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Erythrocyte ATPase activity in relation to hyperglycemia and hyperlipidemia in diabetic urban Indian population

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Abstract
This study examines the relationship between; erythrocyte membrane bound Na^+K^+- ATPase activity, HbA_{1c} level, Hb-AGE (hemoglobin advanced glycation end product) level and lipid profile in Indian diabetic population. The aim was to look at the dependence of this parameter on glycemic and lipid indices in general. Control subjects were volunteers with no diabetic history but were either normolipidemic or dyslipidemic. The diabetic patients were categorized on the basis of glycemic control (HbA1c values) and further into normolipidemic or dyslipidemic groups. HbA_{1c} was analyzed by affinity chromatography while all other parameters including hemoglobin, plasma glucose and lipid profile was estimated using kits on automated analyzer. Hb-AGE was measured in terms of fluorescence intensity and RBC-ATPase activity was measured using an assay kit. Among the diabetic groups, patients in the uncontrolled category (Group 3) were maximally dyslipidemic with high total cholesterol (TC) and triglyceride levels (TG). Observations indicate that even though HbA_{1c} is not significantly different between dyslipidemic and normolipidemic patients in each group, levels of fasting blood sugar, TG and LDL cholesterol were significantly higher in the former. Na^+-K^+ATPase activity was found to decrease as a function of HbA_{1c} in an inverse manner but did not show any significant correlation with TC levels. Our data indicates that erythrocyte membrane Na^+-K^+ATPase activity is significantly related to glycemic status rather than to the lipidemic status of individuals in the Asian Indian population. Glycation of the membrane proteins rather than increase in membrane cholesterol content alters the enzyme function.

Key words: *HbA_{1c}, advanced glycation, erythrocyte, Na^+K^+ATPase, hyperglycemia, hyperlipidemia*

Introduction

Type 2 diabetes (T2D) or non-insulin dependent diabetes (NIDD) is the most prevalent form of diabetes caused due to either impaired secretion of insulin or insulin resistance or both. Prolonged exposure to hyperglycemia is considered as the major factor for the various complications in the pathogenesis of diabetes. Micro and macro-vascular complications including nephropathy, retinopathy neuropathy, myocardial infarction and stroke are the major cause of morbidity and mortality due to diabetic complication.[1] Asian Indians have been shown to have higher incidence of premature coronary artery disease (CAD) compared to Europeans.[2] It is also reported that migrant Asian Indians have dyslipidemia characterized by high triglyceride and low HDL cholesterol and near normal LDL cholesterol.[3] A positive correlation between LDL and CAD has been reported in Indian population.[4]

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One of the principal features of chronic hyperglycemia is protein glycation and formation of advanced glycated end product (AGEs). Diabetes has been correlated with an increase in the glycation of the erythrocyte membrane proteins that causes alterations in the function of erythrocyte membrane. The membrane fluidity and red blood cell deformability was found to be decreased due to the change in the erythrocyte membrane composition in patients suffering from diabetes.[5,6] Lipid peroxidation and membrane protein glycation also results in alteration of membrane structure which affects all membrane bound enzymes. These studies suggest that there may be a link between structural changes of RBC membrane and glycation of the membrane proteins. Additionally RBCs are also exposed to oxidative stress caused by AGEs.

Erythrocyte ATPase enzyme is a membrane bound enzyme with significant role in the intra and extracellular cation homeostasis. Alteration of this enzyme is thought to be linked to several complications of diabetes mellitus. Three different types of ATPase enzyme are present in the erythrocyte membrane *viz.* Na^+/ K^+ -ATPase, Ca^{++} -ATPase and Mg^{++}-ATPase. Na^+/K^+-ATPase catalyzes the hydrolysis of ATP and couples it to the transport of Na^+ and K^+ across the cell membrane.[7] ATPases activity is essential for the control of hydration, nutrient uptake and fluidity of cells.[8]

Dyslipidemia results in increased lipid concentration in blood which has been found to decrease Na^+/K^+-ATPase activity, and is one of the major factor for consequences of diabetes mellitus.[9] The rheological property of the erythrocyte membrane is also changed due to alteration in the lipid composition of the membrane. All these changes reduce life span of the erythrocytes in diabetes.[10]

