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Specific radioimmunoassay of glucitol-lysine – application to lens proteins in streptozotocin-diabetic rats

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Summary. A radioimmunoassay using antibody against glucitol-lysine was developed to quantitate glycated proteins in the lens of diabetic rats. The amount of glycated protein was expressed as molar equivalents of reduced glycated hippuryl lysine (Glc_{RED}-Hip-Lysine). Significant differences ($p<0.01$) were found in the amounts of glycated protein in the lenses of rats with streptozotocin-induced diabetes (3.92 ± 0.59 nmol/mg protein, $n=5$), those with streptozotocin-induced diabetes treated with insulin (2.94 ± 0.36 nmol/mg protein, $n=4$) and normal rats (1.23 ± 0.22 nmol/mg protein, $n=5$). There was a significant correlation between the concentration of glycated protein in the lens and the HbA_{1c} level at the end of the 12 week experiment ($r=0.957$, $p<0.001$). These results indicate that glycation of lens protein is parallel with the severity of diabetes in rats.

Key words: Glycation, diabetes, radioimmunoassay, glucitol-lysine.

Nonenzymatic glycation of plasma and lens proteins, collagen and various other proteins in the human body has been demonstrated in healthy subjects and to a greater extent in diabetic patients [1, 2]. This glycation is due to condensation of glucose with amino groups of proteins to form Schiff's bases. The resulting labile aldimine may then either dissociate or undergo a slow Amadori rearrangement to form a more stable ketoamine [3]. Protein glycation has been reported to be increased in cataractous lenses of diabetic patients [4, 5] and rats [6], supporting the idea of a relationship between glycation of lens protein and cataract formation. However, experimental data that do not support this idea have been reported [7]. In these previous studies, glycated proteins were measured by rather unspecific and insensitive chemical methods, such as a colorimetric method [4, 5] or use of $[^3H]NaBH_4$, which is highly reactive with the ketoamine linkage between a reducing sugar and the ε-amino group of lysine [7]. In this work we developed a radioimmunoassay (RIA) specific for glucitol-lysine residues. Then we used this assay to determine the amounts of glycated proteins in the lens of control and diabetic rats and the relationship between the extent of glycation of lens protein and the severity of diabetes mellitus.

Material and methods

Animals

Male Wistar rats weighing approximately 300 g were fed ad libitum. Streptozotocin was injected at a dose of 65 mg/kg body weight into 9 rats via a jugular vein. Four weeks later 4 of these rats received Lente insulin s.c. at a dose of 12 U daily in the evening until they were killed in week 12. Five untreated littermates served as controls. Blood was taken from the jugular vein in weeks 0 and 12 for measurement of blood glucose and HbA_{1c}. At the end of the experiment, after blood sampling, lenses were excised and frozen until use.

Materials

The following materials were used for development of a RIA for glycated protein: hippuryl-L-lysine (Hip-Lysine; the Peptide Institute, Inc., Osaka, Japan), bovine serum albumin (BSA) and low density lipoprotein (LDL; Sigma Chemical Co., St. Louis, Mo, USA), human serum albumin (HSA; Behring Institute, Mannheim, FRG), sodium cyanoborohydride ($NaCNBH_3$; Aldrich Chemical Co., Milwaukee, Wis, USA), glucose and sodium borohydride ($NaBH_4$; Wako Pure Chemical Industries Co., Osaka, Japan), and $Na^{125}I$ (New England Nuclear, Boston, Mass, USA).

Radioimmunoassay of glycated protein

Preparation of immunogen. Proteins were glycated and reduced by the method of Curtiss and Witztum [8] with slight modification.

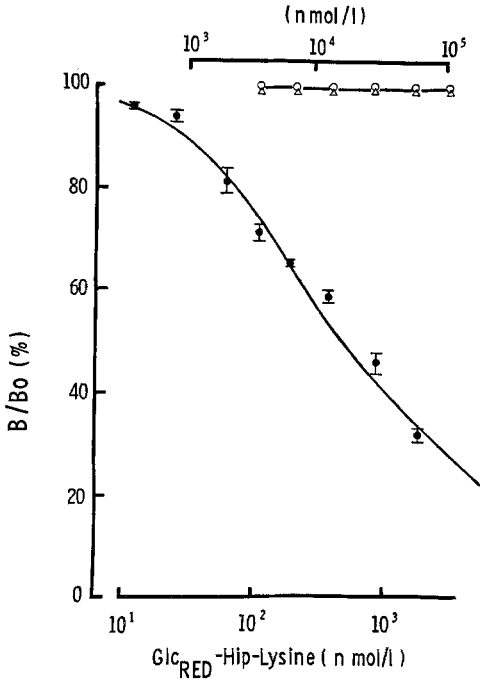

Fig. 1. Displacement of ^{125}I-reduced glycated bovine serum albumin (^{125}I-Glc_{RED}-BSA) from antibody against glucitol-lysine by reduced glycated hippuryl lysine (Glc_{RED}-Hip-Lysine) ⚲, LDL, BSA, human serum albumin, lysine, glucose, sorbitol O△-----O△. Each point on the displacement curve for Glc_{RED}-Hip-Lysine is the mean of six determinations. Bars represent ± SD. B_0 and B are radioactivities of the precipitate in the absence and the presence of Glc_{RED}-Hip-Lysine, respectively

