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Research Article

The Protein Binding of Phenytoin, Propranolol, Diazepam, and AL01576 (an Aldose Reductase Inhibitor) in Human and Rat Diabetic Serum

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The extent of serum protein binding of AL01576, phenytoin (DPH), diazepam (DIAZ), and propranolol (PRO) was evaluated in a group of nondiabetic and a group of insulin-dependent diabetic subjects, as well as in streptozotocin-treated rats. Both serum glucose and glucosylated protein levels were elevated in the diabetic patient population (179 and 150% of control values, respectively). The mean free fractions (f_p) of AL01576, DPH, and PRO were not statistically different for the two human groups. The DIAZ f_p was slightly elevated ($P < 0.05$) in the diabetic patients (mean = 0.016) compared to the control group (mean f_p = 0.014). An acute (<3 days) and chronic (>20 days) diabetic rodent model was evaluated using Sprague–Dawley rats following streptozotocin administration (60 mg/kg i.p.). Both diabetic rat groups exhibited substantial increases in serum glucose, free fatty acids (FFA), and protein glucosylation compared to controls. The f_p of AL01576 was increased in both the acute (mean = 0.248) and the chronic (mean = 0.202) condition compared to controls (mean = 0.163). The f_p of DPH was also markedly increased in the acute (mean = 0.348) and the chronic (mean = 0.280) models compared to untreated controls (mean = 0.207). DIAZ and PRO binding was largely unaffected by the streptozotocin treatment. *In vitro* studies of purified human albumin suggest that a considerable degree of glucosylation would need to be present in diabetic serum before it would effectively alter drug binding. Our data suggest that only minor drug–serum binding changes occur in diabetic patients who are otherwise healthy and whose disease is well controlled.

Key Words: protein binding; diabetes; streptozotocin-treated rats; glucosylated albumin; AL01576; phenytoin; diazepam; propranolol.

INTRODUCTION

AL01576 is an aldose reductase inhibitor which is currently undergoing evaluation for the treatment of some of the medical complications of diabetes (e.g., prevention of cataract formation and peripheral neuropathy). AL01576 is structurally similar to phenytoin (DPH) and appears to exhibit similar serum protein binding characteristics (1).

Recent reports in the literature suggest that the binding of certain drugs may be altered in diabetic serum due to the presence of glucosylated albumin and/or elevated free fatty acids (2–4). These observations may be relevant given the therapeutic use of AL01576 in diabetic patients. Therefore, the following studies were undertaken to compare the binding of AL01576 along with model drugs [DPH, diazepam (DIAZ), and propranolol (PRO)] in diabetic and nondiabetic serum obtained from both human subjects and a diabetic animal model.

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MATERIALS AND METHODS

Radiolabeled (^{14}C) AL01576 was supplied by Alcon Laboratories (Fort Worth, Tex.). AL01576 was determined to be both radiochemically pure (>98%; 42.3 mCi/mmol) and stable during dialysis (1). Radiolabeled (^{3}H) DPH (sp act, 46 Ci/mmol; >95% radiochemically pure), DIAZ (70 Ci/mmol; >99%), and PRO (20 Ci/mmol; >98%) were obtained from Amersham (Arlington Heights, Ill.). Human serum albumin (Fraction V, lot No. 1051-9328), streptozotocin, 2-thiobarbituric acid, and 5-(hydroxymethyl)-2-furaldehyde (HMF) were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Human Study. Following an overnight fast, blood samples were obtained from 10 healthy, adult male volunteers and 10 diabetic male patients. These diabetic patients were otherwise healthy adults who had been diagnosed as type I diabetics and had received insulin therapy for at least 2 years. All subjects had given informed consent prior to their participation in the study. Following the blood collection, the blood was allowed to clot and the resulting serum was harvested. An aliquot of the serum was used for clinical chemistry analysis (SMAC profile and protein electrophoresis); the remaining serum was stored at −20°C until used.

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Animal Study. Twenty-one male Sprague–Dawley rats were obtained from Harlen Industries (Indianapolis, Ind.). The rats were made diabetic by intraperitoneal injections of streptozotocin (60 mg/kg). The rats were divided into three treatment groups; chronic diabetic, dosed 20 days prior to blood sampling ($N = 7$); acute diabetic, dosed 3 days prior to blood sampling ($N = 7$); and control ($N = 7$). All of the blood samples were obtained on the same day via venipuncture of the abdominal aorta under light ether anesthesia. Following collection, the blood was allowed to clot and the resulting serum was harvested. An aliquot of the serum was used for chemical analysis; the remaining serum was stored at −20°C until used in the binding studies. Serum glucose and creatinine were determined spectrophotometrically with the use of two commercial kits (Sigma; glucose No. 315 and creatinine No. 555). Total proteins were assayed using the Biuret method (6) and free fatty acids (FFA) were measured colorimetrically (7).

