The Effects Ex Vivo and In Vitro of Insulin and C-Peptide on Na/K Adenosine Triphosphatase Activity in Red Blood Cell Membranes of Type 1 Diabetic Patients

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The decrease in Na/K adenosine triphosphatase (ATPase) activity observed in several tissues of type 1 diabetic patients is thought to play a role in the development of long-term complications. Infusion of insulin may restore this enzyme activity in red blood cells (RBCs), and recent arguments have been developed for a similar role of C-peptide. The aims of this study were to determine whether insulin acts directly on the RBC enzyme and to evaluate the effect of C-peptide on Na/K ATPase activity. Thirty-nine C-peptide–negative type 1 diabetic patients were studied (blood glucose, 11.2 ± 1.49 mmol/L; hemoglobin A_{1c} [HbA_{1c}], 8.9% ± 0.1%, mean ± SEM). Blood samples were obtained in the morning, before breakfast and insulin injection. Intact and living RBCs were resuspended in their own plasma and incubated with or without insulin (50 μU/mL) or C-peptide (6 nmol/L). Ex vivo by microcalorimetry, the heat produced after 1 hour by the enzyme-induced hydrolysis of adenosine triphosphate (ATP), was measured in a thermostated microcalorimeter at 37°C. The results showed that Na/K ATPase activity was significantly increased by insulin (12.4 ± 0.5 *v* 15.4 ± 0.9 mW/L RBCs, $P < .05$, n = 23) but not by C-peptide (11.9 ± 0.7 *v* 12.9 ± 0.9 mW/L RBCs, NS, $P = .26$, n = 12). In another experiment, RBC suspensions were incubated at 37°C in a water bath with or without insulin (50 μU/mL) or C-peptide (6 nmol/L) for 10 minutes. RBC membranes were isolated and Na/K ATPase activity was assessed by measuring inorganic phosphate release at saturating concentrations of all substrates. The results showed that insulin and C-peptide significantly increased RBC Na/K ATPase activity (342 ± 25, $P < .005$ and 363 ± 30, $P < .005$, respectively *v* 255 ± 22 nmol Pi · mg protein^{-1} · h^{-1}, n = 14). We conclude that insulin and C-peptide act directly on RBC Na/K ATPase, thus restoring this activity in type 1 diabetic patients. The stimulatory effect of C-peptide observed in vitro on RBC Na/K ATPase activity confirms that C-peptide plays a physiological role.

THE ENZYME Na/K adenosine triphosphatase ([Na/K ATPase] EC 3.1.6.37) is a ubiquitous transmembrane enzyme that maintains cellular homeostasis, ie, low Na^+ and high K^+ concentrations in cytosol. This homeostasis is crucial for preservation of the membrane potential of all cells,[1] and acts by operating an active Na^+ and K^+ transport through the cellular membrane. Type 1 diabetes mellitus induces a decrease in Na/K ATPase activity in several tissues in rats[2,3] and in red blood cells (RBCs)[4] and nerves[5] of human patients. This decrease is considered an important factor in the development of diabetic neuropathy.[6-8] Restoration of membrane Na/K ATPase activity by various types of interventions in experimental diabetes is associated with the restoration of nerve conduction velocity[9,10] and of the anatomical integrity of nerve tissue.[10] It thus appears important to restore this enzymatic activity in type 1 diabetes mellitus patients.

With in vitro animal studies, insulin increased Na/K ATPase activity in adipocytes,[11] soleus[12] and skeletal[13,14] muscle, bladder,[15] skin,[16] astrocytes,[17] hepatocytes,[18] and nerve.[19] RBC Na/K ATPase activity is lower in diabetic rats versus normal rats,[3] and insulin treatment restores this activity.[20] Infusion of insulin for 24 hours in type 1 diabetic patients normalized the blood glucose level and restored Na/K ATPase activity in RBCs.[21] It was difficult to determine whether this restoration was a direct effect of insulin on RBC Na/K ATPase activity or a result of various regulations induced by insulin during the 24 hours.