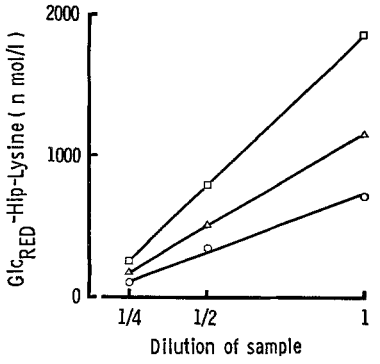

Fig. 2. Linear relationship between measured glycated protein for Glc_{RED}-Hip-Lysine and serum samples with different concentrations of glycated protein (low O, medium △, high □) diluted two- and four-fold with our buffer as described in Materials and methods

Briefly, 1 mmol/l of bovine LDL was dissolved in 10 ml of 50 mmol/l phosphate buffer containing 0.15 mol/l NaCl (PBS) and incubated with 80 mmol/l D-glucose in the presence of 200 mmol/l of freshly prepared sodium cyanoborohydride ($NaCNBH_3$) at room temperature for seven days. Then the mixture was acidified with acetic acid to stop the reaction, dialysed extensively against distilled water and lyophilised. BSA and HSA were also glycated by the same method.

Preparation of reduced glycated (Glc_{RED})-Hip-Lysine

A solution of 200 mg of Hip-lysine and 129 mg of glucose in 10 ml of a mixture of H_2O and dioxane (1:1) was prepared and incubated with 100 mg of $NaCNBH_3$ at room temperature for 4 days. The reaction was stopped by adjusting the mixture to pH 3.0 with acetic acid. The product was washed with 10 ml of 100% methanol, evaporated to dryness, and purified by high performance liquid chromatography on a column of TSK 120 T (Toyosoda Co, Osaka, Japan). For elution, two solutions were prepared: 50 mmol/l trifluoroacetic acid in 90% acetonitrile (solution A), and 5% acetonitrile (solution B). The column was eluted with a linear gradient of solutions A and B at ratios of from 1:9 to 6:4 in 30 min at room temperature at a flow rate of 2 ml/min. Fractions of eluate were collected every minute, and their absorbance at 280 nm was measured. Glc_{RED}-Hip-Lysine, which was eluted just before the elution position of authentic Hip-Lysine, was collected and evaporated in a centrifugal concentrator. The recovery was 58.2% of the starting material by weight.

Immunisation of rabbits with reduced glycated (Glc_{RED})-LDL

Five rabbits were immunised s.c. with 100 μg of Glc_{RED}-LDL emulsified in complete Freund's adjuvant at 2-week intervals for 5 months.

Radiolabelling of reduced glycated (Glc_{RED})-BSA

Glc_{RED}-BSA was iodinated with ^{125}I-Na by the chloramine-T method [9]. The iodinated product was purified by gel chromatography on a column (1.0 × 60 cm) of Sephadex G-50 developed with 50 mmol/l phosphate buffer, pH 7.4, containing 0.15% gelatin and 0.01% NaN_3. The specific activity of the iodinated Glc_{RED}-BSA was about 8 μCi/μg protein.

Radioimmunoassay procedure

Each incubation mixture contained 100 μl of anti-Glc_{RED}-LDL serum (initial dilution 1:5,000), 100 μl of Glc_{RED}-Hip-Lysine as a standard or a test sample which was reduced with $NaBH_4$ as described later and 300 μl of assay buffer (50 mmol/l PBS, pH 7.4 containing 0.15% gelatin and 0.01% NaN_3). The mixture was incubated for 24 h at 4 °C and then ^{125}I-Glc_{RED}-BSA (100 μl about 10,000 cpm) was added and incubation was continued for 24 h. Volumes of 100 μl of anti-rabbit IgG goat serum (1:10 dilution) (Eiken Immunochemical, Tochigi, Japan) and normal rabbit serum (1:50 dilution) were added to the mixture. After further incubation for 24 h at 4 °C, the mixture was centrifuged at 1800 g for 30 min at 4 °C and radioactivity of the precipitate was counted in a gamma counter. Figure 1 shows the displacement curves of ^{125}I-Glc_{RED}-BSA by Glc_{RED}-Hip-Lysine and several other materials used to prepare glycated proteins, such as sorbitol, glucose, lysine and native protein. The lowest detectable concentration of Glc_{RED}-Hip-Lysine was 13.7 nmol/l, as estimated from the concentration that produced a response of 2SD above the zero-dose response.