In Vitro *Glucosylation of Human Serum Albumin.* Aliquots of a 40-g/liter albumin solution (in 0.13 *M* sodium–potassium phosphate buffer, pH 7.4) containing various concentrations of glucose (0–200 m*M*) were filtered (0.22-μm Sterivex-GV filters; Millipore) and then incubated for 24 hr at 37°C. Protein binding experiments were performed subsequently on these aliquots. Glucosylated protein was measured using a modification of the thiobarbituric acid procedure as presented by Mereish *et al.* (2). This assay measures 5-(hydroxymethyl)-2-furaldehyde (HMF) released upon the hydrolysis of ketoamine adducts of proteins (2).

Protein Binding. The binding of AL01576 (1 μg/ml), DPH (10 μg/ml), DIAZ (1 μg/ml), and PRO (100 ng/ml) was determined by equilibrium dialysis methods as previously established (1,8,9). Postdialysis samples of both the buffer and the serum side were obtained. No significant volume shifts were detected. Postdialysis serum and buffer were analyzed for drug concentration by liquid scintillation counting; previous experiments have determined these drugs to be stable under dialysis conditions (1,8,9). Quench correction was performed using the external standards methods. The binding results are expressed as the fraction unbound (f_p).

Statistical Analysis. Differences between human diabetic and human nondiabetic mean values for serum chemistry and drug–protein binding results were assessed by unpaired t test. The influence of acute and chronic diabetes on rat serum chemistry and drug-binding values was evaluated by analysis of variance; subsequent differences were then identified by Dunnett's test. Statistically significant differences were assessed at the $P < 0.05$ level.

RESULTS

The values for the clinical chemistry analysis of the serum from diabetic and nondiabetic human subjects are presented in Table I. There was a significant difference in serum glucose and protein glucosylation. No difference was observed for any other serum chemistry parameter. The results of the binding of AL01576, DPH, DIAZ, and PRO to diabetic and nondiabetic human serum are presented in Table II. AL01576, DPH, and PRO binding was comparable, whereas DIAZ exhibited a small, but statistically significant higher f_p for DIAZ in diabetic patients.

The serum glucose concentrations for both the acute and the chronic rat model were substantialy elevated compared with controls (Table III). Serum FFA concentrations were also higher in the two diabetic animal groups. Mean creatinine and total protein concentrations were comparable to those of controls. There was a modest increase (10–20%) in the glucosylation of protein in the diabetic rat groups, which reached statistical significance for the acute group. The binding of AL01576 was less in the two diabetic rat groups, with a mean f_p value which was 50 and 24% higher than the control value for the acute and chronic model, respectively (Table IV). The mean f_p for DPH was also considerably higher in the two diabetic groups compared to the controls (Table IV). The mean f_p values of DIAZ and PRO were comparable among all of the groups, with the exception that the binding of PRO was slightly increased for the chronic diabetic model (Table IV).

The results of the *in vitro* glucosylation study are presented in Table V. Although there was a fourfold increase in HMF (i.e., glucosylated protein), there was no increase in the f_p for AL01576, DPH, DIAZ, or PRO during the *in vitro* glucosylation of albumin.

DISCUSSION

Glucosylated albumin and hemoglobin are being used clinically as a measure of sustained elevations in blood glucose (10–13). The nonenzymatic glucosylation of proteins is a relatively slow process and thereby reflects a sustained elevation in blood glucose as opposed to serum glucose itself, which can fluctuate widely in response to a variety of stimuli. Realization of the potential clinical implications of altered albumin structure on drug binding has prompted the

Table I. Clinical Chemistry Profile in Control and Diabetic Human Serum

Subjects	Glucose (mg/dl)	FFA (mg/dl)	ALBUMIN (g/dl)	AAG (mg/dl)	Total protein (g/dl)	HMF (μg/mg protein)
Control ($N = 10$)						
Mean	89*	17.0	3.93	69.7	7.1	0.10*
(±SD)	(6)	(3.5)	(0.34)	(17.2)	(0.3)	(0.02)
Diabetic ($N = 10$)						
Mean	159*	19.2	3.73	66.4	6.8	0.15*
(±SD)	(61)	(5.2)	(0.32)	(15.4)	(0.4)	(0.03)

* Statistically significant difference, Student's t test ($P < 0.05$).