The aim of our study was to examine the relationship between, erythrocyte membrane bound total Na^+/K^+-ATPase activity and glycemic indices *viz.* HbA_{1c} and Hb-AGE (hemoglobin advanced glycation end product) levels and lipid profile (serum lipid composition) in Indian diabetic population. Understanding the magnitude and association between different lipid profile markers and ATPase activity may provide an indicator of the progress of diabetes to cardiovascular complications. Few studies have been done to relate diabetes and erythrocyte membrane fluidity by examining the fatty acid composition and cholesterol content of red cell membrane. They report a decrease in the membrane fluidity and increase in the ratio of saturated to unsaturated fatty acid for the diabetic patients compared to the control.[11]

Methods

Data for this pilot study was drawn from samples taken from 51 type 2 diabetic patients and 10 normal non diabetic individuals who visited the Department of Diabetes and Metabolic Diseases at Fortis hospital. All patients attending the clinic were included in the study after obtaining informed consent. The protocol was cleared by the Hospital Ethics Committee. For all tests, 5 ml blood sample was drawn after 12 hour overnight fast by venipuncture, into heparinized vacutainers. All the diabetic patients were further subdivided into three subgroups based on HbA_{1c} (%) values; Group 1 (good glycemic control $HbA_{1c} < 7$), Group 2 (moderate glycemic control HbA_{1c} 7-9), Group 3 (poor glycemic control HbA_{1c} >9). Further grouping of the population was done according to the total cholesterol (TC) levels into two groups; Group A and B with TC >200 and TC ≤ 200 mg/dl respectively.

Analysis of various blood parameters

HbA_{1c} was analyzed by affinity chromatography while all other parameters including hemoglobin, plasma glucose and lipid profile was estimated using kits on automated analyzer. Hb-AGE and RBC-ATPase activity was measured as described below.

Hb-AGE measurement

Hb-AGE was measured by the fluorescent technique developed previously.[12] Briefly, one ml of anti-coagulated blood samples was hemolysed into 8 ml of distilled water. The hemolysate was then centrifuged and supernatant taken and diluted up to 1 mg/ml protein concentration in 0.4 M sodium phosphate buffer (Ph-7.4) for fluorescence measurement. The fluorescence intensity will be measured at excitation and emission wavelength 308 nm and 345 nm respectively, using spectrofluorimeter (LS 50B Perkin Elmer, Cambridge, UK).

RBC-ATPase

(i) Erythrocyte ghost membrane preparation

The blood samples were centrifuged at 1,500 g at 25°C for 10 minutes; plasma and buffy coat were removed and packed RBC taken for erythrocyte ghost membrane preparation. A simplified procedure developed by Iwalokun and Iwalokun (2007) was adapted.[13] Briefly, 10 volumes of ice cold 5 mM Tris /0.1 mM Na_2EDTA, pH 7.6 were added to packed erythrocytes for osmotic lyses. Lysed cells were centrifuged at 18,500g for 30 minutes at 4°C to pellet down the RBC membranes; membranes were washed three times in 0.017 M NaCl/5mM Tris-HCl, pH 7.6 and finally three to four times with 10mM Tris-HCl (pH 7.5) until the pellet become white. The hemoglobin free membrane suspension was stored at -80 °C in 10mM Tris buffer before assay.

(ii) Erythrocyte ghost membrane protein concentration determination

Protein concentration was determined by Bradford method.[14] Bovine serum albumin (BSA) in the range of 100 to 1000 µg /ml was used as standard. For the assay, 50 µl of ghost membrane protein and 50 µl of Tris buffer (pH 7.5) was taken into a test tube, 5 ml of protein reagent (Coomassie brilliant blue G-250 (100 mg) in 95% ethanol and 85% (w/v) phosphoric acid) was added and kept in dark for 1 hour at room temperature under shaking condition. The absorbance was taken at 595 nm after 1 hr of incubation against a reagent blank prepared from 0.1 ml of the Tris buffer and 5 ml of protein reagent.

