INSULIN OR SULFONYLUREA TREATMENTS OF THE DIABETICS DIFFERENTIALLY AFFECT ERYTHROCYTE MEMBRANE AND SERUM ENZYMES AND EXTENT OF PROTEIN GLYCOSYLATION

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ABSTRACT

Erythrocyte membrane protein glycosylation increased by 3.4 fold in diabetes. Insulin or sulfonylurea treatment did not reduce the extent of glycosylation. The serum protein glycosylation was comparable in all the groups including control. Erythrocyte membrane Na^+, K^+ -ATPase activity decreased in the diabetics; only insulin treatment partly restored the activity. Erythrocyte membrane acetylcholinesterase activity decreased only in the sulfonylurea treated group. Serum butyrylcholinesterase activity was relatively low in the diabetic and insulin treated diabetic groups. The K_m and V_{max} of the two components of Na^+, K^+ -ATPase from erythrocyte membranes were differently affected in the diabetic and the two treatment groups. The V_{max} of acetylcholinesterase decreased only in the sulfonylurea treated group. Diabetic states resulted in decreased V_{max} of components I and II of serum butyrylcholinesterase. In insulin-treated diabetics, component II was absent. Sulfonylurea group resembled diabetics. *In vitro* incubation with insulin differentially affected the Na^+, K^+ -ATPase and serum butyrylcholinesterase activities.

KEYWORDS : Diabetes mellitus, Erythrocyte membranes, Na^+, K^+ -ATPase, Acetyl choline esterase, Butyryl choline esterase, Glycosylation

INTRODUCTION

Increased blood glucose level is known to result in increased content of glycosylated hemoglobin - HbA1c (1). Additionally, non-enzymatic glycosylation of ϵ -amino group of lysine in proteins gives rise to a product called 'fructosamine' via Amadori rearrangement (1). HbA1c fructosamine levels serve as useful indices for monitoring the glucose control (1). However, there are practical limitations for their clinical application (2, 3). Hyperglycemia is also known to cause non-enzymatic glycation of Na^+, K^+ -ATPase results in its inactivation (5).

Diabetic condition is known to result in decreased Na^+, K^+ -ATPase activity in kidney cortex and medulla,

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glomerulus, brain and red blood cell membranes (1, 6, 7). However in the kidney, the activity increases significantly by the sixth week (1). Decreased acetylcholinesterase (AChE) activity in the erythrocyte membranes from alloxan-diabetic rats and type I human diabetic patients have also been reported (8, 9). By contrast, the serum butyrylcholinesterase (BChE) activity in streptozotocin-diabetic rats and in human type I and type II diabetic patients increased significantly (10,11). Interestingly *In vitro* incubation with insulin can significantly influence the Na^+, K^+ -ATPase and AChE activities in the erythrocyte membranes from control as well as diabetic groups (8, 12).

Insulin therapy or treatment with sulfonylurea type drugs (13) have differential effect on lipoprotein metabolism at least in the NIDDM patients (14). In view of the above it was of interest to find out if the extent of glycosylation of erythrocyte membrane proteins and serum proteins, as well as the activities

and kinetic properties of Na^+, K^+ ATPase, AChE and BChE are differentially affected under these treatment conditions. The results of these investigations are described in the present communication, which confirm that the effects of the two therapies are indeed differential.

MATERIALS AND METHODS

Chemicals

Acetylthiocholine iodide (ACTI), butyrylthiocholine iodide (BCTI) and ethopropazine hydrochloride (ETPZ.HCl) were purchased from Sigma Chemical Co., USA. 5,5' Dithio-bis (2-nitrobenzoic acid) (DTNB) and vanadium free adenosine 5' triphosphate, sodium salt (ATP) were purchased from SRL, India. Thiobarbituric acid (TBA) was purchased from LOBA Chemicals, India, and NPH insulin from Knoll Pharmaceuticals Ltd., India. All other chemicals were of analytical reagent grade and were purchased locally.

