

RELATIONSHIPS BETWEEN ERYTHROCYTE Na^+/K^+ -ATPase ACTIVITY, OXIDATIVE STRESS AND HIGH BLOOD PRESSURE IN HYPERTENSIVE DIABETIC PATIENTS

ALEXANDRA TOTAN^{1*}, MARIA GREABU¹, NICULINA MITREA²,
MARIA MOHORA³, ANDREEA LETIȚIA ARSENE²

¹University of Medicine and Pharmacy "Carol Davila", Faculty of Dental Medicine, Department of Biochemistry

²University of Medicine and Pharmacy "Carol Davila", Faculty of Pharmacy, Department of Biochemistry

³University of Medicine and Pharmacy "Carol Davila", Faculty of Medicine, Department of Biochemistry

*corresponding author: alexandratotan@yahoo.com

Abstract

Diabetes mellitus is a chronic disease frequently associated, at molecular level, with an imbalance between reactive oxygen species (ROS) generation and antioxidant defence systems in the body. ROS production can damage arterial walls including an impairment of the endothelium-dependent vasodilatation or endothelial dysfunction. Na^+/K^+ -ATPase is an antioxidant plasma membrane-associated protein complex whose activity has been found to be altered in various cell types under diabetic conditions. The aim of the present study was to test if there are relationships between the activity of erythrocyte Na^+/K^+ -ATPase and high blood pressure in the context of diabetes mellitus.

Rezumat

Diabetul zaharat este o afecțiune cronică, deseori asociată, la nivel molecular, cu dezechilibrul între producerea speciilor oxigen reactive și sistemele antioxidante, protectoare ale organismului. Speciile oxigen reactive alterează, structural și funcțional, endoteliul vascular. Na^+/K^+ -ATPaza este un complex enzimatic membranar, a cărei activitate este modificată în cazul diabetului. Studiul descris în această lucrare și-a propus corelarea activității Na^+/K^+ -ATPazei eritrocitare cu hipertensiunea arterială, în cazul diabetului zaharat.

Keywords: erythrocyte Na^+/K^+ -ATPase, high blood pressure, diabetes mellitus

Introduction

Diabetes mellitus is a chronic disease frequently associated, at molecular level, with an imbalance between reactive oxygen species (ROS) generation and antioxidant defence systems in the body. ROS production can damage arterial walls including an impairment of the endothelium-

dependent vasodilatation or endothelial dysfunction [7]. The interaction of ROS with biological membranes produces a variety of functional modifications due to either direct interaction with the molecular mechanisms or to oxidative modification of the environment of biological macromolecules [8]. Lipid peroxidation in the context of hyperglycaemia contributes to the loss of cellular functions through the inactivation of membrane enzymes and cytoplasmatic proteins [9, 12].

Na^+/K^+ -ATPase is an antioxidant plasma membrane-associated protein complex whose activity has been found to be altered in various cell types under diabetic conditions. Na^+/K^+ -ATPase couples the energy released from the intracellular hydrolysis of ATP to the transport of cellular ions, a major pathway for the controlled translocation of sodium and potassium ions across the cell membrane. Therefore, Na^+/K^+ -ATPase controls directly or indirectly many essential cellular functions, for example, cell volume, free calcium concentrations, and membrane potential [1, 2, 5]. It is a member of the P-type family of ATPases and is closely related to the Ca^{2+} -ATPase family and the H^+/K^+ -ATPase. Na^+/K^+ -ATPase is composed of two major subunits, the catalytic and the glycosylated subunit.

The aim of the present study was to assess if there are relationships between the activity of erythrocyte Na^+/K^+ -ATPase and high blood pressure in the context of diabetes mellitus [11, 12].

Materials and methods

Patients

This study included two groups of patients:

- 30 hypertensive diabetic patients between 45 and 65 years old;
- 20 healthy normotensive non-diabetic subjects, between 30 and 45 years old.

All patients gave their informed consent prior to joining this study.

