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ELECTROLYTES AND SODIUM TRANSPORT MECHANISM IN DIABETES MELLITUS

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The metabolic derangements and disturbances and their consequences in diabetes mellitus are well known more or less in details too. However, knowledge on the diabetic disorders in membrane functions and transport mechanisms is limited which is an essential factor in progression of the disease. Serum electrolytes were measured by flame photometer (Corning 410) and spectrophotometer (Spectro SC) in 60 diabetic patients with stable glycemic control (aged 38 $\pm$ 2.5 years) and in 60 age-matched normal subjects with no known history of hyperglycemia as control. Erythrocytes were isolated from samples, washed and used for the estimation of sodium and potassium concentrations using flame photometer. Erythrocyte membranes were prepared for the estimation of Na^+-K^+-ATPase activity in terms of inorganic phosphate released/mg protein/hour. Na^+-K^+-ATPase activity, Intra-erythrocyte potassium and serum magnesium levels were significantly low in diabetic patients than in the controls. Serum and intra-erythrocyte sodium and serum potassium levels were increased significantly in patients as compared to control subjects. A significant effect of sex and interaction was observed on serum sodium, potassium and magnesium. A significant effect of sex, disease and interaction on red cell sodium, potassium and Na^+-K^+-ATPase activity was observed in male and female subjects. Na^+-K^+-ATPase dysfunction and changes in intra-erythrocyte and serum sodium, potassium and magnesium induced by diabetes may be implicated in the pathogenesis of neuropathy, nephropathy and vascular diseases in humans. It is suggested that male diabetic patients are at high risk of diabetic complications than females.

Keywords: Sodium, Na^+-K^+-ATPase, potassium, magnesium, diabetes mellitus.

INTRODUCTION

The pathogenesis of neuropathy, nephropathy, vascular diseases and other late degenerative complications of diabetes remain unresolved. Metabolic derangement thought to be responsible for their developments are induced by chronic hyperglycemia. However role of hyperglycemia and hyperinsulinemia in the pathogenesis of these metabolic changes are unknown. The metabolic disturbances and their consequences in diabetes mellitus are well known but still our knowledge on the diabetic disorders in membrane functions are limited (Somogyi *et al.,* 2001).

In insulin dependent diabetes mellitus (IDDM) it has recently been reported that diabetic neuropathy occurs sometimes in family clusters. In addition neuropathy has been reported to be more common in patients with family history of hypertension. These observations indicate that susceptibility to neuropathy cannot be explained solely by differences in metabolic control and that genetic predisposition to hypertension may be an important factor in the development of diabetic neuropathy (Jannot *et al*., 1999).

Recent studies indicate that most diabetic complications like neuropathy, nephropathy and hypertension are vascular originated. It has been reported that there is an inverse relationship between serum sodium and potassium levels in diabetic coma. This dissociation may be based on the movement of electrolytes between intra and extra cellular space dependent on impaired insulin action (Saito *et al*., 1999).

The red cell Na^+-K^+-ATPase plays a central role in the regulation of intra and extra cellular cationic homeostasis. Alterations of this transport enzyme are thought to be linked to several complications of diabetes mellitus (Totan and Greaby, 2002). In diabetic animals Na^+-K^+-ATPase activity is decreased in various tissues including erythrocytes. A decrease in this enzyme activity has been implicated in the pathogenesis of diabetes poly neuropathy.

Ca^+-Mg^{++}-ATPase is an important regulator of intracellular calcium and magnesium concentration and therefore of erythrocyte deformability (Midgalis *et al*., 2000). In clinical practice diabetes mellitus is the most significant cause of hypomagnesaemia and magnesium depletion (Rattanata-yarom *et al.,* 2001).

The aim of the present study is to evaluate the potentiality of red cell transport mechanism with reference to sodium and other electrolytes as a marker to progression of diabetes mellitus especially to nephropathy, neuropathy and vascular disease.

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MATERIALS AND METHODS

Study population

Sixty diabetic patients with stable glycemic control and with out intercurrent illness or sever diabetic complications were studied on the occasion of a regular follow-up after informed consent was obtained. Twenty of these patients were women, their mean age was 38±2.5 years. None were taking any medication known to influence Na^+-K^+-ATPase, such as drugs like calcium blockers, thyroxin, glucocorticoid, mineralocorticoid or digitalis. Sixty age matched healthy normal subjects with no known history of hyperglycemia were selected as controls.