Subjects

Total 39 subjects were chosen for the study. These included 10 healthy controls (4 males and 6 females; mean age 52 yrs.), 11 untreated diabetic (4 males and 7 females; mean age 53 yrs.), 10 insulin treated diabetic (5 males and 5 females; mean age 55 yrs.) and 8 oral hypoglycemic drug (either Glynase [glipizide] or Sorbitrate [tolbutamide]) treated diabetics (6 males and 2 females; mean age 55 yrs.) as shown in Table 1. The insulin / sulfonylurea treatment was for variable durations of 2 to 8 years. All the subjects gave informed consent and the study was approved by the local ethics committee.

Preparation of erythrocyte membranes

Erythrocyte membranes were isolated according to the method described previously (15). Briefly, saline (0.9%) washed RBDs were lysed in hypotonic (14mM) Tris-HCl buffer pH 7.4 (15). And the membranes were sedimented at 30,000 X g for 35 min and washed repeatedly to obtain in hemoglobin-free membranes. The pellet was finally suspended in the same buffer to give erythrocyte membrane suspension containing approximately 2 mg protein/ml.

Enzyme assays

Na^+, K^+ -ATPase activity

Na^+, K^+ -ATPase activity was measured by the method of Kurnthekar and Katyare (15) and the liberated inorganic phosphorous was estimated according to method of Fiske and Subba Row (16). For kinetic studies, the substrate (ATP) concentration range used was from 0.1 to 4.0 mM.

The data were analyzed by the Lineweaver Burk, Eadie-Hofstee and Eisenthal and Cornish-Bowden methods (plots not shown) for the determination of K_m and V_{max} (17, 18). The values of K_m and V_{max} obtained by the three methods of analysis were in close agreement and were averaged. The results are given as mean $\pm$ SEM of the averaged values. The data were computer analyzed employing Sigma Plot version 5.0.

Assay of AChE and BChE activities

The AChE / BChE activities were determined essentially according to the procedure of Ellman et. al. (19) as described previously (18). For AChE activity, the assay system contained in a total volume of 1 ml : 100 mM potassium phosphate buffer pH 8.0, 0.32 mM DTNB, 0.1 mM ETPZ-HCl and 10 to 50 μ g erythrocyte membrane protein as the source of the enzyme. For substrate kinetics studies concentration of substrate i.e. ACTI was varied from 0.05 to 10 mM. For determination of BChE activity the assay system was the same as above except that 50 mM Tris-HCl buffer pH 8.0 replaced the potassium phosphate buffer, ETPZ-HCl was omitted and BCTI was the substrate. 20 μ l of 1: 10 diluted serum was used as the source of the enzyme (19). The kinetic data were analyzed as described above for Na^+, K^+ ATPase.

In vitro incubation with Insulin

For studying the *in vitro* effect of insulin, the enzymes (i.e. either erythrocyte membranes or serum) were pre-incubated in the respective assay system for 30 min at 37°C using three different insulin concentrations : 10^{-9} , 10^{-8} and 10^{-7} M. The enzyme activities were then determined as described above.

Serum glucose was estimated by glucose oxidase - peroxidase method. Extent of glycosylation was determined by the thiobarbituric acid method (20). Protein estimations were carried out according to the procedure of Lowry, Rosebrough, Farr and Randall (21) with bovine serum albumin used as the standard.

RESULTS

The data in Table 1. show that the mean age of all groups was well matched. From the data in Table 2, it is clear that insulin and sulfonylurea treatments resulted in lowering the blood sugar levels when compared with those in the untreated group but were still higher than in the control group. The extent of glycosylation of serum proteins was somewhat higher (+23 %) in the diabetic group and decreased in insulin treated diabetic group. However within the four groups the overall changes did not present any clear picture. As against this, the data on the glycosylation of erythrocyte membrane proteins revealed dramatic

changes. Thus in the untreated diabetic group the extent of glycosylation was 3.4 fold higher. In insulin treated group there was further increase (5.4 fold) in glycosylation. Sulfonylurea treatment was also ineffective in lowering the extent of glycosylation.