For the first studied group we recruited hypertensive diabetic patients that were not under pharmacological treatment for hypertension. Inclusion criteria were diabetic patients with blood glucose higher than 140 mg/dL and with a blood pressure higher than 135 mm Hg systolic and 80 mm Hg diastolic. Exclusion criteria were smoking, heart, liver or kidney disease, and the use of any current medication.

The second studied group (control group) included normotensive volunteer subjects. Potential participants were subjected to clinical history evaluation, physical examination, and laboratory screening (serum glucose,

glycated haemoglobin, creatinine, cholesterol, triglycerides, high-density lipoproteins (HDL), aspartat aminotransferase (AST), alanin transaminase (ALT) and microalbuminuria) in order to ensure that both normotensive subjects and hypertensive diabetic patients met the inclusion and exclusion criteria.

Blood pressure levels were determined through ambulatory blood pressure monitoring on a regular workday (three times during 24 h from 8:30AM). The mean day-time value of the blood pressure was registered.

Sample collection

Venous blood samples (<10 mL) were collected for preparation of red blood cell lysates (in vacutainers containing disodium EDTA), of erythrocyte membranes (in vacutainers containing heparin) and for preparation of serum. All blood samples were analysed the same day they were collected.

Erythrocyte membranes were isolated by ultra-centrifugation at 10000 g for 45 min and stored in Eppendorf tubes containing TRIS – HCl buffer (pH 7.4) at -75°C until used for erythrocyte $\text{Na}^{+}/\text{K}^{+}$ -ATPase activity measurements.

Biochemical methods

The activity of erythrocyte $\text{Na}^{+}/\text{K}^{+}$ -ATPase was measured by the method of Vague et al [12]. The assay mixture consisted of 140 mM NaCl, 28 mM KCl, 3mM ATP (vanadium free), 10mM MgCl_2 and 10 mM EDTA buffer (pH=7.4).

$\text{Na}^{+}/\text{K}^{+}$ -ATPase activity was calculated from the difference between the amount of inorganic phosphate released in the presence and in the absence of 1mM ouabain in the incubation medium. Protein concentration in the membrane suspension was measured by the Lowry method. The specific activity was expressed as micromol inorganic phosphate released (Pi) /mg protein/h.

Blood glucose, glycated haemoglobin, total cholesterol, triglycerides, high density lipoproteins, creatinine, ALT, AST were performed using an automatic biochemistry analyser using analysing kits from Diasys (Germany). Glutathione peroxidase and glutathione reductase were performed on the same analyser using analysing kits from Randox (UK). For testing microalbuminuria we used qualitative tests from Dialab (Austria).

Statistical analysis

The source of variation between the control group and the hypertensive diabetic subjects was assessed by the unpaired Student t-test, for normally distributed parameters, $p < 0.05$ for statistical significance. The association of variables was studied by the Pearson correlation test due to their Gaussian distribution.

Results and discussion

Biochemical and clinical characteristics for the two studied groups of patients are shown in Table I. Data are expressed as means $\pm$ SD.

Table I
Biochemical and clinical characteristics of hypertensive diabetic patients (n = 30) and healthy normotensive subjects (n = 20)

Parameter	Control group	Hypertensive diabetic group	p
Blood glucose(mg/dL)	91 $\pm$ 15	178 $\pm$ 23	< 0.05
Glycated haemoglobin (HbA1c) (%)	5.1 $\pm$ 0.3	8.49 $\pm$ 0.5	< 0.05
Creatinine (mg/dL)	0.8 $\pm$ 0.05	0.82 $\pm$ 0.06	> 0.05
Albuminuria	negative	negative	
Total cholesterol (mg/dL)	186 $\pm$ 12	230 $\pm$ 34	< 0.05
High density lipoproteins (HDL) (mg/dL)	47.95 $\pm$ 8	35.8 $\pm$ 7.1	< 0.05
Triglycerides (mg/dL)	60 $\pm$ 18	155.5 $\pm$ 16.5	< 0.05
Alanin transaminase ALT activity (U/L)	21 $\pm$ 8	24 $\pm$ 12	> 0.05
Aspartat aminotransferase AST activity (U/L)	19 $\pm$ 11	26 $\pm$ 9	> 0.05
Systolic blood pressure (mm Hg)	118.9 $\pm$ 1.4	138.1 $\pm$ 2.4	< 0.05
Diastolic blood pressure (mm Hg)	75.8 $\pm$ 1.8	92.5 $\pm$ 1.7	< 0.05