Sample collection

Fasting blood samples were collected from control and diabetic subjects in lithium heparin coated tubes. A portion of blood was collected to obtain serum.

Intra-erythrocyte sodium and potassium estimations

Heparinized blood was centrifuged and plasma was separated. Buffy coat was aspirated and discarded. Erythrocytes were washed three times at room temperature by suspension in the magnesium chloride solution (112 mmol/L), centrifugation at 450 x g at 4°C for 5 minutes and aspiration of the supernatant as described earlier (Fortes and Starkey, 1977). Final supernatant was retained for the estimation of intra-erythrocyte sodium and potassium concentration, neither electrolyte was detectable in the final wash. Washed erythrocytes were then lysed and used for the estimation of intra-erythrocyte sodium and potassium (Tabassum., *et al* 1996).

Erythrocyte membrane preparation

The red cell pack extracted by centrifugation at 4°C were resuspended and diluted in 25 volumes of tris buffer at pH 7.4. The hemolyzed cells were then centrifuged at 12,000 rpm at 4°C and the membrane pellet was suspended in 30 ml of 0.11 mol/L tris-HCl buffer. This centrifugation step was repeated three times. The final concentration of the membrane suspension was ~4 mg protein/ml of Tris buffer. The membrane suspension was stored at –80°C until the assay was performed.

Erythrocyte Na^+-K^+-ATPase activity measurement (Raccah et al., 1996)

ATPase activity was measured in a final volume of 1 ml as follows: Membrane (400ug) was preincubated for 10 minutes at 37°C in a mixture containing 92 mmol/L tris-HCl (pH=7.4), 100 mmol/L NaCl, 20 mmol/L KCl, 5 mmol/L $MgSO_4$ .H_2O and 1 mmol/L EDTA. Assays were performed with and without 1mmol/L ouabain, a specific inhibitor of Na-K-ATPase. After incubation with 4 mmol/L ATP (vanadate free, Sigma) at 37°C for 10 minutes, the reaction was stopped by adding ice-cold trichloroacetic acid to a final concentration of 5%. After centrifugation at 4°C, 5500g for 10 minutes. The amount of inorganic phosphate in the supernatant was determined (Dryer and Tammes, 1957). Na^+-K^+-ATPase activity was calculated as the difference between inorganic phosphate released during the 10 minute incubation with and without ouabain. Activity was corrected to a nanomolar concentration of inorganic phosphate released/milligram protein/hour. The concentration of protein was estimated by Biuret method.

All assays were performed in duplicate, and blanks for substrate, membrane and incubation time were included to compensate for endogenous phosphate and non-enzyme related breakdown of ATP. Under these experimental conditions, the coefficient of variation was 7.5%.

Serum electrolyte estimation

Serum sodium and potassium were estimated by flame photometer (Corning 410). Serum magnesium was estimated by the method of Hallry and Sky Peck, 1964).

Statistical analysis

Results are presented as mean ± SD. Statistical significance and difference from control and test values evaluated by Student's t-test. Two way ANOVA was utilized to analyze the effect of sex, disease and interactive effect of sex and disease. Post-hoc analysis was done by using Newman Keul's test.

RESULTS

Results are presented in tables 1-4. Table 1 shows concentration of sodium, potassium and magnesium in serum of diabetic and control subjects. Sodium was elevated (142.07 ± 42.02 vs 136.82 ± 16.22 mEq/L) in diabetic patients but not significantly whereas potassium was increased significantly (7.41 ± 1.8 vs 5.03 ± 1.5 mEq/L) ($p<0.005$) but serum magnesium level was found significantly decreased in diabetic patients (0.84 ± 0.31 vs 1.24 ± 0.68 mmol/L) ($p<0.0005$) as compared to control subjects.

Intra-erythrocyte sodium levels were increased significantly in diabetic patients as compared to control group (15.91±7.02 vs 9.44±3.47 mEq/L) ($p<0.05$) (table 2). The levels of intra-erythrocyte potassium were decreased in diabetic group (100.92±25.62 vs 108.43±28.05 mEq/L) (table-2) but this difference was not significant. Erythrocyte Na-K-ATPase activity was significantly lowered in diabetic patients than in the control group (100.6 ± 43.8 vs 443.4 ± 282.2 nmol pi/mg protein/hr) ($p<0.0005$) *(*table 2).

