Diabetologia (1998) 41: 1080–1084

Diabetologia

Erythrocyte Na/K ATPase activity and diabetes: relationship with C-peptide level

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Summary Erythrocyte Na/K ATPase activity is decreased in Type I diabetic patients; for Type II diabetic patients, literature data are controversial. Therefore, we have compared this enzymatic activity in 81 patients with Type I diabetes mellitus, 87 with Type II diabetes mellitus and 75 control subjects. Mean erythrocyte Na/K ATPase activity was lower in the Type I diabetic patients (285 ± 8 nmol Pi · mg protein^{-1} · h^{-1}) than in the control subjects (395 ± 9 nmol Pi · mg protein^{-1} · h^{-1}) whereas that of the Type II diabetic patients did not differ from that of control subjects. Sex, age, body mass index, and HbA_{1c} levels did not influence erythrocyte Na/K ATPase activity. The 25 Type II diabetic patients treated with insulin, however, had lower Na/K ATPase activity than the 62 on oral treatment (264 ± 18 vs 364 ± 16 nmol Pi · mg protein^{-1} · h^{-1}, $p < 0.001$) but similar to that of Type I diabetic patients. Among the Type II diabetic patients, stepwise regression analysis showed that fasting C-peptide level was the only factor independently correlated with Na/K ATPase activity; it explained 23 % of its variance. In fact, in the insulin-treated patients, those with almost total endogenous insulin deficiency (C-peptide < 0.2 nmol · l^{-1}) had the lower Na/K ATPase activity (181 ± 21 vs 334 ± 17 nmol Pi · mg protein^{-1} · h^{-1}, $p < 0.0001$). The biological effects of treatment with C-peptide have recently led to the suggestion that this peptide could have a physiological role through the same signalling pathway as insulin, involving G-protein and calcium phosphatase and thus restoring Na/K ATPase activity. The relationship we describe between endogenous C-peptide and this activity is a strong argument for this physiological role. [Diabetologia (1998) 41: 1080–1084]

Keywords Na/K ATPase activity, C-peptide, Human erythrocyte, Type I diabetes mellitus, Type II diabetes mellitus.

Na/K ATPase (EC 3.1.6.37) activity is low in various tissues of animals with streptozotocin-induced diabetes [1, 2] and in the erythrocytes of Type I diabetic patients [3, 4]. It has been shown that this impaired enzyme activity plays a role in the pathogenesis of diabetic polyneuropathy [1, 3, 5, 6].

The mechanism leading to the impairment of this enzyme activity in Type I diabetes mellitus is still unclear. It does not seem to be directly linked to hyperglycaemia itself as no correlation has been observed between actual glycaemic or HbA_{1c} levels and erythrocyte enzyme activity in Type I diabetic patients [5, 6]. In diabetic animals, C-peptide stimulates Na/K ATPase activity in renal tubule cells [7]. C-peptide infusion improves some renal and autonomic nerve functions in Type I diabetic patients [8, 9]; this has been attributed to a restoration of Na/K ATPase activity. We therefore hypothesized that the low Na/K ATPase activity in the erythrocyte membranes of Type I diabetic patients could be related, at least in part, to the lack of C-peptide secretion. If this hypothesis is plausible, diabetic patients with persistent C-peptide secretion would have more or less normal Na/K ATPase values. This study was therefore intented to compare erythrocyte Na/K ATPase activity in

Received: 6 November 1997 and in final revised form: 10 April 1998

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Table 1. Characteristics of subjects

	Controls subjects $n = 75$	Type I diabetic patients $n = 81$	Type II diabetic patients whole group $n = 87$	Type II diabetic patients on oral treatment $n = 62$	Type II diabetic patients on insulin treatment $n = 25$
Female/male ratio	58/17	39/42	37/50	26/36	11/14
Age (years)	36.1 ± 1.2	39 ± 1.2	60.7 ± 1	59.2 ± 1.1	64.3 ± 1.9
Diabetes duration (years)		22.5 ± 0.9	14.2 ± 0.9	12.5 ± 1	18.6 ± 1.5
BMI (kg/m^2)	22.8 ± 0.9	23.1 ± 1.5	29.2 ± 0.6	29.4 ± 0.6	28.5 ± 1.2
HbA_{1c} (%)		8.9 ± 0.1	8.8 ± 0.2	8.5 ± 0.2	9.6 ± 0.2
C-peptide concentration ($nmol \cdot l^{-1}$)		undetectable	0.75 ± 0.05	0.88 ± 0.06	0.40 ± 0.1
Neuropathy (absent/present)		50/31	43/44	35/27	5/17
Erythrocyte Na/K ATPase activity ($nmol\ Pi \cdot mg\ protein^{-1} \cdot h^{-1}$)	395 ± 9	285 ± 8	335 ± 13	364 ± 16	264 ± 18