(iii) Total RBC-ATPase activity assay

Total RBC ATPase activity was measured by Quantichrom ATPase/GTPase assay kit. Briefly, 10 µL of ghost membrane suspension was incubated for 30 min with 10 µL of ATP 4mM in 20 µL of assay buffer at 37 °C. ATP is hydrolyzed into ADP and inorganic phosphate (Pi) by the ATPase enzyme. After incubation, total inorganic phosphate liberated by the reaction was measured by incubating 200 µL of reagent for 30 minutes at room temperature. A stable dark green coloured product with the liberated inorganic phosphate is formed which was measured at 620 nm with the help of a micro plate reader (Eon, BioTek), the intensity of colour is proportional to the concentration of inorganic phosphate liberated. Total ATPase activity was expressed as micromole of inorganic phosphate liberated per milligram membrane protein per hour (µM Pi $mg^{-1}H^{-1}$).

Statistical Analysis

Data were analyzed with paired t-test, ANOVA (two-tailed) and rank correlation. All statistical analysis was performed through interactive calculations on StatPages.org.

Results

The analysis was made in a cohort of subjects within 50 years of age with age matched non-diabetic volunteers. Table 1 shows the mean values of the different clinical parameters *viz.* HbA_{1c}, Hb-AGE, total cholesterol (TC), fasting blood glucose (FBG), triglycerides (TG), high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL) and the ratio of LDL/HDL.

Table 1: Comparison of clinical parameters in non-diabetic and diabetic patients grouped on the basis of HbA_{1c} values. Group 1 (HbA_{1c} < 7), Group 2 (HbA_{1c} 7-9), Group 3 (HbA_{1c} >9).

Clinical parameters	Non diabetic group (n=10) (Mean ± SD)	Diabetic group (n=51) (Mean ± SD)	Group 1 (n=16) (Mean ± SD)	Group 2 (n=20) (Mean ± SD)	Group 3 (n=15) (Mean ± SD)
Age	44±11	46 ± 10	46±10	46±9	46±10
FBG (mg/dl)	95±8	151± 52	121±13	128±26	205±55
HbA1c (mg/dl)	5.7±0.4	8.2 ± 1.9	6.5±0.4	7.7±0.5	10.7±1.3
Hb-AGE (A.U)	13.9±1.6	13.0±2.0	13.6± 2.0	13.0± 2.2	12.4±1.6
TC (mg/dl)	202±37	195±42	185±45	197±45	200±54
TG (mg/dl)	197±87	185±90	155±69	182±103	214±83
LDL	110±30	117±38	107±41	121±33	122± 43
HDL (mg/dl)	37±7	42± 7	45± 6	43± 8	39± 7
LDL/HDL	3.1±0.9	2.9± 1.0	2.4±1.0	2.9±0.8	3.2±1.0
ATPase activity (µM Pi/mg/h)	2.10±0.36	1.29±0.33	1.53±0.34	1.27±0.28	1.04±0.14

Table 2: Summary of clinical parameters in sub-groups of diabetic patients based on total serum cholesterol level (TC ≤ 200 mg/dl (A) and TC > 200mg/dl (B))