Two way ANOVA showed a significant ($p<0.01$) effect of sex, disease and interaction on red cell sodium, potassium and Na^+-K^+-ATPase activity in male and female subjects. Membrane Na^+-K^+-ATPase activity was decreased in both

male and female diabetic patients but the decrease was more pronounced ($p<0.01$) in male patients than in females (table-3).

Table 1
Serum Na^+, K^+ and Mg^{++} in control and diabetic subjects

	Control	Patients
Serum Na^+ (mEq/L)	136.82±16.22	142.07±42.02
Serum K^+ (mEq/L)	5.03±1.5	7.41 ± 1.8*
Serum Mg^{++} (mmol/L)	1.24±0.68	0.84±0.31**

*P < 0.005, ** P < 0.0005

Table 2
Intra-erythrocyte Na^+, K^+ and Na^+-K^+-ATPase activity in control and diabetic subjects

	Control	Patients
RBC Na^+ (mEq/L)	9.44±3.47	15.91±7.02 *
RBC K^+ (mEq/L)	108.43±28.05	100.92±25.62
Na^+-K^+-ATPase activity (nm Pi/mg protein/hr)	443.4±282.2	100.6±43.8**

*P < 0.05, ** P < 0.0005

A significant ($p<0.01$) effect of sex and interaction was observed on serum sodium (table 4). The effect of disease was non significant. The effect of sex disease and interaction was found to be significant ($p<0.01$) in both serum potassium and serum magnesium (table 4).

DISCUSSION

The increased intra-erythrocytes sodium and serum potassium levels with decreased intra-erythrocyte potassium and serum magnesium in diabetic subjects is a consequence of decreased Na^+-K^+-ATPase activity as observed during the presented study (tables 1 & 2). Na^+-K^+-ATPase is a ubiquitous enzyme that ensures that the transmembrane gradients of sodium and potassium concentrations are maintained. Alterations of this transport enzyme are thought to be linked to several complications of diabetes mellitus (Totan and Greaby, 2002). In humans, this enzyme activity is mainly studied in the erythrocyte membranes because these cells are easily accessible. A significant decrease has been reported previously in uncontrolled diabetic type I patients (Issautier *et al.,* 1994). This enzyme dysfunction is probably connected with the relative insulinopenia of hyperglycemic diabetic type I patients. Intensive insulin therapy from an artificial pancreas for 24 hr restores erythrocyte Na^+-K^+-ATPase activity in diabetic patients (Raccah *et al.,* 1994). In another connection disturbances of the membrane lipid organization can also explain the decease in Na^+-K^+-ATPase activity (Ruiz-Guttierez *et al.,* 1993). A dysfunction of Na^+-K^+-ATPase is implicated in the pathophysiology of the diabetic neuropathy. A decrease of this enzyme activity (as observed in the present study) induces an increase of the intra axonal sodium concentration, disturbing the depolarization and leading to a decrease in nerve conduction.

Table 3
Sex differences in intra-erythrocytes Na^+, K^+ and Na^+-K^+-ATPase activity in diabetes mellitus

	Male		Female		Two Way ANOVA		
	Control	Test	Control	Test	*Sex*	Disease	Sex X Disease
RBCs Na^+	9.19 ± 3.48	15.53** ± 7.2	9.47 ± 3.26	15.53^{+}** ± 6.69	F=323 P<0.01	F=39.8 P<0.01	F=324 P<0.01
RBCs K^+	102.44 ± 24.92	97.56 ± 19.6	109.47 ± 24.18	99.4 ± 23.02	F=1268 P<0.01	F=3.1 P<0.01	F=1270 P<0.01
Na^+/K^+ ATPase	435.84 ± 320.7	91.29 ± 51.5	450.93 ± 248.5	116.69^{++}** ± 40.7	F=60 P<0.01	F=101 P<0.01	F=60 P<0.01

Values are mean ± SD (n=120)
Significance difference by Newman Keuls test;
From respective controls **p<0.01, *p<0.05
From similarly treated male subjects +p<0.05, following two way ANOVA ++p<0.01