As it can be seen in table I, there were significant differences ($p < 0.05$) between the two groups regarding the blood glucose, glycohaemoglobin (HbA1c), total cholesterol, triglycerides, high density lipoproteins and the mean day value of blood pressure.

Tabel II and figure 1 present the mean activity of erythrocyte membrane Na^+/K^+ -ATPase in the two groups included in the study.

Table II
The mean activity of erythrocyte membrane Na^+/K^+ -ATPase in the two groups included in the study

Parameter	Control group	Hypertensive diabetic group	p
Na^+/K^+ -ATPase ($\mu\text{mol Pi/mg/h}$)	38.255 $\pm$ 4.1	12.55 $\pm$ 2.02	< 0.05

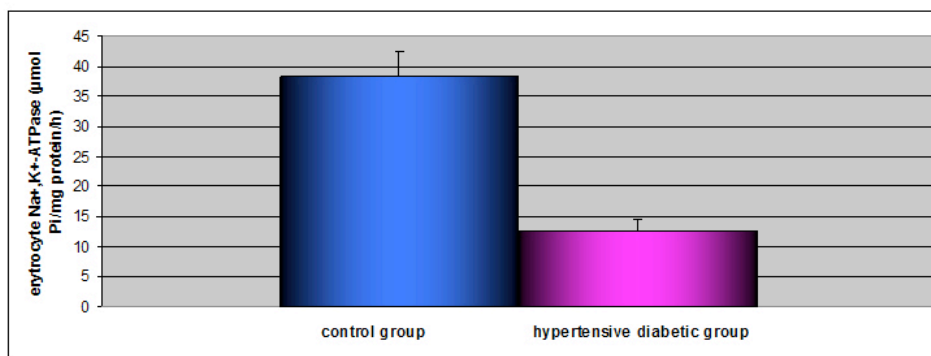


Figure 1

Mean activity of erythrocyte membrane Na^+/K^+ -ATPase in the two groups included in the study

As shown in figure 1 the mean value of Na^+/K^+ -ATPase in the hypertensive diabetic group is significantly decreased ($p < 0.05$) compared with the normotensive healthy group (the control group).

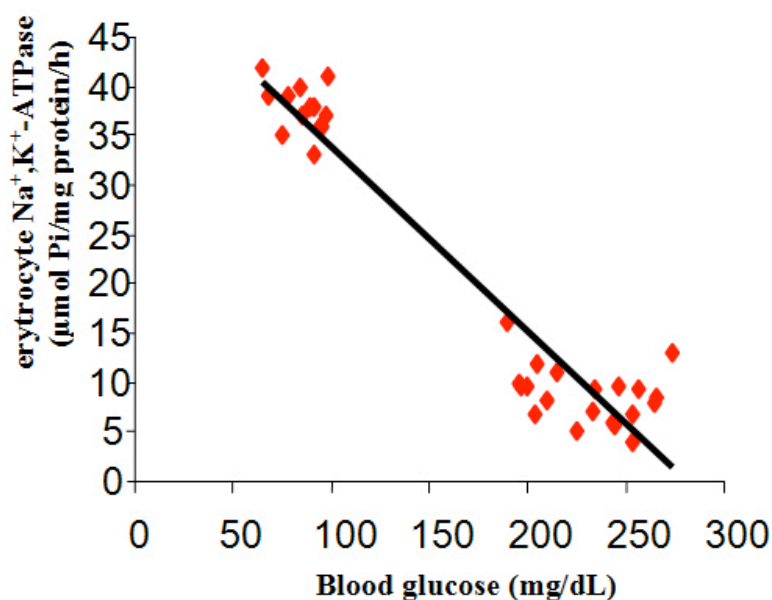


Figure 2

Negative Pearson correlation of erythrocyte Na^+/K^+ -ATPase activity with blood glucose levels in the hypertensive diabetic group ($r = -0.54$; $p < 0.05$)

Figure 2 shows a negative correlation of erythrocyte Na^+/K^+ -ATPase mean activity with blood glucose levels, in the hypertensive diabetic group.