Results as means ± SEM
The * indicates statistically significant difference between the results.

diabetic patients with and without C-peptide secretion. For this purpose, we compared C-peptide negative Type I diabetic patients with a large group of Type II diabetic patients with variable C-peptide levels.

Materials and methods

Study subjects. Subjects were consecutively selected in our unit and were studied after informed consent was obtained. Na/K ATPase activity is influenced by ethnic origin [10] and we have reported previously that erythrocyte Na/K ATPase activity was lower in North-African than in European individuals [3]. Thus, all the subjects were of Caucasian origin to avoid artefactual associations generated by population stratification.

We enrolled 81 patients with Type I diabetes mellitus and 87 patients with Type II diabetes mellitus. None had taken any medication known to influence Na/K ATPase activity (calcium blockers, thyroxine, glucocorticoid or digitalin-like drugs). Type I diabetes mellitus was characterized by diagnosis before 30 years of age, C-peptide negativity, and treatment with insulin. Type II diabetes mellitus was characterized by diagnosis after 30 years of age and more than 3 years without insulin treatment. Of the patients 62 were on oral treatment (biguanides or sulphonylureas or both) and 25 on insulin. In the latter group, the indication for treatment with insulin was either failure to secondary oral agents or severe degenerative complications. We enrolled 168 patients (76 women and 92 men) from 19 to 72 years of age (mean: 50.4 ± 1.1). Mean duration of diabetes was 22.5 ± 0.9 years (range 10–51) in Type I diabetic patients and 14.2 ± 0.9 years (range 3–35) in Type II diabetic patients. As expected Type II diabetic patients were older and heavier than Type I diabetic patients but the degree of glycaemic control estimated by HbA_{1c} value was similar between the two groups.

Absence or presence of neuropathy was defined according to the Diabetes Control and Complications Trial (DCCT) criteria [11]. The criteria consisted of signs, symptoms including numbness, dysesthesias or paresthesias or both, hypersensitivity to touch, burning pain or aching or both, and stabbing pain in hand or feet or both, and decreased or absent deep tendon reflexes. Neuropathy was classified as present if one of the three criteria (signs, symptoms, and reflexes) was observed and absent if none was found. The control group was 75 healthy subjects comprising 58 women and 17 men, enrolled from hospital staff (mean age: 36.1 ± 1.2, range: 18 to 57 years).

All subjects had normal thyrotropin values. Residual endogenous secretion of insulin was estimated by measurement of C-peptide in serum. Subjects had fasted 12 h prior to sampling. Characteristics of the different groups are given in Table 1.

Measurement of erythrocyte Na/K ATPase activity. Venous blood samples were collected from fasting subjects on sodium citrate (0.11 mol) at about 0800 hours before the morning insulin injection. Immediately after collection, leucocytes and platelets were removed by filtering through cellulose micro crystalline column as described by Beutler et al. [12]. Na/K ATPase activity was assayed in isolated erythrocyte membranes and expressed as the difference between inorganic phosphate released from Vanadate-free ATP during separate assays with and without 1 $mmol \cdot l^{-1}$ ouabain, a specific inhibitor of Na/K ATPase as we described previously [3, 5]. Results are given in $nmol\ Pi \cdot mg\ protein^{-1} \cdot h^{-1}$.