Table II. Protein Binding of AL01576, Phenytoin (DPH), Diazepam (DIAZ), and Propranolol (PRO) in Control and Diabetic Human Serum

Subjects	Fraction unbound (×100)			
	AL01576	DPH	DIAZ	PRO
Control (N = 10)				
Mean	16.6	13.5	1.40*	17.7
(±SD)	(2.9)	(1.2)	(0.14)	(2.6)
Diabetic (N = 10)				
Mean	18.5	14.2	1.64*	18.9
(±SD)	(1.8)	(0.9)	(0.20)	(2.4)

* Statistically significant difference, Student's t test ($P < 0.05$).

examination of drug binding in the diabetic population (2–4).

Human Study. The present report appears to conflict with one of the previous reports with respect to the impact of human diabetes on the binding of acidic drugs. Ruiz-Cabello and Erill (3) reported that the mean sulfisoxazole f_p was more than threefold larger in their diabetic patients. These investigators attributed the larger f_p to the extent of glucosylated protein (i.e., albumin). In the present study, the binding of DPH and AL01576 to human serum was unaffected by the diabetic status. The serum protein binding of AL01576 has been previously established to be similar in extent to that of DPH (f_p = 0.15), concentration independent, and apparently bound to the same binding site as DPH on albumin (1). Like DPH, sulfisoxizole is thought to bind to the warfarin sight on human serum albumin (14). The difference between the results of these two reports may be related to the specific patient population studies. In the present study, the diabetic patients were otherwise healthy, adult male subjects whose diabetes was apparently well controlled by insulin. Limited details concerning the diabetic population were provided in the other study (3).

In agreement with the report of Ruiz-Cabello and Erill (3), our studies indicate that DIAZ is bound to serum proteins to a lesser extent in diabetic than in nondiabetic human serum, however, the difference in the present report was small. Although differences between the diabetics and the controls in mean FFA concentrations did not reach statistical significance in the present study, it appears likely that a small increase in serum FFA in the diabetic patients results in the displacement of DIAZ from albumin binding sites.

The binding of PRO was similar in diabetic and nondiabetic serum. Moreover, α-1-acid glycoprotein (AAG) concentrations were comparable in the two populations and could account for a majority of the intersubject variability in PRO binding (PRO bound/free ratio correlated with AAG levels, r = 0.702; $P < 0.05$). This appears to be the first study in which cationic drug binding and α-1-acid glycoprotein concentrations in diabetes have been addressed.

Animal Study. The streptozotocin-treated rat model is used extensively in diabetes research, including the development of aldose reductase inhibitors. Since the glucosylation process is not enzyme mediated, glucosylated albumin is likely to be present in the serum of these rats. Our data (Table III) suggest a slight increase in glucosylated protein in both the acute and the chronic diabetic rat model. Although slightly elevated in the acute diabetic model, serum creatinine concentrations in both models were not statistically different from those in controls. Normal serum creatinine implies intact renal function for these animals. This observation may be relevant to protein binding experiments given the ability of streptozotocin to produce renal damage and the diminished protein binding of drugs in renal disease (15,16). Larger doses of streptozotocin may produce a model which mimics drug-binding behavior representative not only of diabetes, but also of renal failure. Our animal data (Table III) were also consistent with the human data with respect to elevated free fatty acids, which were two to four times higher than in the nondiabetic control animals.

In contrast to the human data, the binding of AL01576 and DPH was dramatically altered in both diabetic rat groups. The diminished binding appeared to be related more closely to FFA concentrations than to the degree of protein glucosylation.

Diazepam binding was unaffected in the diabetic model, which contrasts with the human observation. This lack of effect was somewhat surprising given the substantial elevation in FFA concentrations in the diabetic rats. This difference between rat and human serum with reference to the relative impact of FFA on DIAZ binding has been previously noted (8).

In contrast to the human data, a slight increase in PRO

Table III. Serum Chemistry Values for Diabetic and Control Rats

Group	Glucose (mg/dl)	Creatinine (mg/dl)	FFA (mg/dl)	Total protein (g/dl)	HMF (μg/mg protein)
Control (N = 7)					
Mean	192	0.30	13.9	6.2	0.22
(±SD)	(20)	(0.09)	(3.0)	(0.2)	(0.02)
Acute diabetic (N = 7[a])					
Mean	503*	0.50	45.3*	5.9	0.26*
(±SD)	(23)	(0.22)	(21.8)	(0.5)	(0.03)
Chronic diabetic (N = 7)					
Mean	545*	0.38	38.8*	6.1	0.24
(±SD)	(76)	(0.10)	(20.0)	(0.5)	(0.03)

[a] Insufficient sample size: N = 6 for FFA and HMF; N = 5 for total protein.
* Statistically significant difference from controls, Dunnett's test ($P < 0.05$).