Table III and figures 3 and 4 present the mean activity of erythrocyte

glutathione peroxidase and respectively, glutathione reductase for the two studied groups.

Table III
Mean activity of erythrocyte glutathione peroxidase and glutathione reductase for the two groups

Parameter	Control group	Hypertensive diabetic group	p
Glutathione peroxidase (UI/g Hb)	41 ± 0.5	25.2 ± 0.35	< 0.05
Glutathione reductase (UI/g Hb)	8.0 ± 0.1	4.4 ± 0.09	< 0.05

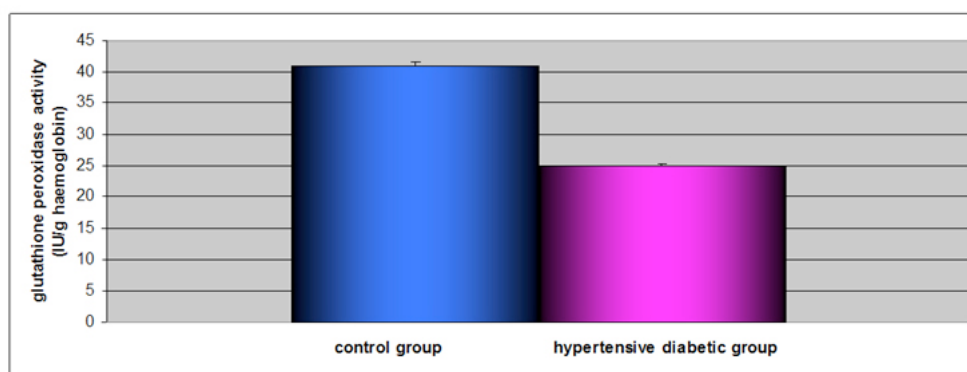


Figure 3

Mean activity of erythrocyte glutathione peroxidase (IU/g haemoglobin)

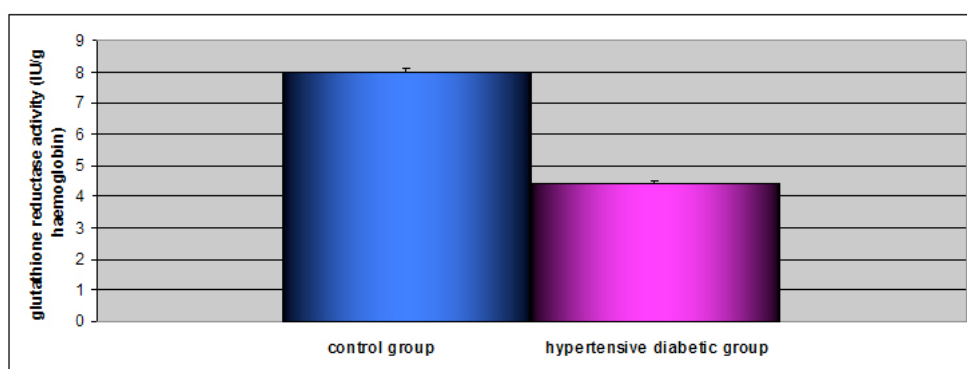
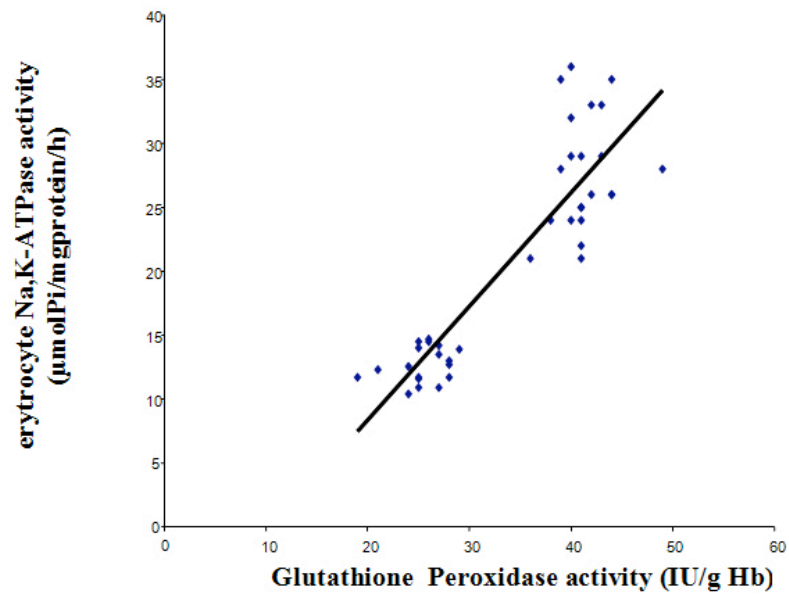