Insulin is synthesized by β cells in the pancreatic islets as a protein chain that is cleaved twice to yield active insulin and a small peptide, C-peptide. Insulin and C-peptide are released in equal amounts in the portal circulation.[22] It has been believed for a long time that C-peptide has only one function—to facilitate the formation of disulfide bonds in the insulin molecule.[23] But in patients with type 1 diabetes mellitus, treated only by insulin, C-peptide administration improves some perturbations induced by diabetes. These improvements include a decrease in renal glomerular filtration and uptake and utilization of glucose,[24] normalization of both blood flow and capillary diffusion in skeletal muscle,[25] and improved nerve function.[26] In a recent study, a dose-dependent stimulation of Na/K ATPase activity was observed in rat renal tubular cells during C-peptide administration.[27]

The objective of this study was to determine whether insulin acts directly on RBC Na/K ATPase activity and to evaluate the effect of C-peptide on this enzymatic activity in patients with type 1 diabetes mellitus. For this purpose, we incubated intact and living RBCs in their own plasma with and without insulin or C-peptide, and assessed variations in Na/K ATPase activity by microcalorimetry (ex vivo study) and by measurement of phosphate release by adenosine triphosphate (ATP) hydrolysis (in vitro study).

SUBJECTS AND METHODS

Study Subjects

Subjects were selected in our department and were studied after informed consent was obtained. We enrolled 39 patients with type 1 diabetes mellitus. None used any medication known to influence Na/K ATPase activity (calcium-channel blockers, thyroxine, glucocorticoid, or digitalin-like agents) and none showed clinical complications. Type 1 diabetes mellitus was defined by diagnosis before 30 years of age, C-peptide negativity, and treatment with insulin. All subjects provided consent in accordance with the Declaration of Helsinki (1989) by the

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World Medical Association. The effect of insulin was tested by the microcalorimetric study for 23 patients and by the in vitro study for 14 patients. The effect of C-peptide was tested in 12 patients by the microcalorimetric study and in 14 patients by the in vitro study. Table 1 shows the characteristics of the patients.

Sample Preparation

Venous blood (40 mL) was collected in Vacutainer tubes (Becton Dickinson, Plymouth, UK) containing lithium-heparin from fasting patients in the morning before insulin injection. After centrifugation ($2{,}000 \times g$ for 10 minutes at 4°C), the plasma was collected and recentrifuged in the same conditions. To eliminate leukocytes and platelets, blood cells were resuspended in NaCl solution (0.9%) and filtered through a cellulose microcrystalline column as described by Beutler et al.[28] Finally, living RBCs from each patient were resuspended in their own plasma to obtain a hematocrit of approximately 10% and incubated with and without insulin or C-peptide. Human insulin (Novo Actrapid, Boulogne-Billancourt, France) and human C-peptide (Lilly, Indianapolis, IN) were tested at physiological postprandial concentrations of 50 µU/mL and 6 nmol/L, respectively.

Microcalorimetric Assay of Na/K ATPase on Living RBCs (ex vivo)

We used an LKB flow Bioactivity Monitor 2277 microcalorimeter set at 37°C. The principle of this method has been described by Issautier et al.[29] Briefly, 3 mL RBC suspension, in a thermostated vessel at 37°C and homogenized under constant magnetic stirring, was pumped into the microcalorimeter cell by a peristaltic pump. Heat production (HP) by the RBCs was recorded versus time. It is important to note that the steady state is obtained after about 1 hour of incubation. When the steady state is reached, ouabain (15 mmol/L specific inhibitor of Na/K ATPase) is added to the suspension and a decrease in HP is noted that corresponds to the inhibition of RBC Na/K ATPase. The difference between the steady state before and after the addition of ouabain corresponds to the RBC Na/K ATPase activity. It was calculated as follows:

$$\text{Na/K ATPase activity (mW/I)} = \frac{\text{HP (µW)}}{\text{V(mL)} \times \text{hematocrit (\%)}} \times 100.$$

Figure 1 shows a typical thermogram.