Consistent with the observations of earlier researchers (1,5,6,7,15), the Na^+, K^+ -ATPase activity decreased significantly in the diabetic group (65 % decrease). Insulin treatment restored the activity considerably. However the sulfonylurea treated was ineffective. The AChE activity in the erythrocyte drastically by 43 % only in the sulfonylurea treated group (Table 3). There was a small but reproducible decrease (28 % decrease) in the BChE activity in the untreated diabetics. Insulin treatment had no beneficial effect but in sulfonylurea treated diabetics, the activity was comparable to the controls (Table 3).

Substrate kinetics analysis studies revealed the presence of two components in the erythrocyte

Table 1. Distribution of male and female volunteers.

Group		Age (Yr.)	
		Male	Female
MeanControl	52.0±1.41 (4)	51.2±0.83 (6)	51.5±0.72 (10)
Untreated diabetic	55.3±1.25 (4)	51.7±1.40 (7)	53.0±1.01 (11)
Insulin treated diabetic	50.8±3.48 (5)	57.6±1.29 (5)	54.2±2.09 (10)
Sulfonylurea treated diabetic	54.8±1.78 (6)	53.5±3.50 (2)	54.5±1.48 (8)

Values are given as mean ± S.E.M. The numbers of volunteers are shown in the parentheses.

Table 2. Serum glucose concentration and protein glycosylation in diabetes.

Group	Serum glucose (mM)	Glycosylation	
		Erythrocyte membrane protein (ng /mg protein)	Serum protein (ng /ml serum)
Control (10)	5.5±0.18	44.2±9.0	7.48±0.84
Untreated diabetic(11)	11.1±1.00 ^a	150.0±26.5 ^c	9.16±1.96
Insulin treated diabetic (10)	7.8±0.41 ^{a,*}	240.5±29.1 ^{a,@}	7.75±0.70
Sulfonylurea treated diabetic(8)	7.2±0.65 ^{a,*}	111.3±19.2 ^b	8.14±0.25

Methods for serum glucose estimation and glycosylation were as described in the text. Results are given as mean ± S.E.M. for the number of volunteers shown in parentheses.

a, p<0.05, b, p<0.01, c, p<0.002, d, p<0.001 compared to control.

@, p<0.05, *, p<0.01, and #, p<0.005 as compared to the Untreated diabetic group.

membrane Na⁺,K⁺-ATPase : one with high affinity (low Km) and the other with low affinity (high Km), in all the groups (Table 4). Thus in the control group,

component I and II had Km of 0.1 mM and 2.24 mM respectively. the different in the Km values being about 20 fold. The Vmax values of the two components were

Table 3. Erythrocyte membrane Na⁺,K⁺ ATPase, AChE and serum BChE activities and the effect of pre-incubation on their activities.

Group	Na ⁺ ,K ⁺ -ATPase		AChE *		BChE	
	0 min	30 min	0 min	30 min	0 min	30 min
Control (10)	170.4±10.1	195.0± 8.21	0.98±.019	1.00±0.10	9.99±0.22	7.67±0.35 ⁺⁺⁺
Untreated Diabetic(11)	58.9±11.5 ^b	102.6±12.04 [†]	1.10±.010 ^c	0.75±0.03 ^{††}	7.19±0.63 ^b	9.30±0.98
Insulin treated Diabetic (10)	113.4±11.8 ^{a*}	90.6±10.98	0.92±0.10	0.95±0.10	7.19±0.63 ^b	6.16±0.51 ^{d#}
Sulfonylurea treated Diabetic (8)	47.1± 4.2 ^b	35.5± 3.10	0.56±0.06 ^b	0.44±0.04	9.43±0.63 [@]	8.25±0.50 [@]

The enzyme assays were carried out as described in the text with or without preincubation of the enzyme with the buffer after 30 min as indicated. The results are given as mean ± S.E.M. for the number of volunteers shown in the parentheses. Na⁺,K⁺ ATPase activity = n moles of Pi liberated / hr / mg protein, AChE activity = m moles / min / mg protein, BChE activity = m moles / min / ml serum, a, p<0.002, and b, p<0.001 compared to the 0 min control group., @, p<0.05, +, p<0.01, and #, p<0.001 compared to the 0 min diabetic., †p < 0.02, ††p < 0.002 and †††p< 0.001 compared to corresponding 0 min. value.