Clinical Parameters	Control (n=10)		Group1 HbA_{1c}<7 n= 16		Group2HbA_{1c}7-9 n = 20		Group 3 HbA_{1c} >9 n = 15	
	TC>200 CA	TC≤200 CB	TC>200 1A	TC≤200 1B	TC>200 2A	TC≤200 2B	TC>200 3A	TC≤200 3B
Age (years)	50.43± 8	35.6 ± 9	49.75±3	43.0±11	44.0 ±9	48.13±11	47.83±13	44.71±9
FBG (mg/dl)	94.62± 7.1	99.10± 12.6	130.0±7.8	118.9±13.3	136.2±25.1	118.6±25.6	224.5±60.4	193.4±51.8
HbA_{1C} (%)	5.71 ± 0.43	5.77± 0.12	6.68 ± 0.15	6.56 ±0.36	7.68 ±0.44	7.69 ±0.6	11.0 ±1.6	10.45 ±1.2
Hb AGE (AU)	14.11 ± 1.7	12.75± 0.8	13.74 ±1.99	12.92 ±1.59	12.92 ±2.73	12.87 ±1.9	11.94 ± 1.2	12.74± 1.8
TC (mg/dl)	225.7± 27.4	156.67± 10	240.75± 28.5	163.2 ±28.08	231.6 ±25.1	158.78 ± 26	258.5 22.3	160.89±21
TG (mg/dl)	176.71±50.1	141.7± 67.6	198.0± 126.5	142.3 ±44.03	236.0± 113.1	123.44 ± 46.1	254.17±75.8	187.3± 80.1
LDL (mg/dl)	128.9 ± 23.6	77.67 ± 11	143.00± 37.64	95.8 ± 37.54	147.4 ± 12.6	93.22 ± 21.7	164.5 ± 19.8	93.58±27.2
HDL (mg/dl)	40.14 ± 7.2	36.33 ± 12	47.33 ± 5.77	44.8 ± 6.53	43.6 ± 3.9	41.56 ± 10.4	42.0 ± 4.8	37.51± 7.9
ATPase activity	2.17 ± 0.39	2.22 ± 0.06	1.40 ± 0.56	1.59 ± 0.28	1.22 ± 0.29	1.33 ± 0.30	1.02 ± 0.14	1.05± 0.15
ATPase protein conc. (mg/ml)	2.0 ± 0.2	1.5± 0.9	1.91 ± 0.22	1.82 ± 0.33	1.85 ± 0.34	1.66 ± 0.29	1.85 ± 0.19	1.97± 0.30
LDL/HDL	3.3 ± 0.8	2.36 ± 1.0	3.11 ± 1.1	2.23 ± 1.01	3.41 ± 0.45	2.36 ± 0.8	3.94 ± 0.4	2.65 ± 1.0

Table 2 summarizes the values of the above parameters in subgroups based on HbA_{1c} and total serum cholesterol. Additionally it also shows the concentration (protein content) and activity of the red cell membrane bound enzyme, Na^+-K^+ ATPase. The control group were normal with respect to diabetic indices i.e. FBG (less than 126 mg/dl) and HbA_{1c} (<6.0) but not with respect to lipid parameters. Both triglycerides (CA and CB) and cholesterol levels (CB) were high in the non-diabetic group of patients (Table 2). Among the diabetic groups, patients in the uncontrolled category (Group 3) were maximally dyslipidemic with high TC and TG levels. When we examine the data in the sub-groups it is observed that even though HbA_{1c} is not significantly different between dyslipidemic and normolipidemic patients in each group, levels of FBG, TG and LDL were significantly higher in the former. Na^+-K^+ATPase activity was found to decrease as a function of HbA_{1c} in an inverse manner. No correlation could be established with Hb-AGE values. Analysis of ATPase enzyme content and enzyme activity data with ANOVA clearly indicates that while the enzyme content in the erythrocyte membrane is not significantly different, the ATPase activity is significantly different ($p< 0.0001$) between the controls and various groups of diabetic patients.

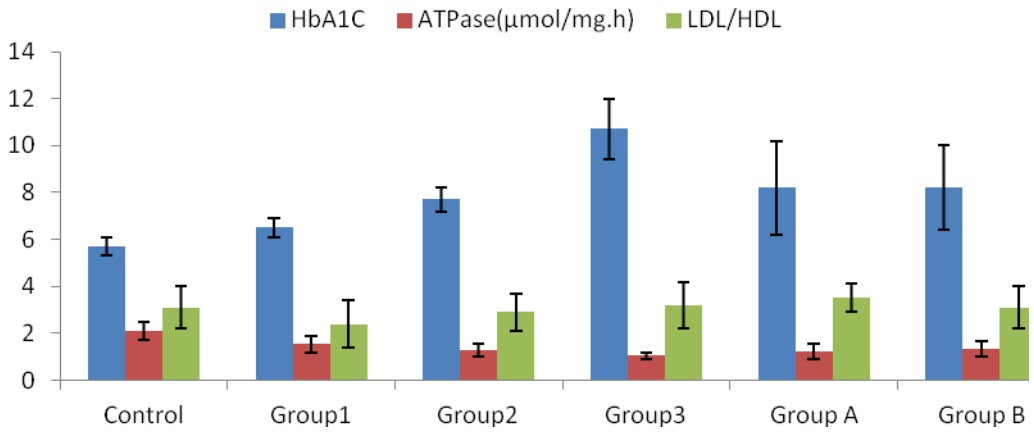

Figure 1: Comparison of HbA1c, ATPase and LDL/HDL ratio for different groups (Group 1 (HbA_{1c} < 7), Group 2 (HbA_{1c} 7-9), Group 3 (HbA_{1c} >9), Group A diabetic patients with TC > 200mg/dl, Group B diabetic patients with TC< 200mg/dl)