Precision

Two samples with low and high concentrations of glycated protein were measured 10 times, in duplicate, during a single analytical run to determine the within-assay variation. For evaluating the between-assay variation, we measured samples in duplicate in 10 successive analytical runs. For samples with low and high concentrations of glycated protein, the within-assay variations were 5.9, and 4.4%, and the between-assay variations were 6.4 and 5.2%, respectively.

Recovery studies

Several concentrations of Glc_{RED}-HSA were added to serum samples reduced with $NaBH_4$. The reduction of serum samples was done as follows; 8 mg $NaBH_4$ was added to each ml of serum and the mix-

Table 1. Body weight, blood glucose and HbA_{1c} in weeks 0 and 12 in normal rats and insulin-treated (STZ+Ins) and untreated (STZ) rats with streptozotocin-induced diabetes

	Body weight (g)		Blood glucose (mmol/l)		HbA_{1c} (%)	
Week	0	12	0	12	0	12
Normal	321± 9.7	554±36.5[d]	7.7±2.66	9.4±0.59	1.3±0.68	0.9±0.18[d]
STZ+Ins	308±20.5	417±35.9[a, d]	6.6±0.91	25.6±1.20[a, d]	1.2±0.12	2.4±0.24[a, d]
STZ	311±13.6	356±25.6[a, c, e]	8.8±1.65	27.2±1.54[a, d]	1.2±0.14	3.7±0.45[a, b, d]

Blood glucose and HbA_{1c} were determined by the ways as described in Materials and methods. All values represented as mean±SD. [a] vs normal ($p<0.01$); [b] vs STZ+Ins ($p<0.01$); [c] vs STZ+Ins ($p<0.05$); [d] vs week 0 ($p<0.01$); [e] vs week 0 ($p<0.05$).

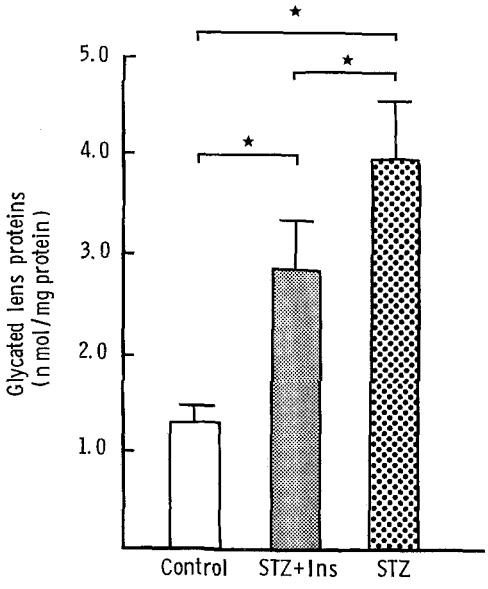

Fig. 3. Mean concentrations of glycated protein in the lens of control (□) ($n=5$), insulin treated (▒) (STZ+Ins, $n=4$) and untreated streptozotocin-induced diabetic (▒) (STZ, $n=5$) rats determined by radioimmunoassay (RIA). Vertical bars represent SD. ★ $p<0.01$

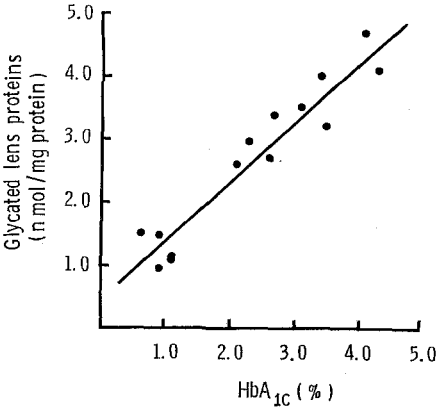

Fig. 4. Correlation of the amount of glycated protein in the lens with the HbA_{1c} level at the end of the experiment (12 weeks). Points represent values for individual animals ($r=0.957$, $p<0.001$, $y=0.96x+0.45$). HbA_{1c} was measured by the high performance liquid chromatography method as described in Materials and methods

ture was incubated for 30 min at 37 °C; subsequently, the reaction was stopped by 0.5 ml of 5% acetic acid and the mixture was diluted with the buffer. The average of analytical recovery of the glycated protein added to serum samples was 110.6% (90.5%–126.7%). When three reduced serum samples with low, medium and high concentration of glycated protein were diluted two- and four-fold with the buffer, the dilution-corrected glycated protein values were those expected (Fig. 2).