Table 4. Substrate kinetics analysis of erythrocyte membrane Na⁺,K⁺ ATPase, AChE and BChE.

Group/Enzyme	Component I		Component II		Component III	
	Km	Vmax	Km	Vmax	Km	Vmax
Na⁺,K⁺ ATPase						
Control (10)	0.101±0.024	80.5± 2.94	2.24±0.19	239.2±11.95		
Untreated diabetic(11)	0.106±0.008	30.0± 3.24 ^a	0.71±0.05 ^a	45.7± 2.54 ^a		
Insulin treated diabetic(10)	0.290±0.049 ^{a*}	57.1±13.34	3.49±0.33 ^{a*}	193.3±29.55 ^a		
Sulfonylurea treated diabetic(8)	0.160±0.050	37.6± 2.97 ^a	0.87±0.10 ^a	61.8± 6.94 ^a		
AChE						
Control (10)	0.059±0.007	0.91±0.18	0.90±0.12	1.45±0.28		
Untreated diabetic(11)	0.052±0.013	0.89±0.07	1.01±0.16	1.54±0.09		
Insulin treated diabetic(10)	0.058±0.009	0.73±0.03	1.18±0.16	1.39±0.08		
Sulfonylurea treated diabetic(8)	0.059±0.012	0.44±0.03 ^{a*}	1.24±0.24	0.77±0.07 ^{a*}		
BChE						
Control(10)	0.056±0.006	6.29±0.16	0.259±0.018	9.97±0.23	1.41±0.14	13.34±0.31
Untreated diabetic(11)	0.046±0.005	4.65±0.48 ^a	0.171±0.019 ^c	6.72±0.60 ^a	1.31±0.12	11.22±1.07
Insulin treated diabetic(10)	0.047±0.004	4.26±0.55 ^a	—	—	1.06±0.13	8.81±0.75
Sulfonylurea treated diabetic(8)	0.038±0.005 ^a	3.83±0.69 ^a	0.154±0.010 ^a	6.88±0.88 ^a	0.81±0.05 ^{a*}	10.79±0.99

The results are given as mean ± S.E.M. for number of volunteers shown in the parentheses. The experimental details are as given in the text. Unit Km = mM, Vmax = m moles / min / ml serum. a, p<0.05, b, p<0.01, c, p<0.005 and d, p<0.001 compared to the control. @, p<0.002 compared to the untreated diabetic.

81 and 239 n moles / hr / mg protein respectively. The K_m of component I was not influenced by diabetic state but insulin and sulphonylurea treatment caused 3.0 and 1.6 increase. For component II the K_m decreased significantly in the untreated diabetic group and was restored in insulin treated diabetics. In the sulphonylurea treated diabetic group the K_m was comparable to untreated diabetics.

The AChE activity was also resolved in two components (Table 4). The K_m of component I and II did not change under any of the conditions while the V_{max} value decreased only in the sulphonylurea treated diabetic group significantly (47-52 %).

In the control group three components of BChE differing in their kinetic properties were present (Table 4). Similar pattern was seen for other groups excepting for the one treated with insulin. For component I the K_m decreased significantly in sulphonylurea treated diabetic group while the V_{max} decreased from 26 to 39 % in all the groups; maximum effect was seen in the sulphonylurea treated diabetic group. For component II the K_m as well as V_{max} were lower by about 31 to 41 % in the untreated diabetic and sulphonylurea treated diabetic groups; the insulin treated diabetic group proved to be unique in that component II was altogether absent. The K_m for component III decreased by 25 to 43 % in the sulphonylurea treated diabetic and untreated diabetic groups while V_{max} was lower by 16 to 32 %. The maximum effect on K_m was noted for component III.

