Influence of Blood Proteins on Biomedical Analysis. XII.[1] Effects of Glycation on Gliclazide (Oral Hypoglycemic Drug)-Binding with Serum Albumin in Diabetics

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The sera of diabetic patients showed an inverse correlation ($r=-0.67$, $n=57$) between free gliclazide (oral hypoglycemic drug) level and the fructosamine value. The binding capacity of the primary binding site for gliclazide in the albumin molecule was increased from 4.5×10^{-4} to $8.0\times10^{-4}\,M^{-1}$ by glycation of albumin, but not that of the secondary binding site ($1.2\times10^{-4}\,M^{-1}$). This suggests that the glycation of albumin increases its total binding capacity for gliclazide, resulting in a low free gliclazide level. Therefore, a low hypoglycemic activity of the drug is observed when it is administered to diabetic patients with hyperglycemia.

Keywords oral hypoglycemic drug; gliclazide; protein binding; glycation; glycated albumin; fructosamine; free drug level; diabetes mellitus

Many kinds of proteins are nonenzymatically glycated with glucose in circulating blood,[2-4] and the extent of glycation is accelerated in uncontrolled diabetic patients with hyperglycemia. Albumin, a typical drug-carrier protein in the blood, is glycated in serum, and it has been reported to alter the binding activity with a drug by glycation.[5-8] The level of glycated albumin in diabetic patients is known to correlate well with the fructosamine value.[9-11] Uncontrolled diabetic patients show a high value of fructosamine because of high blood glucose levels.[12-16] Gliclazide, 1-(3-azabicyclo[3.3.0]-oct-3-yl)-3-(*p*-tolylsulfonyl)urea, is a "second generation" oral potential hypoglycemic drug having the sulfonylurea structure. In uncontrolled diabetic patients administered a therapeutic dose (80 mg/d) of gliclazide, a reduced hypoglycemic effect is sometimes observed. This led us to consider the glycation of albumin in the serum from the viewpoint of drug–protein binding.

In the preliminary study, we examined the relationship between the level of free gliclazide, which has a hypoglycemic action, and the fructosamine values in the gliclazide-added sera of diabetic patients. The effects of glycation on the gliclazide-binding activity of albumin were studied by Scatchard analysis.[17]

Materials and Methods

Chemicals Gliclazide was purchased from Dainippon Pharmaceutical Industries Co., Ltd., Japan. Human serum albumin (fraction V) was obtained from Sigma Chemical Co., U.S.A. Märtrex PBA-10 (phenyl boronated agarose) for affinity chromatography was obtained from Amicon Corp., U.S.A. The dialysis membrane used was seamless cellulose tubing (20/32 inch inflated diameter, Visking Co., U.S.A.). A collodion bag (SM-13200) was obtained from Sartorius GmbH, 3400 Göttingen, Germany. Ultrafree (C3LGC, Japan Millipore, Ltd., Japan) was used for ultrafiltration. All other chemicals were of analytical reagent from Wako Pure Chemical Industries, Ltd. Osaka, Japan.

Subjects Serum samples were collected from 57 diabetic patients undergoing insulin or drug treatment at Hyogo College of Medicine Hospital. Blood was sampled preprandially without an anticoagulant. After the blood had clotted, the tube was centrifuged at $1400\times g$ for 10 min and the supernatant serum was separated and stored at −20 °C until use.