Analyses

Each lens was thawed and decapsulated. The lens tissue was solubilised by the method of Lee et al. [4] with a slight modification. Briefly, the tissue was solubilised in 2 ml of 0.05 N sodium hydroxide by incubation at 40 °C for 2 h with stirring. The mixture was neutralised with hydrochloric acid, and the neutralised solution was reduced with $NaBH_4$ in the same way as described above. Then the reduced mixture was diluted (1:20) with the buffer, and used for determination of glycated protein by RIA as described above. A portion of the neutralised solution was used for protein determination by the method of Lowry et al. [10]. The recovery of Glc_{RED}-Hip-Lysine treated in the same way as the lens tissue was approximately 50%. Blood glucose was measured by the glucose oxidase method and HbA_{1c} by high performance liquid chromatography. All values are expressed as mean±SD.

Statistical analysis

Statistical significance was analysed by Student's t-test.

Results

Table 1 shows the body weights and blood glucose and HbA_{1c} levels of the experimental groups. In week 0, there were no significant differences in the values of any of these parameters in the three groups. Untreated diabetic rats (STZ group) gained little weight during the experiment, while diabetic rats treated with insulin (STZ+Ins group) gained appreciable weight, although their mean non-fasting blood glucose concentration was as high as that of the STZ group. The differences in the mean HbA_{1c} levels in the three groups in week 12 were statistically significant ($p<0.01$). As shown in Figure 3, significant differences ($p<0.01$) were also found in the mean concentrations of glycated protein in the lenses in the three groups (STZ, 3.92± 0.52 nmol/mg protein; STZ+Ins, 2.94±0.36 nmol/mg protein; control, 1.23±0.22 nmol/mg protein). The concentrations of glycated protein in the lenses of individual rats were well correlated with their HbA_{1c} levels in week 12 (Fig. 4; $r=0.957$ $y=0.96x+0.45$, $p<0.001$).

Discussion

In the present study we developed a RIA for measuring glycated protein. The antibody obtained in this work was not specific for reduced glycated bovine LDL, which was used as an antigen, since it also bound to reduced glycated derivatives of a variety of other proteins, including bovine serum albumin and human serum albumin (data not shown). Judging from these results and the finding that it reacted with glucitol-lysine but not with any of the materials used for preparing glycated protein, we concluded that it was specific for the glucitol-lysine residue. This method of immunoassay of glycated proteins has the advantage of being sensitive and specific, and suitable for use with large numbers of samples. But it has the disadvantage of showing little reactivity with products of nonreductive glycation, which are believed to be the only forms of ketoamine and hemiketal Amadori protein adducts in the body. Therefore, before RIA, these compounds must be reduced with $NaBH_4$ to glucitol-lysine, which the antibody specifically recognises.

Our results obtained by this RIA showed that glycation of lens proteins is significantly greater in diabetic rats than in nondiabetic control rats. This is consistent with the findings of others [4, 5] obtained by different analytical methods. Moreover, our values for glycated protein in the lens are similar to those obtained by Lee et al. [4]. There has been no report of the relation of the degree of glycation of lens proteins to the severity of diabetes mellitus. Our data showed a significant correlation between glycation of lens protein and the level of HbA_{1c} in experimental animals. This fact indicates that the glycation of lens protein and haemoglobin are correlated, or, in other words, that glycation of lens protein depends on the severity of diabetes mellitus. This explains why the amount of glycated protein in the lenses of diabetic rats treated with insulin was intermediate between those of normal rats and untreated diabetic rats.

Lens crystallin, a structural protein of the lens, has a much longer life-span than other proteins [11], and accordingly accumulates glycation products for a long period, even though the susceptibility of amino groups of this protein for glucose are weaker than those of other proteins such as albumin [12]. The HbA_{1c} levels in normal rats were approximately a quarter of those in man. These results are comparable with those reported by Rendell et al. [13]. The lower level in rats might be due to lower permeability of glucose [14] and a shorter life-span of rat erythrocytes [15]. The pathogenesis of cataract formation in diabetes is still controversial [4, 5, 7, 16, 17]. But if glycation of lens crystallin proteins has a role in the development of diabetic cataract, our finding that glycation of lens protein in diabetic rats increases in proportion to the severity of their diabetic state indicates that diabetes should be kept under control to prevent cataract formation. Our RIA for precise and specific determination of the glycated proteins should be helpful in discovering the causes of diabetic complications.

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