Table IV. Protein Binding of AL01576, Phenytoin (DPH), Diazepam (DIAZ), and Propranolol (PRO) in Control and Diabetic Rat Serum

Group	Fraction unbound (×100)			
	AL1576	DPH	DIAZ	PROP
Control (N = 7[a])				
Mean	16.3	20.7	15.9	18.9
(±SD)	(0.7)	(2.7)	(1.4)	(1.1)
Acute diabetic (N = 7[b])				
Mean	24.8*	34.8*	16.8	18.0
(±SD)	(4.3)	(2.1)	(1.9)	(1.6)
Chronic diabetic (N = 7)				
Mean	20.2*	28.0*	14.7	16.2*
(±SD)	(2.2)	(4.4)	(1.8)	(2.1)

[a] Insufficient sample size: N = 6 for DPH.
[b] Insufficient sample size: N = 5 for DIAZ; N = 4 for DPH.
* Statistically significant difference from controls, Dunnett's test ($P < 0.05$).

binding was noted for the chronic diabetic rat model compared to the control animals. This difference was not present for the case of the acute model. Although the difference is small, the decrease in the PRO f_p may suggest an increase in the AAG concentration. Our results could be misleading since propranolol is bound to both albumin and AAG in the rat and the large decreases in albumin binding may be offsetting an even greater increase in AAG binding. Rat AAG cannot be measured using human AAG antibodies (8,18); indirect measurements of AAG [i.e., serum sialic acid concentrations or tris butoxyethylphosphate displacement (8,18)] were not attempted due to insufficient sample volume. AAG is an acute-phase protein which increases in concentration as a response to inflammation or physiological stress (17,18). Streptozotocin treatment produces a number of adverse effects (i.e., physiological stress) in the rat, therefore, an acute-phase response is possible. The reported time course of AAG induction (18) may have precluded a similar observation being made in the acute model.

Glucosylated Albumin. Our *in vitro* glucosylation study, as well as the work of others (2), suggests that any serum protein binding differences observed in the diabetic population are unlikely to be attributable to glucosylated albumin. Mereish *et al.* (2) reported diminished salicylate binding to "completely" glucosylated albumin but observed no alteration in salicylate binding to albumin which was 35% glucosylated. The data in Table V suggest no influence of glucosylation on drug binding up to 0.32 μg HMF/mg protein (approx. 20% molar ratio). Several studies suggest that nondiabetic serum proteins are 5–10% glucosylated, whereas diabetic serum can be up to two to three times that value (10–30%) (9–12). Ruiz-Cabello and Erill (3) reported glucosylated protein concentrations of up to 8 mg glucose/g protein (comparable to 6 μg HMF/mg protein, a value yet to be reconciled with our own data, i.e., normal value = 0.10 μg HMF/mg protein) and found that the percentage of free sulfisoxazole correlated with protein glucosylation. Glucosylated protein levels were 50% higher than in nondiabetic serum in our diabetic population (Table I). However, our results (Tables I and V) suggest that no change in drug binding would be observed at this relatively low degree of glucosylation. In the light of the present data, it remains unclear whether albumin, perhaps glucosylated to a greater extent in a patient population whose serum glucose may have been less well controlled, was responsible for the diminished sulfisoxizole binding in the previous study (3). Additional studies will be needed to confirm the present observations and to reconcile them with the previous studies.

Table V. Protein Binding of AL01576, Phenytoin (DPH), Diazepam (DIAZ), and Propranolol (PRO) to Human Serum Albumin Following 24-hr Incubation at 37°C with Various Concentrations of Glucose[a]

Glucose solution (mM)	Glucosylated protein (μg HMF/ mg protein)	Fraction unbound (×100)			
		AL01576	DPH	DIAZ	PRO
0	0.092	26.0	36.6	13.9	54.5
5	0.129	24.4	36.2	13.9	55.6
50	0.169	24.9	36.7	12.9	56.8
100	0.263	26.1	36.4	12.3	57.2
200	0.317	26.2	35.5	12.5	55.7

[a] Each value represents the mean of three determinations.

In summary, we have examined the binding of several model compounds to serum proteins in diabetic and nondiabetic human subjects, as well as an animal model of diabetes. In our relatively healthy diabetic population, we found little change in the protein binding of these agents, which contrasts with the findings of several literature reports of diminished binding in diabetic serum. We feel that the difference between studies is probably related to the specific drugs which were studied and to the diabetic population involved. The less well-controlled diabetic patients exhibit more extensive protein glucosylation, lower albumin concentrations, and higher FFA concentrations, which result in diminished drug–protein binding. The streptozotocin-treated rat appeared to mimic the human situation, with increases in serum FFA and glucosylated protein, suggesting that it may provide additional insight into the mechanism and consequences of *in vivo* alterations in protein binding in diabetes.

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