Preparations of Glycated and Nonglycated Albumins Human serum albumin (2 g) was dissolved in 0.067 M phosphate (Na) buffer (pH 7.4, 20 ml) containing glucose (2 g) and then was incubated at 37 °C for 48 h. The reaction mixture was then adequately dialyzed against 0.025 M phosphate (Na) buffer (pH 8.5) until the glucose level was less than 0.05 mg/ml. Märtrex PBA-10 column (280 × 16 mm i.d.) was equilibrated with 0.025 M phosphate (Na) buffer (pH 8.5), and the reaction mixture (2 ml) was subjected to affinity chromatography. After the column was washed with 0.025 M phosphate (Na) buffer (pH 8.5, 150 ml), glycated albumin was eluted with 0.025 M phosphate (Na) buffer (pH 8.5) containing 0.1 M sorbitol (Fig. 1).[6] The conditions of affinity chromatography were as follows: column temperature, ambient (25—27 °C); detection wavelength, 280 nm; flow rate, 1.0 ml/min; fraction volume, 5 ml. After the fraction of glycated albumin has been concentrated using the collodion bag, it was dialyzed against 0.067 M phosphate (Na) buffer (pH 7.4, 1.0 l) twice to remove sorbitol. The concentration of albumin in the fraction was adjusted to 38.9 g/l. The same procedure was used to prepare the nonglycated albumin sample. The fructosamine values in the glycated and nonglycated albumin samples were 2800 and 170 μM, respectively. Concentrations of glucose and albumin were measured with a glucose analyzer (glucose-oxidase method, Auto & STAT™ GA-1120, Kyoto Daiichi Kagaku Co., Ltd. Kyoto, Japan) and an automated clinical analyzer (BCG method,[18] JCA RX-30, Japan Electronic Optical Laboratory, Ltd., Tokyo, Japan), respectively. Fructosamine was assayed by the nitroblue tetrazolium (NBT)-reducing method.[9]

Binding of Gliclazide with Serum Proteins, Glycated and Nonglycated Albumins To study the binding of gliclazide with serum proteins, a serum sample (180 μl) was incubated in 0.067 M phosphate (Na) buffer (pH 7.4) solution (20 μl) with or without 0.5 mg/ml gliclazide at 37 °C for 24 h. To obtain the binding parameters, glycated or nonglycated albumin sample (180 μl) and gliclazide (0—50 mg/ml) was added to 0.067 M phosphate (Na) buffer (20 μl). The mixture was incubated at 37 °C for 24 h. Free gliclazide in the sample was separated by ultrafiltration using a filter membrane, Ultrafree (C3LGC). The mixture was placed into an Ultrafree tube and centrifuged at $1400\times g$ for 20 min. A 10 μl portion of the filtrate was injected into a high performance liquid chromatography (HPLC) column to measure the free gliclazide level. HPLC was performed with small modifications based on the method of Kimura *et al.*[19] under the following conditions: HPLC, model Twincle (Japan Spectroscopic, Tokyo, Japan); column, Diaion CDR-10 (250 × 4.6 mm i.d., Mitsubishi Chemicals, Tokyo, Japan); mobile phase, 1.2 M NH_4ClO_4–methanol–acetonitrile (7 : 3 : 3.5, v/v/v); flow rate, 1.0 ml/min; detector, UVIDEC-100-VI with recorder and chromatocorder (Sic Chromatocorder 12) (Japan Spectroscopic, Tokyo, Japan); detection, 227 nm; temperature, ambient (25—27 °C).

Calculations The binding data obtained in the present study were analyzed according to Scatchard *et al.*[17] The following equation for a single class of n equivalent binding sites was used:

$$r/(\text{free drug}) = nK - rK \quad (1)$$

where r is the number of moles of bound drug per mole of protein, and K and (free drug) are the association constants for the drug–protein complex and the concentration of free drug (gliclazide), respectively. If the Scatchard plot is not linear, the existence of multiple classes of sites on the protein molecule can be assumed, and we can generalize Eq. 1 to 2.[20]

$$r/(\text{free drug}) = \sum n_i K_i - \sum r K_i \quad (2)$$

Here, the subscript i denotes the i-th class of binding sites. Extrapolation of the limiting straight line drawn by the least-squares method for the linear portion of the Scatchard plot to the y axis gives $\sum n_i K_i$ (intercept on the y axis, total binding capacity). Similarly, extrapolation of the limiting

straight line to the x axis gives $\sum n_i$ (intercept on the x axis, total number of binding sites), and the apparent K_i (slope of the straight line) can be calculated. The values of n_1K_1 and n_2 are obtained from the differences between $\sum n_iK_i$ and n_2K_2, and $\sum n_i$ and n_1, respectively. Statistical significance was assessed by Student's t-test for paired data.

