Increased Glycosylation of Proteins from Cataractous Lenses in Diabetes

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Summary. The rates of glycosylation of lens proteins were determined in extracts of human 'diabetic' and 'senile' cataractous lenses by a method employing thiobarbituric acid. Incubation of soluble lens proteins (6,500 × g supernatant of homogenates) in vitro with various concentrations of D-glucose in sodium phosphate buffer (50 mmol/l, pH 7.2) resulted in a gradual glycosylation which was time and concentration dependent. Glycosylated proteins from the cataractous lenses of 21 senile and 12 diabetic subjects afforded 0.72 ± 0.22 and 1.84 ± 0.44 nmol 5-hydroxymethylfurfural/mg protein (mean ± SD), respectively. The value is significantly higher in

the diabetic than in the senile group ($p < 0.001$), although the mean age of the diabetic patients (67 years) was significantly younger than that of senile subjects (75 years; $p < 0.01$). These results indicate that human lens proteins can be glycosylated both in vitro and in vivo, and that hyperglycaemia can accelerate the non-enzymatic glycosylation of lens proteins in diabetic patients.

Key words: Lens proteins, human diabetic cataracts, non-enzymatic glycosylation, 5-hydroxymethylfurfural, thiobarbituric acid.

The progressive secondary complications of diabetes mellitus now account for most of the morbidity and mortality associated with this disease [1–3]. The mechanisms by which hyperglycaemia may induce the secondary complications are unknown. Recent studies have demonstrated that several proteins undergo post-ribosomal, non-enzymatic glycosylation in vivo and in vitro [4–11], and suggest that these glucose-dependent chemical alterations may cause changes in protein structures and functions which could lead to the pathophysiology of diabetic complications [12, 13]. Tentative evidence suggests that non-enzymatic glycosylation of lens crystallins may be involved in the formation of sugar cataracts in experimental animals [6, 14]. The present study was carried out to examine whether human lens proteins can be glycosylated in vitro and whether human diabetic cataractous lenses show activated glycosylation.

Subjects and Methods

Cataractous lenses were surgically extracted from both diabetic and senile patients. The extracted lenses were rinsed once with 154 mmol/l saline, decapsulated and stored at -20°C until used. A portion of lens was homogenized with sodium phosphate buffer (0.5–1.0 ml, 50 mmol/l, pH 7.2) and sonicated using a Sonifier Mod-

el 200 (Branson, Connecticut, USA) for 30 s at 4°C . The homogenate was centrifuged at 6,500 g for 30 min and the resulting supernatant was used as soluble lens proteins for determination of the rate of glycosylation. For this purpose the method of thiobarbituric acid-assay [15, 16] was employed with slight modification. Glycosylation of lens proteins gathered from 15 senile cataractous lenses was performed in vitro in sodium phosphate buffer (50 mmol/l, pH 7.2) containing NaN_3 (3.1 mmol/l) and various concentrations of D-glucose (0–55.6 mmol/l). Incubations were performed at 37°C and continued for 2, 4 and 7 days, at which times, the respective incubation mixture (1.2 ml) was added to cold 18% trichloroacetic acid (3.3 ml). After centrifugation at 1,500 g for 30 min at 4°C to remove unbound glucose, the resulting precipitates were resuspended in 13% trichloroacetic acid (4.5 ml) and the centrifugation was repeated. The final precipitates were suspended in 0.33 mol/l oxalic acid (4.5 ml) and hydrolyzed at 100°C for 4.5 h in an oil bath. The hydrolysates were cooled on ice, cold 40% trichloroacetic acid (3 ml) was added and the mixtures were centrifuged as before. Aliquots (2 ml) of the supernatants were incubated with thiobarbituric acid (0.05 mol/l, 0.5 ml) for 30 min at 37°C . Released 5-hydroxymethylfurfural (5-HMF) was estimated from the absorbance of the samples at 443 nm, using water (0.5 ml) in place of thiobarbituric acid for blank determinations.

For comparison of glycosylation rates, cataractous lenses from 12 diabetic (eight females and four males, 67 ± 5 years; mean ± SD) and 21 senile (14 females and seven males, 75 ± 8 years) subjects were used. Seven out of 12 diabetic patients were under treatment with insulin and the others were treated with oral hypoglycaemic agents. The mean ± SD fasting blood glucose levels were 12.1 ± 0.5 mmol/l on admission and 7.7 ± 2.1 mmol/l at operation in these diabetic patients. In senile subjects, the value on admission was 5.1 ± 0.5 mmol/l. The mean total amounts (± SD) of buffer soluble lens proteins were

Table 1. Glycosylation of lens proteins in vitro with varying concentrations of glucose

Incubation time (days)	Glucose concentration in the medium (mmol/l)			
	0	13.9	27.8	55.6
0	0.64	—	—	—
2	0.63	0.83	1.08	1.56
4	0.63	0.93	1.23	1.83
7	0.63	0.93	1.36	1.98