Paired t-test between the sub groups also indicates significant difference (p< 0.0001). Figure 1 depicts the comparative values for HbA_{1c}, ATPase activity and LDL/HDL ratio for all the groups compared with control.

In the overall data from diabetic patients, a fairly strong negative correlation is indicated between HbA_{1c} and ATPase activity, as analyzed with the non-parametric Spearman coefficient (R = -0.64, p< 0.0001). On the other hand, a weak negative correlation was observed between ATPase activity and total blood cholesterol (TC) levels which was not statistically significant (R= -0.26, p< 0.08). In case of control, the weak negative correlation of ATPase activity with both parameters was not significant (R= -0.14 and -0.23 with HbA_{1c} and TC).

Discussion

The onset and development of secondary complications in Type 2 diabetes may not depend on the duration and control. The predisposition factors vary between different racial and ethnic groups.[15] Reduced Na^+-K^+ATPase activity has been linked to diabetic neuropathy via an activation of the polyol pathway.[16] Beutler E. et al have implicated that the level of this enzyme activity is genetically determined based on ethnicity.[17] They have reported relatively high levels in non-Jewish white subjects, particularly those with some Scandinavian ancestry, while in African, Asian, and Jewish white subjects the activity has been found to be lower. They have also ruled out any relation between erythrocyte ATPase activity and obesity. Lipid abnormalities in diabetic patients have long been recognised to play a key role in macrovascular complications like atherogenesis. Both quantitative and qualitative abnormalities of lipoproteins are potentially atherogenic and are encountered in non-insulin-dependent diabetes (NIDDM). The main quantitative abnormalities are increased triglyceride (TG) levels. Qualitative changes in lipids occur by way of lipid peroxidation and glycoxidation of these molecules under elevated glucose levels and resultant oxidative stress. High levels of circulating lipids are likely to find their way into the cells of the body and thus into the lipid component of the cell membrane.

Keeping this scenario in mind, the present study was undertaken to examine the variation in the activity of red cell membrane bound Na^+-K^+ATPase as a function of both diabetic indices and lipid profile indices. First and foremost, the enzyme activity did not depend on its concentration in the membrane, since the difference in protein content was not significantly different across the groups while the enzyme activity was significantly different. Contrary to the observations made in an earlier study[8] that increased lipid concentration directly lowered the enzyme activity, our results clearly indicate that the decreased enzyme activity may not be a function of dyslipidemia. It was however found to be directly related to glycemic control as seen in non-diabetic but dyslipidemic control group (CA). Even though the lipid profile was abnormal in this group, these patients did not have diabetic history. Na^+-K^+ATPase activity in this group was higher than all the diabetic patient groups irrespective of the latter's lipid profile and was found to be not significantly different from group CB (non-diabetic and normolipidemic). On the other hand in case of diabetic groups (good, moderate and poor control of glycemia), the enzyme activity was found to be lowest in the poorly controlled high TC group. The LDL/HDL ratio was significantly higher in case of all the high TC categories including the non diabetic patients, but the ATPase activity was low only in the diabetic patients. Previous studies have reported that in IDDM patients Na^+/K^+ATPase activity was neither dependant on degree of glycemic control nor was it correlated with HbA_{1c} in these patients. It was found to be less impaired or near normal in NIDDM patients.[18]

In our study comprising Indian patients, Na^+/K^+ATPase activity was found to be significantly impaired in NIDDM patients which were further dependent on the glycemic control. The high blood cholesterol levels did not show any direct influence on the enzyme activity however. It was not possible to obtain enzyme protein in sufficient quantity from the RBC membrane to enable us to check for its glycation also because of the low blood sample volume (2ml) that was available for all the clinical investigations.

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