Na/K ATPase Activity Assay on RBC Membranes (in vitro study)

The RBC suspension was incubated under stirring at 37°C for 10 minutes with or without insulin or C-peptide. The reaction was stopped by the addition of Tris buffer 0.011 mmol/L (pH 7.40). The hemolyzed cells were centrifuged ($30{,}000 \times g$ for 30 minutes at 4°C) and the membrane pellet was resuspended in 30 mL Tris buffer 0.011 mmol/L. This centrifugation step was repeated 3 times, and the final concentration of the membrane suspensions was approximately 3 mg protein $\cdot$ mL^{-1} Tris buffer.

RBC Na/K ATPase activity was measured in isolated RBC membranes and expressed as the difference in inorganic phosphate release from Vanadate-free ATP with versus without ouabain 1 mmol/L (specific inhibitor of Na/K ATPase) as previously described.[7] The results are given as nanomoles of P_i per milligram of protein per hour. All assays were performed in triplicate, and blanks were routinely included to compensate for endogenous phosphate and non–enzyme-related breakdown of ATP.

Table 1. Characteristics of the Type 1 Diabetic Patients (N = 39)

Characteristic	Mean ± SEM
Age (yr)	37.2 ± 3.2
Diabetes duration (yr)	13.9 ± 2.7
Blood glucose (mmol/L)	11.2 ± 1.49
HbA_{1c} (%)	8.9 ± 0.1

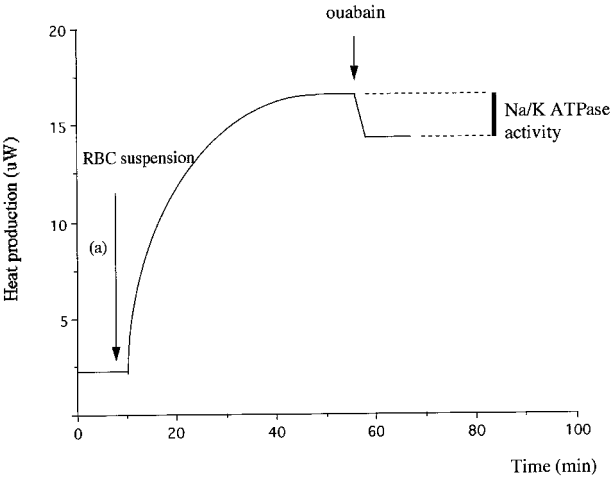

Fig 1. Typical thermogram. Heat production by RBC suspension was recorded versus time. The difference between steady states before and after ouabain addition corresponds to RBC Na/K ATPase activity. (a) Introduction of sample.

Statistical Analysis

Data are given as the mean ± SEM. We performed a Kolmogorov-Smirnov test for normality and a Barlett test for homogeneous variance for each group. Since the data were not normally distributed, differences between groups were identified by the nonparametric Wilcoxon test and were considered significant at a p value of less than .05.

RESULTS

Effect of Insulin

Microcalorimetric study. Insulin (50 µU/mL) was tested in 23 patients with type 1 diabetes mellitus. After about 1 hour of incubation, RBC Na/K ATPase activity was significantly stimulated with insulin in comparison to incubation in the absence of insulin (15.4 ± 0.9 *v* 12.4 ± 0.5 mW/L RBCs, respectively, $P = .02$, n = 23) (Fig 2A).

In vitro study. The RBC suspension was incubated in a water bath at 37°C with insulin (50 µU/mL) for 10 minutes. Again, insulin significantly stimulated RBC Na/K ATPase activity (342 ± 25 *v* 255 ± 22 nmol Pi $\cdot$ mg protein^{-1} $\cdot$ h^{-1}, $P = .003$, n = 14) (Fig 2B).

Effect of C-Peptide

Microcalorimetric study. C-peptide (6 nmol/L) was tested in 12 patients with type 1 diabetes mellitus by incubating RBCs for about 1 hour with or without C-peptide. RBC Na/K ATPase activity did not significantly change (12.9 ± 0.9 *v* 11.9 ± 0.7 mW/L RBCs, $P = .26$, n = 12) (Fig 3A).

In vitro study. RBC suspensions were incubated in a water bath at 37°C with C-peptide (6 nmol/L) for 10 minutes. RBC Na/K ATPase activity was significantly increased in the presence of C-peptide versus its absence (363 ± 30 *v* 255 ± 22 nmol Pi $\cdot$ mg protein^{-1} $\cdot$ h^{-1}, $P = .002$, n = 14) (Fig 3B).

