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ASSOCIATION OF HBA1C AND HOMA-IR LEVELS WITH NA+/K+ ATP-ASE ACTIVITY IN TYPE 2 DIABETES MELLITUS PATIENTS.

Biochemistry

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ABSTRACT

Background: Na+/K+ ATP-ase enzymes are one of the major integral membrane proteins that are involved in signal transduction, metabolic activities and maintenance of the membrane potential. Reports have suggested significant adverse effects of hyperglycemia of type 2 diabetes mellitus (DM) on the Na+/K+ ATPase activity predicting future complications of the disease.

Aims: The present study aimed to assess the relative importance of insulin resistance and glycated haemoglobin on reduction of Na+/K+ ATP-ase activity in type 2 DM.

Methods: Fasting blood glucose (FBG), glycated haemoglobin (HbA1c), serum insulin levels and Na+/K+ ATP-ase activity were measured in 60 cases and 60 control subjects in a hospital based, cross sectional study. HOMA-IR was calculated as a marker of insulin resistance from the values of FBG and serum insulin using HOMA calculator.

Results: Mean values of Na+/K+ ATP-ase was significantly lower with increased values of FBG, HbA1c and HOMA-IR in the case group (P values < 0.001). Although, Na+/K+ ATP-ase activity showed significant negative correlation with both HOMA-IR (r = -0.353, P = .006) and HbA1c (r = -0.266, P = .04), its dependence was significant only on the HOMA-IR values (β = -0.295, P = .04).

Conclusion: Na+/K+ ATPase activity is significantly reduced in type 2 DM. In spite of marked increase in both glycated haemoglobin and insulin resistance in type 2 DM, the latter determines the membrane dynamics of receptor proteins and transporter like the Na+/K+ ATPase pump more effectively.

KEYWORDS

Type 2 diabetes mellitus, Na+/K+ ATPase, HOMA-IR, HbA1c.

INTRODUCTION:

Micro and macro-vascular complications including nephropathy, retinopathy neuropathy, myocardial infarction and stroke are the major causes of morbidity and mortality due to diabetic complication (1). Different studies in humans indicate that uncontrolled diabetes mellitus causes change in membrane protein structure, its organization and also its function which play major role in the pathogenesis of diabetic complications (2, 3). Na+/K+ ATP-ase enzymes are one of the major integral membrane proteins that are involved in signal transduction, metabolic activities and maintenance of the membrane potential. The enzyme Na^+/K^+ ATPase couples breakdown of ATP to the simultaneous movement of both Na^+ and K^+ against their electrochemical gradients. It is necessary for proper cellular function since it helps to preserve the ionic gradients across the cell membrane and thus the membrane potential and osmotic equilibrium of the cell (4). Reports have suggested a significant adverse effect of type 2 diabetes mellitus on the Na^+/K^+ ATPase activity in biomembranes. For some authors, lowered erythrocyte membrane Na^+/K^+ ATPase activity may be a predictor for future complications. Glycated haemoglobin, a product of spontaneous glycation of the globin chains of haemoglobin due to persistently elevated blood glucose level is a strong indicator of long term glycemic control (for about 3 months) in these patients. Study shows that insulin resistance in skeletal muscles alter the expression of Na^+/K^+ ATPase pump subunits and a direct relationship between reduced pump activity and advanced glycation end products (AGEs)(5). With the background of this knowledge about derangement of Na^+/K^+ ATPase activity and increased insulin resistance in type 2 diabetes mellitus, it becomes important to assess whether there is any relationship between the degree of insulin resistance and AGEs with the reduction in Na^+/K^+ ATPase activity in these patients. However, till now studies related to the dependency of Na^+/K^+ ATPase activity on insulin resistance and glycated haemoglobin have not been explored in details. Based on this lacunae in the present knowledge we hypothesized that there is an inverse correlation between the degree of severity of poor glycemic control as marked by HOMA-IR and glycated haemoglobin with Na^+/K^+ ATPase activity in the biomembranes. The present study was designed and carried out to test this hypothesis in a cross sectional observational study.

MATERIALS AND METHODS:

Study design:

It was a hospital based non interventional, case-control observational study.

Selection of cases and control subjects:

Cases were selected from the type 2 Diabetes mellitus (DM) patients attending the Department of Medicine in this tertiary care medical college of Kolkata by the method of convenience. After attaining proper clinical history, final inclusion was made after confirming the diagnosis of type 2 DM by estimation of fasting blood glucose (FBG) and glycated haemoglobin (HbA1c) in the Dept. of Biochemistry. Patients suffering from any other metabolic, endocrinological or chronic diseases, any malignant or premalignant condition, any chronic inflammatory diseases, any history of addiction to alcohol and use of tobacco in any form or any other drug were excluded from the study. Pregnant population were excluded to avoid the pregnancy associated endocrinological changes.

Control subjects were selected from the same geographical area with similar socioeconomic and nutritional status in age and sex matched way during the same period. Subjects falling between 25to 50 years were considered for both case and control selection. Following these inclusion and exclusion criteria 60 subjects were finally selected in each case and control group each within a study period of July 2015 to June 2016.