Quantitative measurement of C-peptide and HbA_{1c}. Plasma C-peptide was measured by conventional radioimmuno assay (Diagnostic Systems Laboratories Inc., Webster, Tex., USA)

Table 2. Stepwise regression analysis of the correlations between erythrocyte Na/K ATPase activity and the different parameters in Type II diabetic patients

	Correlation coefficient	f-to enter	
C-peptide	0.48	7.7	$p = 0.002$
Age	–0.2	3.4	NS
Diabetes duration	–0.17	1.9	NS
Type of treatment[a]	0.09	0.04	NS
HbA_{1c}	–0.089	0.04	NS
BMI	–0.087	0.03	NS

[a] insulin or oral hypoglycaemic agents

with a sensitivity of 0.05 nmol · l^{-1}. The intra- and interassay coefficients of variation were 2.8 and 3.9 %.

HbA_{1c} was measured by High Performance Liquid Chromatography (Bio-Rad, Hercules, Calif., USA) (normal range: 4–6.5 %).

Statistical analysis. Data are given as means ± SEM. Statistical analysis was performed with Student's *t*-test and analysis of variance (ANOVA). *P* values less than 0.05 were considered statistically significant. Analysis of correlation between one variable and multiple parameters was performed with Stepwise Regression and a parameter F-to enter value over 4 was considered independently and significantly correlated.

Results

Erythrocyte Na/K ATPase activity was lower in Type I diabetic patients than in control subjects and Type II diabetic patients (Table 1 and Fig. 1). The mean enzymatic activity in Type II diabetic patients did not differ from that of control subjects but the individual values of the former varied widely.

Linear regression analysis showed no correlation between erythrocyte Na/K ATPase activity and sex, body mass index, diabetes duration, HbA_{1c} or age in any group.

In Type I diabetic patients, presence of neuropathy was associated with a lower erythrocyte Na/K ATPase activity (188 ± 18 vs 300 ± 12 nmol Pi · mg protein^{-1} · h^{-1}, $p = 0.01$).

The association between erythrocyte Na/K ATPase activity and neuropathy was also found in Type II diabetic patients. Mean erythrocyte Na/K ATPase activity was lower in 44 Type II diabetic patients suffering from neuropathy than in 43 Type II diabetic patients without neuropathy (305 ± 16 vs 367 ± 19 nmol Pi · mg protein^{-1} · h-, $p = 0.01$).

Because of the heterogeneity in Type II diabetic patients at least in terms of type of treatment and C-peptide values, we looked for Na/K ATPase activity in the patients treated or not by insulin. Patients on insulin were older, had had diabetes for longer and had higher HbA_{1c} values and lower C-peptide levels than those on oral treatment (Table 1). Erythrocyte

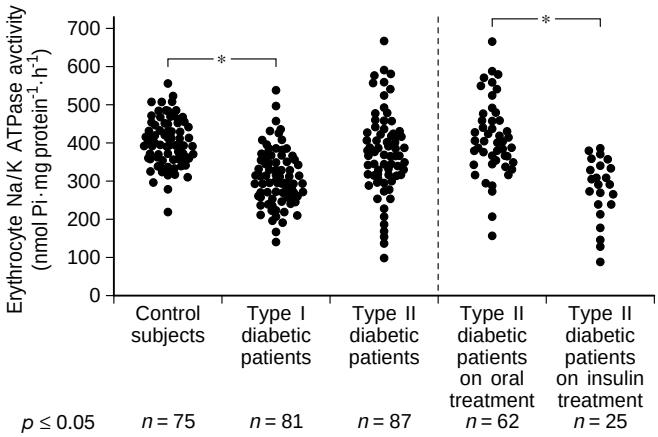

Fig. 1. Erythrocyte Na/K ATPase activity in the different groups of subjects. Mean erythrocyte Na/K ATPase activity was lower in Type I diabetic patients group ($p < 0.0001$) and in Type II diabetic patients on insulin treatment ($p < 0.001$) compared with control group

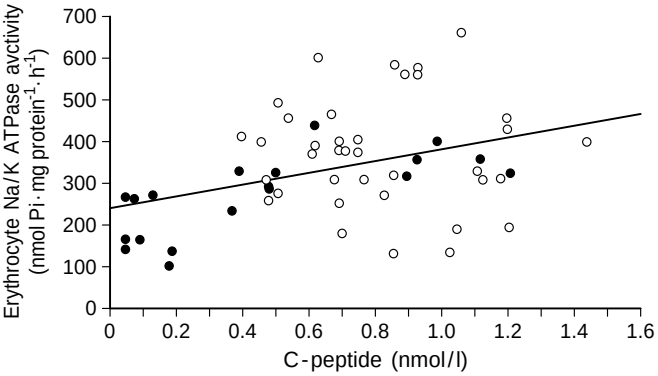

Fig. 2. Correlation between fasting C-peptide and erythrocyte Na/K ATPase activity among the Type II diabetic patients. In the whole group, there was a positive correlation ($r = 0.4$, $p < 0.01$). In Type II diabetic on insulin treatment, this correlation was even stronger ($r = 0.67$, $p < 0.001$). ○ Type II diabetic patients on oral treatment. ● Type II diabetic patients on insulin treatment

