Alteration of Phenytoin Binding by Glycosylation of Albumin in IDDM

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SUMMARY

We measured glycosylated albumin and hemoglobin and serum protein binding of phenytoin in 57 children and adolescents with insulin-dependent diabetes mellitus (IDDM). Serum was incubated with phenytoin to yield concentrations of 15 and 25 mg/L, and a serum ultrafiltrate was prepared from an aliquot of each sample. We observed a linear correlation between glycosylated albumin and the free fraction of phenytoin at serum phenytoin concentrations of 15 mg/L (r = .35, P = .03) and 25 mg/L (r = .40, P = .003). A better correlation existed between the free fraction of phenytoin and total albumin concentrations for both serum concentrations (r = .45, P = .005 for 15 mg/L; r = .56, $P = 10^{-5}$ for 25 mg/L), whereas the best linear correlation resided between the free fraction of phenytoin and the concentration of nonglycosylated albumin (r = .54, P = .0005 for 15 mg/L; r = .63, $P < 10^{-6}$ for 25 mg/L). There was no correlation between the free fraction of phenytoin and the concentration of glycosylated albumin. Incubation of solutions of glycosylated and nonglycosylated albumin demonstrated significantly lower binding to the glycosylated fraction ($P = 8.1 \times 10^{-6}$). These results indicate that glycosylation of albumin diminishes the affinity of the phenytoin binding site on albumin. This alteration may have clinical significance in that it may alter the disposition of phenytoin in patients with IDDM and produce free phenytoin serum concentrations that are not accurately reflected by total serum phenytoin concentrations. *Diabetes* 36: 505–509, 1987

Chronic hyperglycemia results in nonenzymatic glycosylation of many proteins, including hemoglobin (1), albumin (2,3), collagen (4), and transferrin (5,6). Recently, Ruiz-Cabello and Erill (7) demonstrated that when serum from adults with diabetes mellitus was incubated with sulfisoxazole, the free fraction of the drug was directly related to the extent of serum protein glycosylation. We investigated the relationship between serum albumin concentrations, glycosylation of albumin, and the binding of the commonly used anticonvulsant phenytoin in 57 patients with insulin-dependent diabetes mellitus (IDDM).

MATERIALS AND METHODS

Study subjects. Serum from 57 children (24 boys, 33 girls) with IDDM was collected during the course of a routine clinic visit. The ages of these subjects ranged from 2.1 to 22.4 yr (mean ± SD, 11.7 ± 5.1 yr). This investigation was approved by the University of Arkansas for Medical Sciences Committee on Human Investigation.

Venous blood (5 ml) was collected into a non-anticoagulant-containing glass tube, the rubber stopper was removed, and the tube was capped with Parafilm (American Can, Greenwich, CT). Samples were permitted to clot for 30 min at 37°C and were then subjected to centrifugation at 5000 × *g* for 10 min. The serum was then separated and immediately frozen at −70°C. At the same time, an aliquot of the blood (2 ml) was placed in a tube containing EDTA for determination of glycosylated hemoglobin.

Determination of glycosylated proteins. Glycosylated hemoglobin was determined by boronate affinity chromatography (8) with columns obtained from Pierce Chemical (Rockford, IL). Glycosylated serum proteins were separated with boronate-agarose gel (Glyc-Affin, Isolab, Akron, OH), as previously described (5), and albumin concentrations were measured by the bromocresyl green method (9).

Preparation of glycosylated and nonglycosylated albumin. Purified glycosylated and nonglycosylated albumin was prepared from human albumin (Sigma, St. Louis, MO) by

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TABLE 1
Effect of albumin and glycosylated albumin on phenytoin binding

Total phenytoin concentration (mg/L)	x	y	r	P(F)	Regression equation
15	Gly-Alb (%)	DPH(FF)	.35	.03	$y = 8.9 + 0.2x$
15	[ALB(T)]	DPH(FF)	.45	.005	$y = 29.6 - 4.4x$
15	[NGly-Alb]	DPH(FF)	.54	.0005	$y = 27.6 - 4.6x$
25*	Gly-Alb (%)	DPH(FF)	.40	.003	$y = 6.7 + 0.19x$
25	[ALB(T)]	DPH(FF)	.56	10^{-5}	$y = 20.2 - 2.6x$
25†	[NGly-Alb]	DPH(FF)	.63	$<10^{-6}$	$y = 19.1 - 2.8x$

Gly-Alb, glycosylated albumin; NGly-Alb, nonglycosylated albumin; DPH, phenytoin; FF, free fraction; T, total.
* See Fig. 1.
†See Fig. 2.

boronate affinity resin chromatography (Glyc-Affin, Isolab). Both fractions were dialyzed versus 0.5 M HEPES buffer (pH 7.4) and were concentrated with an Amicon concentrator (Amicon, Danvers, MA) with a YM-5 ultrafiltration membrane. Final concentration of each solution was adjusted to 3.2 g/dl.