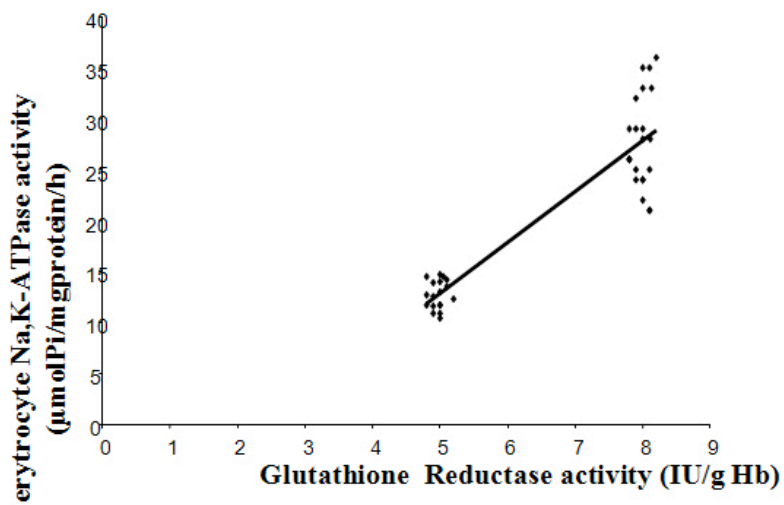
Figure 4

Mean activity of erythrocyte glutathione reductase (IU/g haemoglobin)

Figure 3 and 4 illustrate a statistically significant ($p < 0.05$) decrease of both glutathione peroxidase and glutathione reductase mean activities versus controls.

**Figure 5**

Pearson correlation of erythrocyte Na^+/K^+ -ATPase activity with glutathione peroxidase activity ($r = 0.3$; $p < 0.01$)

**Figure 6**

Pearson correlation of erythrocyte Na^+/K^+ -ATPase activity with glutathione reductase activity ($r = 0.1$; $p < 0.01$)

Furthermore, analysing fig. 5 and 6, it can be observed that the mean activities of erythrocyte glutathione peroxidase and glutathione reductase are positively correlated with the mean activity of Na^+/K^+ -ATPase, in the hypertensive diabetic group.

Conclusions

The findings of the present study confirm previous data reporting the association of diabetes mellitus with both antioxidant status and oxidative stress-related parameters [9, 10, 11].

Furthermore, our data provides a new point of view related to erythrocyte membrane Na^+/K^+ -ATPase activity in hypertensive patients in the context of diabetes mellitus.

The ubiquity of Na^+/K^+ -ATPase in mammalian cells and its function in the vascular wall cells and erythrocyte suggested its important role in hypertension pathogenesis.

The pathophysiology of hypertension involves several abnormalities in cardiovascular homeostatic mechanisms including endothelial and erythrocyte dysfunctions. Reactive oxygen species (ROS) are increasingly implicated (directly and indirectly) in hypertension occurrence. This suggests that understanding and controlling the direct and indirect implications of ROS will help to control mediating vasoconstriction in hypertensive patients.