Na/K ATPase was lower in Type II diabetic patients on insulin treatment than in patients on oral treatment (264 ± 18 nmol Pi · mg protein^{-1} · h^{-1} vs 364 ± 16, $p < 0\ 001$) (Fig. 1). No differences were observed between Na/K ATPase activity in the groups of subjects treated with biguanides or with sulphonylureas or both (346 ± 26, 409 ± 36 and 348 ± 33 nmol Pi · mg protein^{-1} · h^{-1}, respectively).

To investigate what had influenced erythrocyte Na/K ATPase in the whole group of Type II diabetic patients, we analysed by stepwise regression the various parameters that could influence this enzymatic activity (Table 2). After adjustment, only C-peptide remained independently positively correlated with erythrocyte Na/K ATPase activity ($r = 0.48$ and $F = 7.7$); it explained 23 % of its variance (Fig. 2). The cor-

relation was even stronger in Type II diabetic patients receiving insulin treatment ($r = 0.67$, $p < 0.001$).

In Type II diabetic patients receiving insulin treatment, erythrocyte Na/K ATPase was lower in those with severe insulin deficiency than in those with higher residual endogenous secretion of insulin (C-peptide level over 0.2 nmol · l^{-1}) (181 ± 21 nmol Pi · mg protein^{-1} · h^{-1} vs 334 ± 17, $p < 0.0001$).

Discussion

As we showed in previous studies [3, 5, 6], erythrocyte Na/K ATPase activity is lower in Type I diabetic patients than in control subjects. In the entire group of Type II diabetic patients, mean enzymatic activity was similar to that of control subjects. The age of Type II diabetic patients differed from that of Type I diabetic patients by reason of the age at diagnosis characterization. Age though did not significantly influence erythrocyte Na/K ATPase activity. Only one study has shown a significant decrease in lymphocyte Na/K ATPase activity with age in women over 60 years of age [13].

There are only scarce data on erythrocyte Na/K ATPase activity in Type II diabetic patients. In a study of 47 Japanese Type II diabetic patients, erythrocyte Na/K ATPase activity was slightly reduced but only in those presenting with microalbuminuria [14].

In our series, regression analysis showed that age, diabetes duration, and body mass index did not explain the difference in Na/K ATPase activity between Type I and Type II diabetic patients. Therefore, it appears that hyperglycaemia cannot account for the decrease in erythrocyte Na/K ATPase activity in Type I diabetic patients because glycaemic control was similar for Type I and Type II diabetic patients.

Because of the heterogeneity of the Type II diabetic patients and the wide range of individual values of erythrocyte Na/K ATPase activity, we studied this activity in relation to the type of treatment. It appeared that erythrocyte Na/K ATPase activity was significantly lower in Type II diabetic patients on insulin treatment than in those on oral treatment. Also, in the former this activity was similar to that of Type I diabetic patients. It could be hypothesized that the type of treatment influences erythrocyte Na/K ATPase activity except that stepwise regression analysis (Table 2) ruled out such a treatment effect. In addition, we looked for differences in Na/K ATPase activity in the subjects treated with biguanides or sulphonylureas or both but no significant difference was observed. Ribalet et al. [15] have shown that Na/K ATPase activity could be lowered by sulphonylureas in an insulin-secreting tumour cell line. We observed that this enzymatic activity was lower in patients treated by insulin, suggesting that treatment by exogenous insulin could negatively regulate Na/K ATPase activity. A previous study, however, has shown that intensive insulin therapy by means of an artificial pancreas restored the enzyme activity after 24 h in erythrocyte membranes [16].