Preparation of serum ultrafiltrates. Serum samples were brought to 37°C and spiked with sodium phenytoin (Sigma) from a working solution containing 0.5 mg/ml phenytoin in 0.5 M HEPES buffer to yield a final volume of 0.5 ml. After adjusting the pH to 7.4 by the addition of HEPES buffer (final concentration, 0.1 M), samples were incubated for 1 h at 37°C in a shaking water bath. Each sample was divided into two aliquots: the first fraction (200 μl) was reserved for determination of total phenytoin concentration, and the second (300 μl) was subjected to preparation of serum ultrafiltrate (0.1–0.15 ml) with the Amicon micropartition system. After membrane equilibration for 10 min at 37°C, the samples were centrifuged at 1000 × g for 15 min, and the serum ultrafiltrate was harvested and frozen at −70°C. Microprotein leakage was not detected in any of the ultrafiltrate samples (10). This ultrafiltration technique has been validated against standard equilibrium dialysis methods for the study of phenytoin binding (11).

Quantitation of phenytoin. The concentrations of phenytoin in the serum and ultrafiltrate were determined by a fluorescence polarization immunoassay technique (Abbott, North Chicago, IL), as described by Argyle et al. (12). This method has lower detection limits of 0.5 and 0.2 mg/L for total and free serum concentrations of phenytoin, respectively. Between-run precision was consistently <6.7% at low, intermediate, and high concentration points selected within the range of linearity. Analytical recovery for phenytoin from serum was consistently >92%, and recovery across the ultrafiltration membrane was >98% when serum samples ($n = 5$) were compared with HEPES buffer containing 15.0 and 25.0 mg/L of total phenytoin.

Statistical methods. Grouped data were analyzed for statistical significance by a two-tailed unpaired Student's *t* test. Covariance determinations were performed by linear least-squares regression analysis that employed the following tests: *1*) a Student's *t* test, the null hypothesis (H_0) being that the slope of the regression line equals 0; *2*) a correlation coefficient (*r*), H_0 being that *x* and *y* are independent vari-

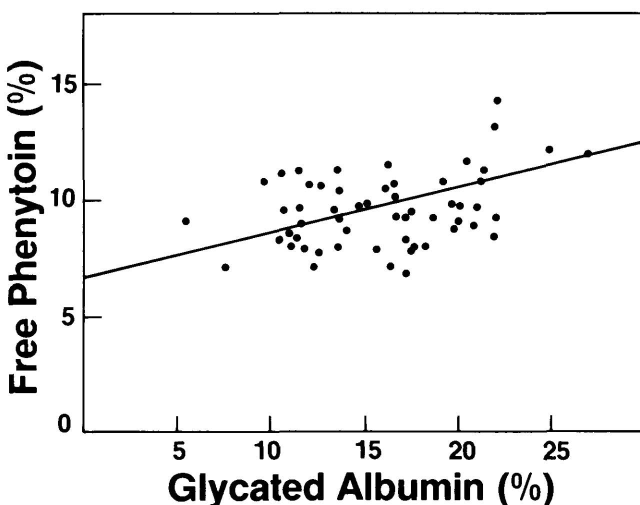

FIG. 1. Correlation of phenytoin free fraction with glycosylated albumin. Glycosylated albumin was compared with free fraction of phenytoin after incubation of phenytoin (25 mg/L) for 1 h at 37°C with serum from 57 subjects with type I diabetes.

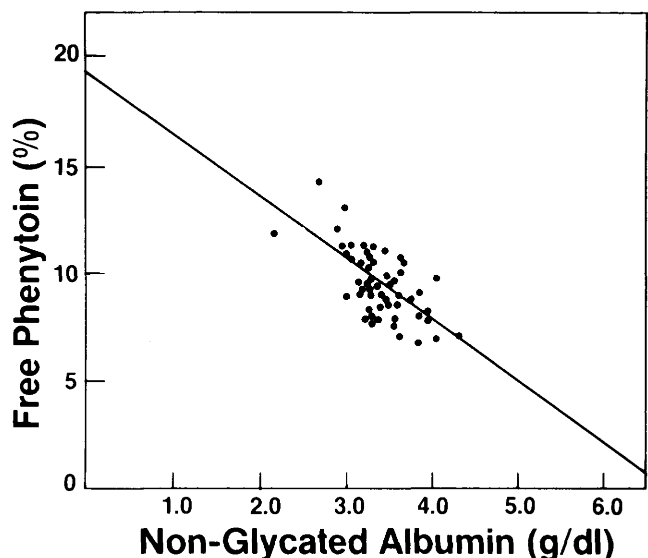

FIG. 2. Correlation of phenytoin free fraction with concentration of nonglycosylated albumin. Phenytoin free fraction was compared with concentration of nonglycosylated albumin (calculated from percent glycosylated albumin and total albumin concentration) after incubation of phenytoin (25 mg/L) for 1 h at 37°C with serum from 54 subjects with type I diabetes.