Results and Discussion

Glycated and nonglycated albumin samples were prepared according to the method of Tsuchiya *et al.* with minor modifications.[6] As shown in Fig. 1, the glycated albumin was completely separated from nonglycated albumin by affinity chromatography using Märtrex PBA-10 column (phenyl boronated agarose). However, the fraction which was eluted with 25 mM phosphate buffer without sorbitol still showed NBT-reducing activity. This activity seems to be due to the sulfhydryl group (−SH) in the albumin molecule.[21]

In the previous report, we stated that the level of gliclazide corresponding to the blood level of gliclazide at a therapeutic dose (oral dose: 80 mg/d) is around 10 μg/ml.[22] As shown in Fig. 2, the free gliclazide level remained at 5—7% in the range of 10—100 μg/ml of total gliclazide. Therefore, by considering the detection limit of the HPLC method, a solution containing 50 μg/ml of gliclazide was used for the present study on gliclazide–albumin binding. Figure 3 shows the correlation profile between the free gliclazide level and the fructosamine value obtained from the experiments *in vitro* using sera from diabetic patients. Fructosamine values ranged from 196 to 628 μM (mean value, 382 μM) and the free gliclazide level ranged from 2.20 to 9.20% (mean value, 4.72%). The free gliclazide level correlated inversely with the fructosamine value ($r=-0.67$, $y=-0.01x+7.65$, $n=57$, $p<0.001$).

Scatchard plots for the interaction of gliclazide with glycated and nonglycated albumins are shown in Fig. 4. The plot for the glycated albumin was a hyperbola like that for nonglycated albumin, showing the existence of two (or more) classes of gliclazide-binding sites in the albumin molecule. They were significantly different between the glycated and the nonglycated albumins at less than 0.01 of r (mole number of bound gliclazide per mole of protein) ($p<0.01$), but not at a higher value (>2.5). Scatchard plot showed that the amounts of gliclazide bound to glycated and nonglycated albumins did not change at higher value of r (>3.0).

Table I shows the binding parameters obtained from Scatchard plots. The number of primary binding sites (high

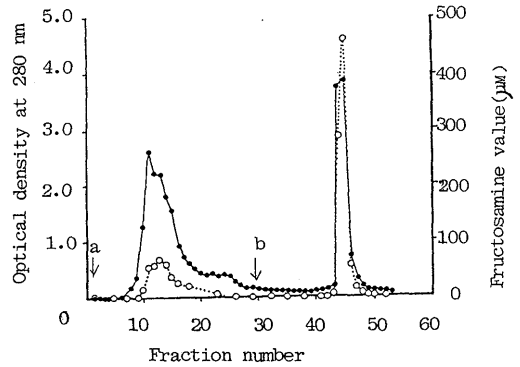

Fig. 1. Separation of Glycated Albumin by Affinity Chromatography (Märtrex PBA-10)

a, 25 mM phosphate buffer (pH 8.5); b, 25 mM phosphate buffer (pH 8.5) containing 0.1 M sorbitol. ●, optical density at 280 nm; ○, fructosamine value (NBT-reducing activity).

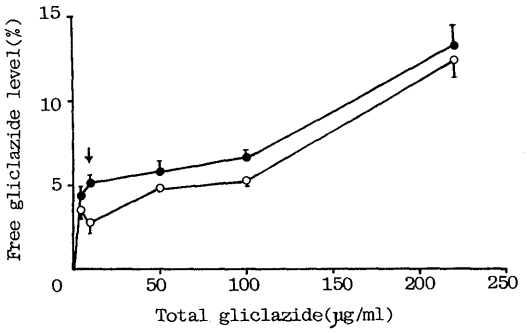

Fig. 2. Relationship between Total and Free Gliclazide Levels

Glycated or nonglycated albumin solution (35.0 mg/ml) containing gliclazide (0—220 μg/ml) was incubated at 37 °C for 24 h. Free gliclazide was separated as described in Materials and Methods. Arrow indicates the therapeutic blood level of gliclazide (around 10 μg/ml). ●, glycated albumin; ○, nonglycated albumin. Each point is the mean (±S.D.) of four separate experiments.