The effect of pre-incubation on the three enzymes in the four different groups (Table 1) are summarized in Table 3. It is evident (Table 3) that 30 min pre-incubation at room temperature only marginally influenced the Na^+, K^+ -ATPase activity in the erythrocyte membranes from the control group, while in the untreated diabetics the activity increased by 74 %. In the insulin and sulphonylurea treated diabetic groups the activity decreased by 20- 25 %. Pre-incubation resulted in 32 % decrease in the AChE activity only in the untreated diabetic group. For BChE, pre-incubation caused 23 and 15 % decrease respectively in the control and sulphonylurea treated diabetic groups. No effect was seen in the untreated diabetic group. Surprisingly, however, in the insulin treated diabetic group the activity increased by 80 %.

Pre-incubation with insulin on the Na^+, K^+ -ATPase activity in the control and untreated diabetic groups, whereas significant decrease was seen in both the treatment groups; the effect being more pronounced in the sulphonylurea treated group (Fig. 1). Pre-incubation with insulin did not have much effect on the erythrocyte membrane AChE activity in any of the groups (data not shown). For BChE activity pre-incubation of serum from control and untreated diabetic groups resulted in 18 to 30 % stimulation of the activity, whereas no effect was seen in insulin treated diabetic group (Fig. 2). In the sulphonylurea treated diabetic group the activity decreased by 10 - 15 % after incubation with insulin.

Fig.1 Effect of *in vitro* incubation with insulin on erythrocyte membrane Na^+, K^+ -ATPase activity. Controls —○—, untreated diabetic —●—, insulin treated diabetic —△—, sulphonylurea treated, diabetic —x—.

Fig. 2. Effect of *in vitro* incubation with insulin on serum BChE activity. Controls —○—, untreated diabetic —●—, insulin treated diabetic —△—, sulfonylurea treated, diabetic —x—.

Discussion

It is clear from the data (Table 2), that both insulin and sulfonylurea treatments were able to control the glucose level almost to the same level. Despite this the extent of serum proteins glycosylation was almost comparable amongst all the groups. As against this, the extent of erythrocyte membrane glycosylation was very high (3.4 fold increase) in the untreated diabetic group. with insulin treatment, the extent of glycosylation increased further (5.4 fold). By contrast sulfonylurea treatment was able to decrease the membrane protein glycosylation to some extent. Thus the effect of two treatment were differential. The data on enzyme activities (Table 3) and kinetic properties of the three enzymes (Table 4) also validate this point. Other researchers have reported elevated serum BChE levels in the diabetic humans and in experimental animals (9-12). We were, however, unable to find such a difference. In the human studies referred to above, the BChE activities were measured in 20 mM Tris-HCl beffer Ph 7.6 containing 10 mM CaCl_2 (9). It is possible that the observed differences could be due to the pH and presence of high Ca_2 concentration in their assay medium (9). Nevertheless, it is quite clear from the data (Tabel 3) that the effects of th two treatemnts. In parallel studies we found that the phospholipid composition of the erythrocyte membranes had altered significantly in all the

experimental groups (Dave, K. R. and Katyare, S.S. unpublished observations). It would therefore appear that the observed decrease in the Na^+, K^+ -ATPase activity may be attributed either to absolute decrease in the content of the enzyme units and/or to altered membrane phospholipid milieu (23).

Likewise, it is possible that the decreased AChE activity and altered membrane glycosylation (22), and/or the absolute content of the enzyme itself might have decreased possible through decreased membrane binding.

In conclusion, the results of our present studies have shown that diabetic state and subsequent treatments can differentially influence not only the activities but also the kinetic properties of the erythrocyte enzymes Na^+, K^+ -ATPase and AchE, and serum BChE. Besides, our studies suggest that erythrocyte membrane glycosylation could be a better index of the glucose control. It may also be pointed out that neither of the treatments could completely rectify the observed enzyme defects i.e. K_m or V_{max} . This is consistent with the general observation that all the maladies of diabetes are not corrected by insulin treatment (24).

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