Type II diabetic patients treated with insulin were older, had had diabetes for longer, poorer glycaemic control and lower C-peptide levels than Type II diabetic patients treated with oral agents. Only C-peptide was independently correlated with erythrocyte Na/K ATPase activity. The correlation between erythrocyte Na/K ATPase activity and C-peptide concentration was much stronger in the insulin treatment subgroup.

Thus, erythrocyte Na/K ATPase activity is positively correlated with C-peptide concentration, which reflects endogenous insulin secretion. This enzyme activity is not directly dependent on diabetes type or antidiabetic treatment. Insulinopenic subjects have a significantly lower activity than patients with significant residual endogenous insulin secretion (C-peptide over 0.2 nmol · l^{-1}) and control subjects. Indeed, in Type II diabetic patients on insulin treatment, erythrocyte Na/K ATPase activity is significantly lower in those with very low C-peptide than in those with nearly normal fasting C-peptide.

Recent studies have suggested that C-peptide could have a biological activity [17]. C-peptide is released from the beta cells into the circulation in equimolar amount with insulin and fulfils an important function in the assembly of the two-chain insulin structure. Now, however, there is evidence that this is not the there is only function of C-peptide. Giving C-peptide to Type I diabetic patients or diabetic rats reduces glomerular hyperfiltration, increases blood flow and oxygen uptake, stimulates glucose transport in skeletal muscle, reduces urinary albumin excretion, decreases blood retinal barrier leakage, and attenuates vascular dysfunction [8, 18–20]. In addition C-peptide infusion improves autonomic nerve function [9]. The mechanism(s) resulting in these effects have not been determined but several of the processes stimulated or modulated by C-peptide relate to membrane permeability or transport, events which are in part dependent on Na/K ATPase activity [9]. Giving C-peptide to diabetic rats stimulates Na/K ATPase in their renal tubule cells in a dose dependent manner [7] and stimulates Na/K ATPase activity in different, including nerve, tissues [21]. These studies showed that C-peptide treatment has a biological effect in humans and animals that are insulinopenic and therefore C-peptide deficient. Our findings of a direct correlation between endogenous C-peptide and erythrocyte Na/K ATPase activity in Type II diabetic patients strongly suggest a physiological role for this circulating molecule.

The effects of C-peptide on Na/K ATPase are similar in many aspects to those of the hormone signal pathway [22]. A recent study has shown that C-peptide action does not require the normal chirality of

the peptide and that this property might be attributable to the glycine-rich central portion [23, 24]. It was suggested that C-peptide may function like some antibiotic peptides and does not follow the usual rules of ligand and receptor chemistry.

Yet, Flatt et al. [25] found evidence for a specific. binding of C-peptide in cultured beta cells indicating the existence of C-peptide receptors. Othomo et al. [22] established the C-peptide signal pathway by using Na/K ATPase as a target protein. In their study, the C-peptide effect was abolished by pertussis toxin or by FK 506, respectively specific inhibitors of G-proteins and the calcium modulin-dependent protein phosphatase. Their results indicate that C-peptide activates G-proteins and Ca^{++} dependent signalling pathways. Intensive insulin therapy by an artificial pancreas restores Na/K ATPase activity after 24 h in erythrocyte membranes [16]. In fact, insulin increases the sodium affinity of Na/K ATPase in skeletal muscle and adipocyte of diabetic rats [26]. The effect of insulin on Na/K ATPase, through the insulin receptor, seems to follow the same signalling pathway that is dependent on G-protein and calcium modulin phosphatase [26, 27]. Thus both C-peptide and insulin appear to act together to stimulate Na/K ATPase activity.

In this study, we confirmed our previous finding [3, 4, 5, 6] that diabetic neuropathy is associated with lower erythrocyte Na/K ATPase in Type I diabetic patients. The same finding was also observed in Type II diabetic patients. The positive correlation between erythrocyte Na/K ATPase activity and fasting C-peptide and that between low erythrocyte Na/K ATPase activity and diabetic neuropathy suggest a putative role for C-peptide.

In conclusion, we have shown that the impairment of erythrocyte Na/K ATPase activity in diabetic patients occurs when blood C-peptide is low, a strong argument for the physiological role of C-peptide in humans. This finding needs to be confirmed by an intervention trial in patients with diabetes to establish if giving C-peptide together with insulin could be beneficial in restoring Na/K ATPase activity.

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