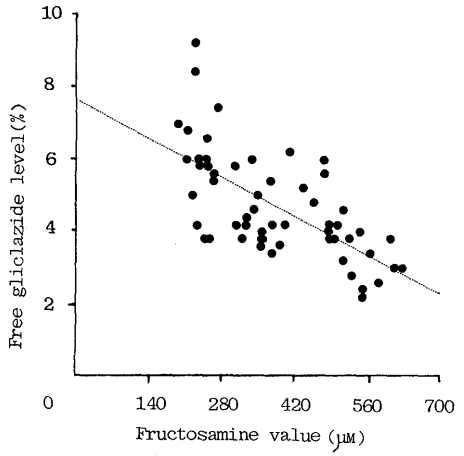

Fig. 3. Correlation Profile between Free Gliclazide Level and Fructosamine Value

$y=-0.01x+7.65$, $r=-0.67$, $n=57$, $p<0.001$.

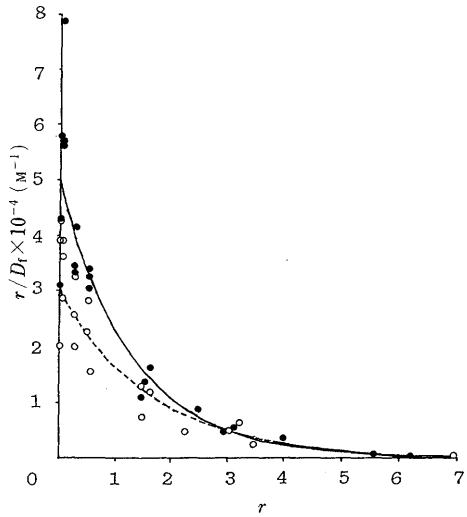

Fig. 4. Scatchard Plots for Interaction of Gliclazide with Glycated and Nonglycated Albumins

●, glycated albumin; ○, nonglycated albumin. Each point is the mean value of two separate experiments.

TABLE I. Binding Parameters for Interaction of Gliclazide with Glycated and Nonglycated Albumins

Binding parameter		Albumin	
		Nonglycated	Glycated
n_1		0.5	0.8
K_1	($\times 10^{-4}$ M^{-1})	9.0	10.0
n_1K_1	($\times 10^{-4}$ M^{-1})	4.5	8.0
n_2		6.0	6.0
K_2	($\times 10^{-4}$ M^{-1})	0.2	0.2
n_2K_2	($\times 10^{-4}$ M^{-1})	1.2	1.2
Σn_iK_i	($\times 10^{-4}$ M^{-1})	5.7	9.2

affinity binding site, n_1) and primary affinity constants (K_1) for gliclazide increased from 0.5 and 9.0×10^{-4} M^{-1} to 0.8 and 10.0×10^{-4} M^{-1}, but that (n_2K_2) of the secondary binding site did not change. Total binding capacity ($n_1K_1 + n_2K_2$) of the glycated albumin was 1.6-fold higher than that of the nonglycated albumin.

The binding ratio of a drug such as salicylate or sulfonylurea with glycated albumin usually decreases with glycation, and the free drug level is correlated with the extent of albumin glycation *in vitro*.[5,6] This occurs because the binding site of the drug is located close to the position of interaction with glucose in the albumin molecule, but the binding ratio of the drug with albumin is not dependent on the glycation of albumin.[8]

In conclusion, we found that the free gliclazide level was inversely correlated with the fructosamine value, which indicates the extent of albumin glycation from *in vitro* experiments using sera from diabetic patients. The total binding capacity of gliclazide at the primary binding site in the albumin molecule was increased by the glycation of albumin. These results suggest that albumin glycation in circulating blood can lower the level of free gliclazide, thus reducing the hypoglycemic effect of gliclazide administered to diabetic patients with hyperglycemia.

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