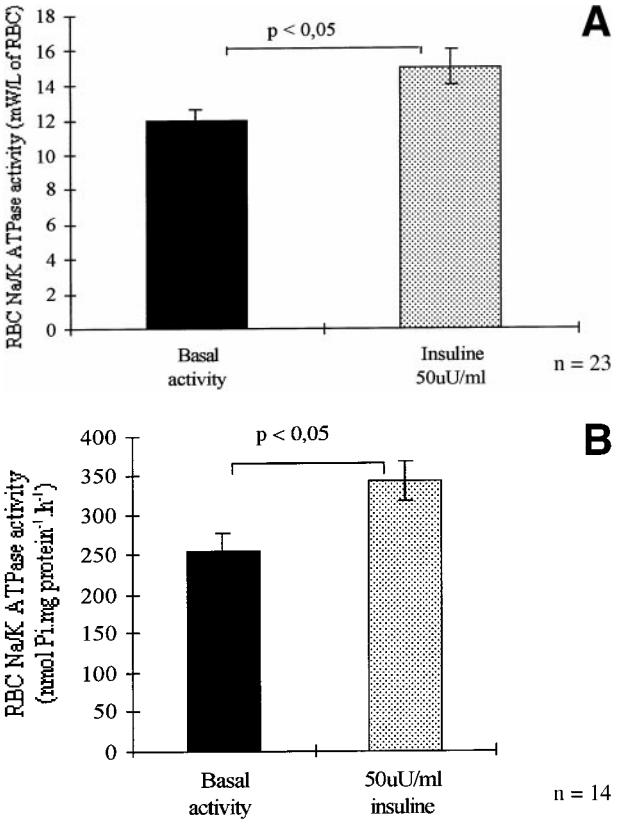

Fig 2. Insulin (50 μU/mL) effect on RBC Na/K ATPase. (A) Ex vivo study by microcalorimetry after an incubation of about 1 hour of RBC suspensions without (■, basal activity) or with (▨) insulin. RBC Na/K ATPase activity was significantly increased in the presence of insulin (12.4 ± 0.5 *v* 15.4 ± 0.9 mW/L RBCs, *P* = .02). (B) In vitro study after an incubation of 10 minutes of RBC suspensions without (■, basal activity) or with (▨) insulin. RBC Na/K ATPase activity was significantly increased with insulin (342 ± 25 *v* 255 ± 22 nmol Pi · mg protein^{-1} · h^{-1}, *P* = .003, mean ± SEM).

DISCUSSION

The results show that insulin and C-peptide act directly on RBC Na/K ATPase and increase its activity in patients with type 1 diabetes mellitus when RBCs are suspended in their own plasma. It has been shown that perfusion with C-peptide at its physiological concentration in patients with type 1 diabetes mellitus restores several parameters of renal function,[24] and the beneficial effect on renal blood flow and protein excretion was maintained when treatment with C-peptide was prolonged for 1 to 3 months.[30] Autonomic nerve function is significantly improved if C-peptide is administered with insulin.[26]

Kunt et al[31] showed that C-peptide restores the decreased deformability of diabetic RBCs. They observed that this restoration of RBC deformability was blocked when RBC Na/K ATPase was inhibited by ouabain. They suggested that Na/K ATPase was implicated in the restoration of RBC deformability by C-peptide. Another study showed that an attenuation of Na/K ATPase activity is correlated with a decrease in the deformability of diabetic RBCs.[32] Recently, Forst et al[33] observed an increase in RBC Na/K ATPase activity in type 1 diabetic patients when C-peptide was infused continuously for 2 hours. Our results confirm that C-peptide directly stimulates Na/K ATPase activity in RBCs.