Ethical consideration: Informed consent were taken from patients and control. The study was initiated after getting written consent from the properly constituted institutional ethical committee.

Measurement of study parameters:

A) *Measurement of Na+K+ATPase activity in RBC membrane: This* was done in two steps:

Step I: Preparation of membrane suspension from RBC hemolysate:
The packed red cells were extracted by centrifugation at 4°C, 450 g for 15min and was resuspended and diluted in 25 volumes of 0.011 mol/L Tris-HCl buffer at pH 7.4. The haemolysed cells was centrifuged for 30

min at 12,000 rpm at 4^0 C and the membrane pellet was resuspended in 30 ml of 0.011 mol/L Tris-HCl buffer. This centrifugation step was repeated three times. The membrane suspension obtained was stored at -80^0 C until the assay will be performed.

Step II Measurement of Na+K+ATPase Activity:
A reaction mixture consisting of 1.15 ml of Tris HCl buffer (pH 7.4), 0.15 ml of ionic solution (30 mmol/L $MgSO_4$, 1 mol/L NaCl, 200 mmol/KCl), 0.025 ml of NADH (10mg/ml), 0.015 ml of pyruvate kinase (3 units), 0.025 ml of phosphoenolpyruvate (0.1 mol/L), 0.005 ml of LDH and 0.045 ml of deionized water was prepared. The reaction mixture was put in a UV spectrophotometer and the baseline was established by measuring the extinction at 340 nm for 1-2 min. At the end of this time, 50µl of suitably prepared sample was added and the reaction was initiated by adding 100µl of sodium ATP solution (0.1 mol/L). Once a good linear rate was established, 100µl of ouabain (10 mmol/L) was added and the rate was measured after this addition for 3 minutes and ΔA/minute was calculated.

The Na+K+ATPase activity was calculated from the differences between two ΔA/minute .Once the difference between the above two rates were measured, the enzyme activity was calculated as follows:

IV) Calculation of enzyme activity:
ΔA/minute for Na+K+ATPase= ΔA/minute before addition of ouabain - ΔA/minute after addition of ouabain.

ØΔA/minute for Na+K+ATPase obtained was then multiplied with 50 to convert it into nmol of ADP.

The final value was expressed in units or milli units (1µmol/min=1 Unit) per mg of tissue protein obtained by using the Lowry's method.

B) Measurement of serum Insulin and HOMA-IR:
Serum insulin levels were measured using the technique of ELISA with the ELISA kit obtained from Accubind, USA. HOMA-IR was calculated using the HOMA calculator from the values of serum insulin and FBG.

C) Measurement of glycated haemoglobin: HbA1c was measure by the technique of latex immunoturbidimetry method in the autoanalyser obtained from Konilab, Scalvo.
D) *Quality control of study procedure:* Coefficient of variation (CV) was monitored for the precision of each assay and was found to be limited within 5 percent.

RESULTS:
After obtaining the data from both the case and control groups, statistical significance for the differences between their mean values and strength of association were calculated using the independent t test, bivariate correlation analysis and multiple linear regression analysis through SPSS software version 20 for Windows.

Table 1: Group statistics showing the distribution of mean values of study parameters in cases (n = 60) and control groups (n = 60)

Parameters	Case (Mean ± SD)	Control (Mean ± SD)	T value	P value
Na+/K+ ATPase activity in mIU/mg of tissue protein	2.65 ± 1.40	7.31 ± 2.09	- 14.39	<0.001*
Fasting Blood Glucose in mg/dl	196.7 ± 55.9	89.4 ± 6.6	13.4	<0.001*
HOMA-IR	8.18 ± 2.87	1.08 ± 0.66	18.63	<0.001*
HbA1c	8.19 ± 1.20	5.27 ± 0.35	17.93	<0.001*

*P value significant at P < 0.05 for 95% confidence interval.
The Table 1 shows the results of independent t test performed between the cases and controls groups in all parameters. Na+/K+ ATPase activity in mIU/mg of tissue protein in case group was significantly decreased in respect to the control group. In contrast, other parameters like HOMA-IR and HbA1c were found to be significantly increased in the case group.

To observe the strength of association between different parameters in the case group Pearson bivariate correlation analysis was performed. It was observed that Na+/K+ ATPase activity was significantly negatively associated with HOMA-IR and HbA1c values. On the other hand, glycated haemoglobin and insulin resistance were significantly positively related to each other.

Table 2: Bivariate Pearson's correlation study showing the relationship between individual study parameters in the case group (n = 60).

		Na+/K+ ATPase activity in mIU/mg of tissue protein	HOMA-IR	HbA1c
Na+/K+ ATPase activity in mIU/mg of tissue protein	r value	1	-0.353*	-0.266*
	P value		0.006	0.040
HOMA - IR	r value	-0.353*	1	0.530*
	P value	0.006		<0.001
HbA1c	r value	-0.266*	0.530*	1
	P value	0.040	<0.001	

*P value significant at P < 0.05 for 95% confidence interval.

