of the same age (P-SH: $12.8 \pm 2.5$ nmol/mg protein; protein carbonyls: $1.88 \pm 0.2$ nmol/mg protein).

**DISCUSSION**

This study quantified the level of protein oxidation in human lenses as a function of age and different types of cataract. An age-related decrease in the concentration of P-SH and increase in the content of protein carbonyls was observed both in clear lenses and in cataracts, although significantly more pronounced in the latter. This was also noticed in diabetic and myopic clear lenses compared with clear lenses from nonmyopic, nondiabetic subjects of the same age and in diabetic and myopic cataracts compared with senile cataracts.

Our data show that the lens levels of P-SH and of protein carbonyls are closely related to the age of the subjects; the lowest content of P-SH and the highest levels of protein carbonyls were found in clear and cataractous lenses of older patients. During senile cataract development, a progressive decrease in the sulfhydril content of the crystallins occurs as the result of unfolding of the macromolecule and consequent oxidation to disulfide bonds. The age-related loss of P-SH in the reduced form is known to be closely dependent on the availability of GSH and on the activity of its connected enzymes.

The values of P-SH we found in our measurements are approximately five times lower than those observed by others and may be explained by the differences in the methods used for measuring P-SH and isolating proteins. In fact, our data are the result of the final subtraction of assessments performed at 412 nm and those at 530 nm on protein suspended in a guanidine solution. Moreover, the procedure of protein assay (Bio-Rad) also provides accurate measurements on very small amounts of proteins. However, the general trends in the results are similar.

The low grade of correlation between P-SH and carbonyls found in this study may depend on several factors. First, it is known that protein carbonyl content shows a significant variation in elevation depending on the maturity of the cataract and on the activity of proteases. This variability might also explain the loss of significance of correlation in the cataractous lenses between carbonyl groups and age. Second, a close correlation between the decrease of P-SH and the increase in
carbonyls often occurs during an acute oxidative stress as a consequence of an abnormal free radical production. This strong correlation may not be found under chronic conditions, such as cataract formation, because in the latter several other events may be involved (loss of antioxidants, fall of protease activity, reduced capacity of ex novo synthesis, and influence of external oxidizing agents).

The oxidative damage of proteins in the lens is an early event in myopic and diabetic patients, occurring prematurely compared with subjects of the same age. This observation is supported by the presence of a lower P-SH content in cataractous and even in clear lenses obtained from myopic and diabetic patients. These changes were less evident for protein carbonyls. Because an increased oxidation of the proteins was observed in clear lenses removed from myopic and diabetic subjects, oxidation may be suggested as the pathogenic mechanism involved in the development of these forms of human cataract. Therefore, our results are in line with the hypothesis that protein oxidation precedes aggregation of these molecules and lens opacification. A reason for the increased susceptibility of lens proteins to sulphydryl oxidation in sugar cataract is thought to be the nonenzymatic glycosylation of these molecules, which is itself an age-dependent reaction. Increased levels of lipid oxidative products have been observed in the lens and vitreous of patients affected by diabetes and myopia, and lipid peroxidation has been indicated as a possible initiator of cataract in these patients. The origin of malondialdehyde (MDA) and other oxidative products from the retinal tissue has been suggested. The latter, rich in polyunsaturated fatty acids and subjected to photic oxidative injury, is an elective place for lipid peroxidation, especially under conditions such as diabetes and myopia associated with chronic hypoxia. In established diabetic retinopathy, transition metals deriving from hemorrhages and laser photocoagulation may increase the oxidative processes. In contrast, a different pathogenic hypothesis and a different origin has been attributed to the increased content of lipid peroxides found in senile cataracts. In the latter, the concentrations of MDA and other peroxidative products, double that observed in clear lenses, did not reach the levels noted in myopic and diabetic cataracts. Thus, it is conceivable that the increased levels of MDA in senile cataract may be mainly linked or secondary to the age-related reduction in the lens antioxidant content (glutathione, vitamin E, and ascorbate). The higher levels of protein oxidative products found in the lenses of diabetic and myopic subjects may be the result of the additional damage caused by external oxidizing agents or by lipid peroxidative products. The latter were found in the vitreous and supposed to be of retinal origin.

The findings of this study are the identification of a threshold of protein oxidation above which clinically significant cataracts develop. We have shown that the accumulation of protein carbonyls over 2 nmol/mg protein and the fall of PSH below 12 to 10 nmol/mg protein is always accompanied by opacification of the lens. A threshold level of protein oxidation has already been proposed. A threshold of green fluorescence, caused by oxidation, in both nuclear and cortical soluble fractions has been supposed to be a marker for cataract. A threshold of oxidation of protein thiol groups was found over which these become insoluble. It was speculated that the reason for this might be the shift from intramolecular to intermolecular disulfide formation.

Borchman hypothesized that a threshold of lipid oxidation might exist above which the opacification takes place and that this could be surpassed earlier in some subjects predisposed to cataract formation. Nevertheless, a level of lipid hydroperoxide higher than the proposed threshold was also found in noncataractous lenses. In all instances, because lens-insoluble proteins were found to be lipid–protein complexes and protein aggregation to lipids may be significant in the genesis of cataracts, a single threshold for lipids and proteins needs to be considered. Structural, immunochemical, and biochemical evidence indicate that plasma membrane disintegrates in senile cataracts. Accordingly, insoluble proteins are bound to the membranes and oxidation has been shown to begin at the membrane. Also, MDA, known to play a role in the lens opacification, can form cross-links between membrane lipids and proteins.

In conclusion, idiopathic senile, diabetic, and myopic cataractogenesis appear to be closely associated with oxidative damage of lens proteins. Protein oxidation initially occurs in clear lenses, and it is not until it reaches a critical level that becomes clinically evident. It is also involved in the onset of the premature alterations of the lens in diabetic and myopic patients, probably because, in these conditions, there is an increased susceptibility to oxidation of these molecules and the presence of external oxidizing agents and lipid peroxidative products.

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References