In our study, we sought to work as close as possible to physiological conditions to test the effect of C-peptide on the enzyme. That is why we used a microcalorimeter, in which C-peptide is incubated with intact and living RBCs in their own plasma. After an incubation of about 1 hour, a weak but nonsignificant effect of C-peptide on the enzymatic activity was observed. The microcalorimetric method is ideal for studying Na/K ATPase activity in RBCs because it measures the changes in activity in real time and in living cells. However, it is difficult to define a precise incubation time, and only a weak effect of C-peptide was observed by this method. Since we could not arrive at any conclusion, this led us to use another technique in which incubations are still performed in ex vivo conditions, but we used a water bath that enabled us to stop the incubation by RBC lysis whenever we wanted. Enzymatic activity was then measured on RBC membranes in the presence of a saturating concentration of substrates.[7] In preliminary studies after testing several incubation times with in vitro conditions, we determined that Na/K ATPase activity was activated by C-peptide after a 10-minute incubation.

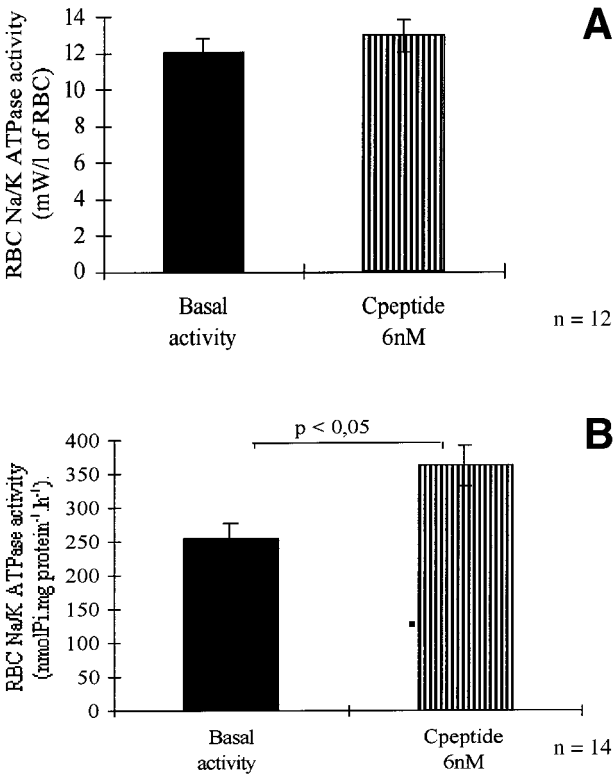

Fig 3. C-peptide (6 nmol/L) effect on RBC Na/K ATPase activity. (A) Ex vivo study by microcalorimetry after an incubation of about 1 hour of RBC suspensions without (■, basal activity) or with (▥) C-peptide. RBC Na/K ATPase activity did not significantly change (11.9 ± 0.7 *v* 12.9 ± 0.9 mW/L RBCs, *P* = .26). (B) In vitro study after an incubation of 10 minutes of RBC suspensions without (▥, basal activity) or with (■) C-peptide. RBC Na/K ATPase activity was significantly increased with C-peptide (363 ± 30 *v* 255 ± 22 nmol Pi · mg protein^{-1} · h^{-1}, *P* = .002, mean ± SEM).

However, Kunt et al[31] observed a restoration of RBC deformability after 4 hours of in vitro incubation of RBCs with C-peptide. These results are not in contradiction to ours if we hypothesize that the effect of C-peptide on Na/K ATPase is fast, while the restoration of RBC deformability, which takes longer, is a consequence of an increase in Na/K ATPase. The restoration of deformability involves different pathways such as calcium homeostasis,[34] interaction of spectrin-dimer-dimer,[35] and interaction of spectrin protein 4.1.[36]

In most studies on the effects of C-peptide, various incubation times have been used: 1 hour for the study of renal function,[24] 3 hours for nervous function,[26] and 4 hours for RBC deformability.[31] This is the first time that we have observed such a rapid effect of C-peptide, 10 minutes, for the stimulation of RBC Na/K ATPase. It has recently been shown that in patients with type 2 diabetes mellitus, RBC Na/K ATPase activity was significantly lower in patients on insulin treatment than in those on oral treatment.[37] Furthermore, in patients with insulin treatment, RBC Na/K ATPase activity was lower when the C-peptide level was very low as compared with those with a nearly normal C-peptide level. The C-peptide level was the only factor independently correlated with RBC Na/K ATPase in type 2 diabetes mellitus. This observation strengthens the idea that C-peptide participates in the regulation of Na/K ATPase in RBCs. Furthermore, during C-peptide perfusions, a positive correlation has been found between the level of C-peptide and RBC Na/K ATPase activity.[33] The mechanism of action of C-peptide is not known, but 2 hypotheses have been suggested. The first hypothesis states that C-peptide has biological activity in its native form, as well as its synthetic reverse sequence, thus suggesting that its effect is mediated by nonchiral interactions.[38] A recent study tested whether C-peptide binds to lipid vesicles. The results showed that C-peptide and lipid vesicles do not comigrate during size-exclusion chromatography and that it did not change its secondary structure in the presence of these vesicles.[39]