In diabetes the glycation reaction seems to have broad pathological significance in diabetic complications, because under hyperglycaemic conditions the production of various reducing sugars such as glucose, glucose-6-phosphate, and fructose, increases through glycolysis and the polyol pathway. All of these reducing sugars are known to promote glycation reactions of various proteins. In diabetic animals, glycation is observed extensively in various tissues and organs, and various kinds of glycated proteins such as glycosylated haemoglobin, albumin, and lens crystalline are produced in a non-enzymatic manner through the Maillard reaction. During the reaction which in turn produces Schiff base, Amadori product and advanced glycosylation end products (AGE), ROS are also produced.

Increased ROS production in the context of diabetes mellitus causes dysfunctions of erythrocyte antioxidant protective mechanisms, illustrated in our study by the significant decrease of glutathione peroxidase and glutathione reductase. Defective antioxidant protection leads to an intracellular oxidative stress that causes increased membrane lipids peroxidation with consequences on erythrocyte plasma membrane integrity.

Membrane lipid composition and integrity is very important in modulating the activity of various ion transport pathways via the changes in membrane microviscosity.

Our results showed a significant decrease of erythrocyte membrane Na^+/K^+ -ATPase and a negative correlation of its activity with blood glucose levels, in the hypertensive diabetic group.

There are also previous data reporting reduced erythrocyte membrane Na^+/K^+ -ATPase in diabetic patients.

Our experimental data suggest an involvement of reactive oxygen species (ROS) in the modulation of Na^+/K^+ -ATPase activity and its role in the pathogenesis of blood pressure elevation. Furthermore, an attenuation of Na^+/K^+ -ATPase activity is strongly correlated with decreased erythrocyte deformability in diabetes.

In agreement with this hypothesis, our data demonstrate that the impairment of erythrocyte Na^+/K^+ -ATPase activity is positively correlated with glutathione peroxidase (figure 4) and glutathione reductase, two very important members of the erythrocyte antioxidant protective equipment. Impairment of Na^+/K^+ -ATPase activity could be due to the loss of its optimal interaction with the membrane components, as a consequence of increased lipid peroxidation. Although a direct inhibition by peroxynitrite, as it occurs in liver plasma membranes, should not be discarded.

Nevertheless, other effects are likely to influence the modulation of Na^+/K^+ -ATPase, due to increased lipid peroxidation and/or protein oxidation, and should not be discarded. Thus, it could be speculated that the above mentioned structural membrane alterations may result in an increased exposure of the endogenous cardiac glycoside binding site of the Na^+/K^+ -ATPase. The cardiac glycoside or endogenous ouabain (EO) is a ligand that behaves as a natural regulator of Na^+/K^+ -ATPase, *in vivo* and thus it may play a role in the mechanism of hypertension. The specific binding site of EO has a strong evolutionary conservation among all species. This ligand has the only known receptor in Na^+/K^+ -ATPase, whose *alpha2* isoform, located in the erythrocyte plasma membrane too, can mediate the development of hypertension either through intracellular ion exchange or signaling cascades. Functionally, EO can exert cardiotonic and vasotonic actions dealing with the development of human hypertension. Consequently, it seems reasonable to assume that structural changes induced by lipid peroxidation in the erythrocyte membrane, as a consequence of an increased level of oxidative stress, as well as in other cell types, could result in increased exposure of EO binding sites, thus, contributing to the elevation of blood pressure by the alternative above mentioned mechanism.

The above mentioned considerations could give an explanation to the finding of lower levels of Na^+/K^+ -ATPase activity in hypertensive diabetic patients, compared with the normotensive, non-diabetic participants.

In conclusion, our results suggest that the modulation of Na^+/K^+ -ATPase activity is influenced directly and indirectly by the presence of intracellular oxidative stress, generated in conditions of hyperglycaemia, and provide an interesting role for this enzyme in the pathophysiology of hypertension in diabetes mellitus.

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