The results are mean values of duplicate determinations of released 5-hydroxymethylfurfural (nmol/mg protein) from soluble proteins of senile cataractous lenses

Fig. 1. Comparison of glycosylation rates of soluble lens proteins for 'senile' and 'diabetic' cataracts. The rates for 21 senile and 12 diabetic subjects are expressed as released 5-hydroxymethylfurfural (5-HMF) nmol/mg protein. The glycosylation rate in the diabetic group is significantly higher than that in the senile group ($p < 0.001$)

33.5 ± 8.5 mg in the senile group and 30.7 ± 9.6 mg in the diabetic group, respectively. There was no significant difference between the two groups. The respective soluble lens proteins were hydrolyzed in 0.33 mol/l oxalic acid at 100 °C for 4.5 h and released 5-HMF was measured by the same procedure as for the glycosylation study. The intra-assay coefficient of variation was 5.2% ($n=5$). Protein concentrations were determined by the method of Lowry et al with bovine serum albumin as standard [17]. Results are expressed as mean $\pm$ SD. Statistical significance was calculated using the Student's *t*-test.

Results

Table 1 shows the glycosylation rate of soluble proteins in vitro from senile cataractous lenses as measured by the release of 5-HMF from the proteins after incubation with varying concentrations of glucose in the media. Glucose gradually associated with proteins during the incubation at 37 °C, depending upon the duration. The rate of glucose incorporation into lens proteins was proportional to the glucose concentration in the media. These results indicate that non-enzymatic glycosylation of human lens proteins in vitro is glucose and time-dependent.

The results of assays for glycosylation of the lens proteins, carried out on cataractous lenses from senile and diabetic patients, are presented in Figure 1. The value in the diabetic group was 1.84 ± 0.44 versus 0.72 ± 0.22 5-HMF nmol/mg protein in the senile group. Although there was a slight overlap between the two groups, the rate obtained from diabetic patients was significantly higher ($p < 0.001$). However, the mean age of 67 ± 5 years in diabetic patients was significantly younger than 75 ± 8 years in senile subjects ($p < 0.01$).

Discussion

Cataract, opacity of the ocular lens, occurs earlier in patients with diabetes mellitus than in non-diabetic subjects [18]. Possible mechanisms by which hyperglycaemia may participate in the development of diabetic cataracts have been suggested. Accumulation of sorbitol in the lens has been reported as one mechanism. Elevated amounts of glucose in the diabetic lens, resulting in an accumulation of sorbitol which may cause osmotic swelling, eventual imbalance in the pump-leak equilibrium and opacification of the lens has been reported [19, 20].

Another mechanism was proposed by Stevens et al [6] and by Monnier et al [14]. They hypothesized that non-enzymatic glycosylation may enhance the susceptibility of lens proteins to sulphhydryl oxidation and formation of high molecular weight aggregates, although several later studies have indicated no obvious correlation between the levels of protein disulphide and the extent of glycosylation [21–23].

In the present study, we have shown that non-enzymatic glycosylation of soluble lens proteins in vitro is dependent on both the glucose concentration in the medium and the incubation time. This result using human lens proteins is compatible with a previous report concerning bovine lens crystallins [6]. Likewise, Cohen et al have reported that rat lens capsule basement membranes were non-enzymatically glycosylated in vitro, the reaction being glucose, time and temperature-dependent [9]. We could not, however, estimate the amount of glycosylation in the membranes because insufficient material was available.

The lens, like other body tissues that develop diabetic complications, does not require insulin for glucose transport. Therefore, hyperglycaemia may increase glucose concentration within the lens, resulting in an accelerated glycosylation of lens proteins. In the present study, we confirmed by the thiobarbituric acid assay that the rates of glycosylation of cataractous lens proteins from diabetic patients were significantly higher than those of senile subjects, but the mean age of the diabetic patients was significantly lower than that of the senile group. Ansari et al. [21] have reported the extent of glycosylation in the diabetic lens to be almost double that found in normal and 'senile' cataractous lenses us-

ing a [^3H]-borohydride reduction assay. Accordingly, the present finding is in agreement with their report, and indicates that hyperglycaemia may induce an activated glycosylation of lens proteins in man. An age-related increase in non-enzymatic glycosylation of long-lived body proteins has been demonstrated in bovine skin collagen [10], bovine lens crystallins [24] and human collagens [25, 26]. Furthermore, Monnier and Cerami [27] have reported that non-enzymatic glycosylation may cause cross-linking of lens proteins and re-arrangement to form yellow-brown pigments (non-enzymatic browning), similar to those reported in aging and cataractous human lenses. Therefore, the present findings suggest that non-enzymatic glycosylation of lens proteins could lead to the acceleration of cataract formation in diabetic patients.

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