The second hypothesis favors an action of C-peptide via a specific receptor. This idea is supported by the study of Flatt et al,[40] who found a specific binding of C-peptide in cultured β cells, suggesting the existence of C-peptide receptors in these cells. In proximal renal tubule segments of diabetic rats, C-peptide activates Na/K ATPase through a G protein, as well as calcium-dependent intracellular pathways,[27] thus inducing an activation of eNOS activity.[33,41] This activation is involved in the improvement of the endothelial-dependent microvascular response in type 1 diabetic patients.[41]

The most recent study shows that C-peptide binds specifically to several human cells. The investigators show that neither the nonnative form (D-C-peptide) nor insulin compete with the fixation of C-peptide.[42]

The effect of insulin, contrary to that of C-peptide, has been studied for many years. The results of our incubation of RBC suspensions with insulin are in line with the findings in other tissues.[43] In RBCs, insulin induces various effects that could account for its action on Na/K ATPase, although this has never been clearly demonstrated. Insulin-receptor interactions could affect membrane fluidity,[44] resulting in an altered membrane microenvironment, which could cause variations in Na/K ATPase activity.[45] Baldini et al,[46] on the other hand, observed that insulin stimulated the sodium pump mechanism and thus increased Na/K ATPase activity, but without modifying membrane fluidity.

It is known that insulin has a time-dependent action which is tissue-specific. An increase in Na/K ATPase activity was observed after an incubation time of 10 minutes in soleus muscle,[12] 10 to 25 minutes in nerve,[19] 20 minutes in adipocytes,[11] and 1 hour in skin.[16] In vivo insulin perfusions restored Na/K ATPase activity in RBCs after 24 hours.[21] In our study, insulin stimulated RBC Na/K ATPase activity after an incubation of about 1 hour in a microcalorimeter. This led us to determine whether this stimulation could be observed after a shorter incubation, as for other tissues. We found that insulin stimulated Na/K ATPase activity in RBCs at 10 minutes, demonstrating that insulin can act rapidly on RBCs.

It is interesting to note that in our in vitro studies, although we have worked at saturating substrate concentrations, we observed a stimulatory effect of insulin and C-peptide on RBC Na/K ATPase. This suggests that insulin and C-peptide cause modifications that are sufficiently stable to be observed after RBC lysis and under saturating conditions. The increase in activity induced by insulin cannot be accounted for by a change in the membrane order; effectively, the modifications in erythrocyte membrane order are observed after an incubation time of at least 30 minutes.[44] We can also exclude the involvement of an increase in sodium affinity induced by insulin as observed in several tissues,[43] because sodium is at saturating concentrations. We thus suggest that the increase in Na/K ATPase induced by insulin or C-peptide activity could be due to an increase in the number of active pumps or an increase in the turnover of these pumps. These hypotheses need further study.

It is well known that in patients with type 1 diabetes mellitus with good metabolic control, microvascular and neuropathic complications cannot be completely prevented.[47] Patients with residual β-cell function seem less prone to develop diabetic microvascular and neuropathic complications than those in whom β-cell function is completely abolished.[48] Maffi et al[49] showed a direct relationship between plasma C-peptide levels and the improvement in neuropathy after 3 years of intraportal islet transplantation in type 1 diabetic patients.

We suggest that a deficiency in C-peptide is an important factor in the appearance of certain complications due to diabetes. Its stimulatory effect on the activity of Na/K ATPase, which is involved in the pathogenesis of neuropathy, indicates that it should be used, in association with insulin in patients with type 1 diabetes mellitus.

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