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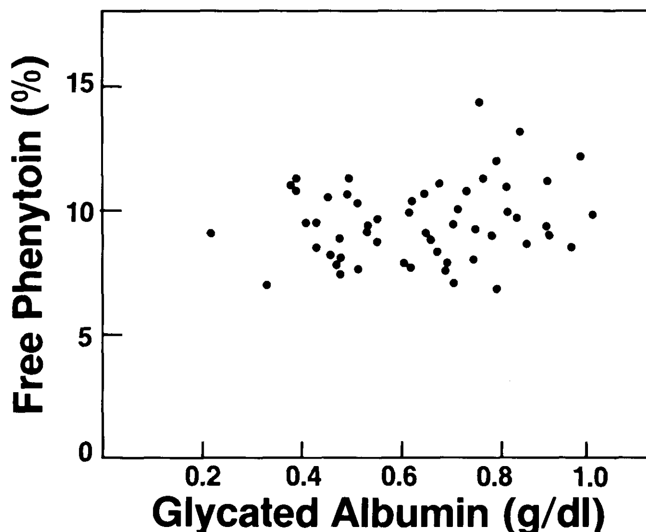

FIG. 3. Correlation of phenytoin free fraction with glycosylated albumin concentration. Free fraction of phenytoin was compared with concentration of glycosylated albumin (calculated from percent glycosylated albumin and total albumin concentration) after incubation of phenytoin (25 mg/L) for 1 h at 37°C with serum from 54 subjects with type I diabetes.

ables; and 3) an analysis of variance, H_0 being that the regression of y on x is not linear. The level of significance chosen for all tests was $\alpha = .05$. Statistical analyses were performed by programs resident on our DEC Professional 350 microcomputer (RS/1 Integrated Data Analysis System, Digital Equipment, Maynard, MA), which employed previously described methods (13,14).

RESULTS

Glycosylated hemoglobin in our patient population ($n = 57$) ranged from 4.8 to 25.2% (mean 14.9 ± 4.4%), and glycosylated albumin ranged from 5.3 to 26.8% (mean 16.1 ± 4.4%). A significant linear correlation was found between the extent of glycosylated hemoglobin and glycosylated albumin ($r = 0.74$, $P < 10^{-6}$). Total serum albumin concentration ranged from 2.95 to 4.63 g/dl (mean 4.01 ± 0.33 g/dl). Based on measured total and glycosylated albumin concentrations, the concentration of nonglycosylated albumin was calculated for each subject. These values ranged from 2.16 to 4.04 g/dl (mean 3.37 ± 0.35 g/dl).

The unbound fraction of phenytoin ranged from 9.2 to 20.1% (mean 12.2 ± 3.1%) after incubation of serum to a total phenytoin concentration of 15 mg/L and from 6.9 to 14.2% (mean 9.1 ± 1.3%) after incubation with phenytoin to a total concentration of 25 mg/L. We observed a significant linear correlation between the free fraction of phenytoin and the percent of glycosylated albumin at both total phenytoin concentrations (Table 1). As shown in Fig. 1, an increase in the extent of albumin glycosylation from 5 to 25% corresponded to an increase of ~1.5-fold in the free fraction of phenytoin. Correlations between the free fraction of phenytoin and total albumin concentration were slightly better (Table 1); however, at both the 15 and 25 mg/L concentrations, the best correlation was found between the free fraction of phenytoin and the total concentration of nonglycosylated albumin (Fig. 2). There was no correlation between the free fraction of phenytoin and the total concentration of glycosylated albumin (Fig. 3). In addition, we observed no correlation between the total serum albumin concentration and the percent of either glycosylated hemoglobin ($r = .09$) or glycosylated albumin ($r = .17$).

To determine whether glycosylation of serum albumin was responsible for changes in binding, solutions of glycosylated and nonglycosylated albumin were incubated with phenytoin. As shown in Table 2, a smaller amount of phenytoin was bound to glycosylated albumin than to nonglycosylated albumin.

DISCUSSION

Nonenzymatic glycosylation of circulating proteins, a result of chronic hyperglycemia, has been used as a measurement of glycemic control in patients with diabetes (1,3,5). Excessive protein glycosylation has also been shown to have biological significance through inactivation of enzymes, alteration of cofactor binding, abnormalities of nucleic acid function, and increased immunogenicity, processes that may be factors in the pathogenesis of diabetic complications (15). Glycosylated hemoglobin in our patient population ranged from 4.8 to 25.2% and reflected various degrees of glycemic control. The correlation observed between the percent of glycosylated hemoglobin and glycosylated albumin was similar to results we have previously reported for patients with IDDM (5). The degree of hemoglobin or albumin glycosylation in the patient samples did not appear to influence total albumin concentrations, as evidenced by a range of values (2.95–4.63 g/dl) that were within normal, age-adjusted limits.