After observing the bivariate association between the study parameters, we proceeded to determine the predictive significance of glycated hemogolobin and insulin resistance on the sodium pump activity through multiple linear regression analysis (Table 3).

Table 3: Simple multiple linear regression analysis showing the dependence of Na+/K+ ATPase activity on other study parameters taken together in the case group (n = 60)

Model	Beta(β)	t value	P value
Constant		4.087	<0.001
HOMA-IR	-0.295	-2.030	0.047*
HbA1c	-0.110	-0.758	0.451

*P value significant at P < 0.05 for 95% confidence interval.

Regression analysis in table no. 3 revealed that although both HbA1c and HOMA-IR showed negative predictive value on the Na^+/K^+ ATPase activity, only that of the HOMA-IR was significant statistically (P = 0.047).

DISCUSSION:
Present study shows, significant reduction in the level of Na^+/K^+ ATPase activity in the type 2 DM patients showing increased insulin resistance and long term hyperglycemia as shown by elevated HbA1c values. Na^+/K^+ ATPase play a central role in the regulation of intra- and extracellular cation homeostasis. So alteration of this transport enzyme is thought to be linked to several complications of DM, like hypertension, nephropathy, peripheral neuropathy, and microangiopathy. Insulin plays a major role in regulation of Na^+/K^+ ATPase activity. Insulin stimulates Na^+/K^+ ATPase activity and induces translocation of Na^+/K^+ ATPase molecules to the plasma membrane. Different insulin regulated intracellular signalling pathways like PI-3K and MAP Kinase play a significant role in controlling Na^+/K^+ ATPase activity.Reduced level of insulin signalling due to development of insulin resistance in type 2 diabetes mellitus is thought to an important cause of decreased sodium pump activity(6-8). Insulin mainly activates the α1 subunit of Na^+/K^+ ATPase pump through different intracellular insulin regulated signalling pathways. Insulin not only promotes translocation of Na^+/K^+ ATPase α1 and α2 subunits to the plasma membrane but it also stimulates α-subunit phosphorylation on serine, threonine and tyrosine residues through protein kinase C(9). Development of insulin resistance in type 2 DM leads to alteration in intracellular insulin mediated signalling pathways which affect the pump activity.

In the present study FBG has been found to be elevated significantly in the case group (Table 1: P < 0.001). Increased IR almost always leads to a persistent hyperglycemia. Hyperglycemia leads to glycation of several tissue proteins, lipids and nucleic acids. Chronic hyperglycaemia during diabetes leads to non-enzymatic glycation of proteins to form advanced glycation end products (AGEs) that contribute to development of different complications. HbA1c gives an indication of chronic hyperglycaemia and is measured primarily to

identify the 3 months average plasma glucose concentration. These structural or enzymatic changes of different proteins resulting from specific glycosylation reactions lead to different complications of type 2 DM. In the present study the effect of glycation on sodium pump activity is observed and the result showed a significant negative correlation between the Na^+/K^+ ATPase activity and HbA1c (Table 2: r = -0.266, P = 0.040).

The Table 2 also showed a significant inverse relationship between the HOMA-IR and sodium pump activity (r = 0.353, P = 0.006). An increased HOMA-IR indicates persistent hyperglycemia that plays a significant contributory role in reducing the sodium pump activity. It has been reported that high glucose levels activate protein kinase C (PKC) which will then mediate the activation of phospholipase A2 (cPLA2) to increase the production of eicosanoid PGE_2 causing an inhibition of Na^+/K^+ ATPase activity (10). Hyperglycemia induced PKC activation can also inhibit Na^+/K^+ ATPase activity by decreasing the synthesis and release of endothelium-derived nitric oxide (11). Other mechanisms like depletion of intracellular pool of myo-inositol , an increased flux through the aldose reductase pathway may also contribute in decreasing the sodium pump activity (12). Excessive production of oxygen free radicals, formation of glycated proteins and the disturbance in metabolism of nerve growth factor in type 2 DM are also responsible for reduced sodium pump activity (13).

Although both of the HOMA-IR and HbA1c showed significant negative correlation with the Na^+/K^+ ATPase activity in bivariate correlation analysis (Table 2), HOMA-IR was found to be the more significant predictor of Na^+/K^+ ATPase activity than the HbA1c (Table 3: Regression coefficient β = -0.293 and -0.110 respectively). These findings suggest that persistently elevated insulin resistance is more specifically linked to derangement in the sodium pump activity than the glycated haemoglobin. The difference between the predictive values of these two indicators of hyperglycemia can be explained by the fact that association of HOMA-IR with increased blood glucose levels is more intricate across the total time span of the whole disease process than the HbA1c levels that mainly represents a periodic elevation of blood glucose level for about 2 to 3 months.

In conclusion, it can be opined that in spite of marked increase in both glycated haemoglobin and insulin resistance in type 2 DM, the latter determines the membrane dynamics of receptor proteins and transporter like the Na^+/K^+ ATPase pump more effectively. Hence, we propose that HOMA-IR being easily measurable can be used as an important cost effective parameter for monitoring the cellular complications found in type 2 DM

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