Table 4
Sex differences in serum Na^+, K^+ and Mg^{++} in diabetes mellitus

	Male		Female		Two way ANOVA		
	Control	Test	Control	*Test*	Sex	Disease	Sex X disease
Serum Na^+	132.32 ± 17.82	142.67 ± 45.20	139.87 ± 14.03	145.65 ± 31.72	F=1411 P<0.01	F=2.4 P<0.25	F=1412 P<0.01
Serum K^+	5.06 ± 1.22	7.45** ± 1.85	4.98 ± 1.78	7.37** ± 1.81	F=87 P<0.01	F=66 P<0.01	F=87 P<0.01
Serum Mg^{++}	1.16 ± 0.48	0.87 ± 0.41	1.34 ± 0.84	0.81 ± 0.2	F=149 P<0.01	F=9.4 P<0.01	F=151 P<0.01

Values are mean ± SD (n=120)
Significance difference by Newman Keuls test;
From respective controls **p<0.01, *p<0.05
From similarly treated male subjects +p<0.05, following two way ANOVA ++p<0.01

Previous studies also showed a correlation between erythrocyte Na^+-K^+-ATPase activity and certain electrophysiological parameters of the peripheral nerves. This suggests that enzyme activity in the red cell membrane reflects that of the peripheral nerve in diabetic humans and also in animals (Raccah *et al.,* 1994).

The hypertension that frequently accompanies diabetes mellitus is characterized by abnormalities of sodium metabolism at all physiologic levels, whole body renal and cellular. The most consistently described abnormality is an expansion of exchangeable sodium, which seems to be closely associated with increased proximal renal tubular sodium reabsorption and suppression of membrane sodium-potassium ATPase activity together with inadequate stimulation of the pump leads to a transitionary sodium cell retention as also observed in present study. Although recent interest has focused almost exclusively on the role of hyperinsulinemia promoting sodium retention and causing expansion of total body sodium and sodium-dependent hypertension (Weder, 1994).

The decrease in the Na^+-K^+-ATPase activity also implicated in the development of vascular diseases in diabetic patients. Previous studies showed that renin angiotensin involvement especially changes in angiotensin converting enzyme (ACE) activity level are considered to be a key factor in the diabetic nephropathy. Since ACE converts angiotensin I to angiotensin II which is a potential vasoconstrictor and plays a vital role in the regulation of blood pressure. Previous studies have indicated that hyper reactivity of adrenal rennin angiotensin system under hypereninemia is associated with sever diabetes mellitus (Ustundag *et al.,* 2000; Biwititi *et al.,* 2000; Nakayama *et al.,* 1998).

Intracellular potassium depletion is a common feature in type II diabetes mellitus and fasting steady state levels are closely linked to calcium and magnesium homeostasis (Resnick *et al.,* 2001). Diabetics differ markedly in their erythrocyte reactions regarding potassium permeability where as patients with renal insufficiency show an efflux of potassium during investigation as there is a decrease of potassium concentration in plasma in diabetic patients (Kraat *et al.,* 1997).

Magnesium, the second most abundant intracellular cation involved in a number of important biochemical reactions, including all ATP-transfer reactions. Possibly because of its relevance to all protein kinases, magnesium appears to mediate hormonal as well as other aspects of cellular glucose utilization (Reinhart, 1988). There was significant decreased level of magnesium found in diabetic patients in the present study as described by various previous workers. Magnesium depletion is a common feature of diabetes mellitus, apparently related to glycemic control (Djurhuus *et al.,* 2000). The magnesium deficiency that has been demonstrated in insulin-resistance such as hypertension and type II diabetes mellitus may thus contribute to suppress glucose metabolism and insulin action (Resnick, 1999; Paolisso and Barbagallo, 1997).

Results show a significant effect of sex on red cell sodium, serum potassium, and magnesium and Na^+-K^+-ATPase activity. A pronounced decrease in Na^+-K^+-ATPase activity in male than in female diabetic patients suggests that male patients are at high risk of diabetic complications.

In conclusion, the results reported here suggest that Na^+-K^+-ATPase dysfunction and changes in intra-erythrocyte and

serum sodium and potassium induced by diabetes is implicated in the complications of diabetes mellitus most commonly in the pathogenesis of neuropathy, nephropathy and vascular diseases in humans. It is also suggested that male patients are at high risk of diabetic complications than female patients.

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