Phenytoin is a weak acid (pK_a 8.3) that is bound primarily to albumin (16,17) and to α-globulins (18). Our studies were carried out at total serum phenytoin concentrations of ~15 and 25 mg/L (0.55 and 0.91 × 10^{-4} M), both of which are in the accepted therapeutic range (16,17). Because the

TABLE 2
In vitro binding of phenytoin to purified glycosylated and nonglycosylated albumin

	Total albumin concentration (g/dl)	Total DPH concentration (μg/ml)	Free fraction DPH (%)	K_a ($\times 10^3$ M^{-1})
NGly-Alb	3.2	14.82 ± 0.06	5.58 ± 0.07	3.9
P		0.04	8.1 × 10^{-6}	
Gly-Alb	3.2	14.55 ± 0.09	7.11 ± 0.09	2.4

Gly-Alb, glycosylated albumin; NGly-Alb, nonglycosylated albumin; DPH, phenytoin; K_a, association constant. *P* values are from two-tailed unpaired Student's *t* test.

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phenytoin concentrations used in our study are approximately an order of magnitude less than the normal serum concentrations of albumin (6×10^{-4} M), binding would not be expected to be concentration dependent (16). Indeed, at both total serum concentrations of phenytoin (Table 1), we observed similar linear correlations between the phenytoin free fraction and the total albumin concentration.

The free fraction of phenytoin in serum also increased in a direct linear relationship to the extent of albumin glycosylation (Table 1). This finding is similar to data reported by Ruiz-Cabello and Erill (7), who demonstrated a direct linear correlation between the free fraction of sulfisoxazole (weak acid) and the percent of glycosylated total serum proteins in 12 adults with diabetes mellitus. These authors suggested that glycosylation of plasma proteins produced a defect in acidic drug binding in patients with diabetes, the magnitude of which is directly influenced by the degree of glycemic control (7). Consistent with this assertion is our finding that the best correlation resided between the phenytoin free fraction and the total nonglycosylated albumin concentration (Table 1). This finding, coupled with the lack of correlation between the phenytoin free fraction and the glycosylated albumin concentration in serum, suggests that the concentration of glycosylated albumin per se does not influence phenytoin binding in pediatric patients with IDDM.

That these changes in binding are the result of glycosylation is substantiated by our demonstration in vitro that phenytoin binds less well to glycosylated than to nonglycosylated albumin (Table 2). Glycosylation of albumin resulted in an increase of ~50% in the free fraction of phenytoin. In the patient samples (Fig. 1), an increase from 5 to 25% in glycosylated albumin also resulted in an ~50% increase in the phenytoin free fraction. Thus, at least one-fourth of the decrease in binding in the samples from patients with diabetes can be attributed to glycosylation of albumin. Garlick and Mazer (19) have shown that nearly half of the glycosylation of human albumin occurs at lysine 525, and such modification decreases the affinity of albumin for bilirubin (20). Other factors that may play a role in phenytoin binding in whole serum include other proteins (such as α-globulins) and fatty acids (the concentrations of which vary with extent of diabetes control).

From our data derived from whole serum, we would estimate the K_a for binding of phenytoin to albumin to be $15–25 \times 10^3$ M^{-1}. This determination is slightly higher than that of 10×10^3 M^{-1} reported by Benedek et al. (21), who also studied whole serum. Our in vitro experiments yielded calculated K_a values that were somewhat lower. Our K_a of 3.9×10^3 M^{-1} for nonglycosylated albumin is close to that of 5.1×10^3 M^{-1} reported for isolated albumin (22). Furthermore, the K_a of 2.4×10^3 M^{-1} that we determined for glycosylated albumin is similar to that of 2.8×10^3 M^{-1} reported for albumin isolated from patients with uremia (22), a situation in which posttranslational carbamylation occurs (23,24). In these patients the free fraction of drug correlates directly with the extent of albumin carbamylation (23–25). Because nonenzymatic glycosylation of albumin also produces a posttranslational alteration in protein structure and physicochemical properties (15,20,26) and because the binding of sulfisoxazole (site I–specific antimicrobial agent) is diminished as a function of plasma protein glycosylation in adults with diabetes (7), a common binding defect between carbamylated and glycosylated albumin has been proposed (7).

Our data from both in vitro and patient specimens indicate that nonenzymatic glycosylation of albumin produces a decrease in the affinity of phenytoin. This alteration in binding may have clinical significance in poorly controlled diabetics, in whom total serum phenytoin concentrations may underestimate free phenytoin.

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