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The effects of captopril and losartan on erythrocyte membrane Na$^+$/K$^+$- ATPase activity in experimental diabetes mellitus

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Abstract
Diabetes mellitus induces a decrease in sodium potassium-adenosine triphosphatase (Na$^+$/K$^+$- ATPase) activity in several tissues in the rat and red blood cells (RBC) and nervous tissue in human patients. This decrease in Na$^+$/K$^+$- ATPase activity is thought to play a role in the development of long term complications of the disease. Angiotensin enzyme inhibitors (ACEi) and angiotensin-II receptor antagonists (ARBs) reduce proteinuria and retard the progression of renal failure in patients with IDDM and diabetic rats. We investigated the effects of captopril and losartan, which are used in the treatment of diabetic nephropathy, on Na$^+$/K$^+$- ATPase activity. Captopril had an inhibitory effect on red cell plasma membrane Na$^+$/K$^+$ ATPase activity, but losartan did not. Our study draws attention to the inhibitory effect of captopril on Na$^+$/K$^+$ ATPase activity. Micro and macro vascular complications are preceeding mortality and morbidity causes in diabetes mellitus. There is a strong relationship between the decrease in Na$^+$/K$^+$ ATPase activity and hypertension. The non-sulphydryl containing ACEi and ARBs must be the choice of treatment in hypertensive diabetic patients and diabetic nephropathy.

Keywords: *Na$^+$/K$^+$- ATPase, captopril, losartan, diabetes mellitus, ACE*

Introduction

Diabetic nephropathy is a clinical syndrome characterised by persistent albuminuria, a relentless decline in glomerular filtration rate (GFR), and elevated systemic blood pressure. The prevalence of abnormality is aproximately 40% in patients with both insulin-dependent diabetes mellitus (IDDM) and non-isulin-dependent diabetes mellitus (NIDDM) [1]. Several studies have shown that certain classses of antihypertensive medications can reduce the risk of developing diabetic nephropathy. Current literature supports the use of angiotensin-converting enzyme inhibitors (ACEIs), angiotensin-II receptor blockers (ARBs), and the non-dihydro-pyridine calcium channel blockers (NCCBs), verapamil and diltiazem, in diabetic patients at risk to developing nephropathy [2].

ACE (EC.3.4.15.1, kininase II), a zinc-requiring metalloenzyme and membrane bound glycoprotein localized mainly in the endothelial cells of pulmonary capillaries, catalyzes the cleavage of histidyl-leucine from the carboxyl-terminal of angiotensin-I, yielding an octapeptide angiotensin II, a known potent vasoconstrictor in the human body. Diabetes is associated with alterations in glomerular filtration rate, kidney mass and renal function, which may elevate blood pressure through altering endogenous fluid and electrolyte balance. This data indicates that an increase in blood pressure is connected with levels of serum ACE activity in diabetes mellitus. High levels of serum ACE activity are also somehow related to the complications of diabetes [3]. Streptozotocin (STZ) is used to induce experimental diabetes in animals and for the treatment of patients with insulinoma [4].

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The sodium-potassium adenosine triphosphatase (Na^+/K^+ ATPase, sodium pump; EC (3.6.1.37) enzyme is an ubiquitous membrane-associated protein complex that is expressed in most eukaryotic cells. The "pump" transduces energy from the intracellular hydrolysis of adenosine triphosphate (ATP) to achieve the countertransport of sodium and potassium across the cell membrane. Its activity is decreased in many tissues of STZ-induced diabetic animals [5]. Alteration of this transport enzyme is thought to be linked to several complications of diabetes mellitus [6]. Captopril, an ACE inhibitor with a short duration of action, has been shown to reduce proteinuria and retard the progression of renal failure in patients with IDDM and nephropathy [7]. The angiotensin II subtype receptor antagonist losartan, reduces albuminuria and mean arterial blood pressure similar to ACE inhibition [8]. In diabetic rats, the decrease of erythrocyte membrane Na^+/K^+ATPase activity is associated with the presence of peripheral neuropathy [9]. Captopril has an inhibitory effect on Na^+/K^+ ATPase [10].

In this study we aimed to investigate the effects of captopril and losartan on erythrocyte membrane Na^+/K^+ ATPase.

Materials and methods

Experimental subjects

Experiments were performed on 250–300 g male Wistar rats. The rats were made diabetic by i.p. injection of 65 mg/kg STZ in pH 4.5 sodium citrate buffer. Eight rats received an equivalent amount of buffer and served as a healthy control. Two days after the administration of STZ, blood glucose levels of overnight fasting rats were measured with accutrend test stripes from the tail vein blood [11,12]. STZ injected rats ($n = 25$) were considered diabetic if blood glucose levels were greater than 12 mM [13,14]. Six weeks following the induction of diabetes, eight rats were given captopril (50 mg/kg/day), eight rats were given losartan (10 mg/kg/day) via orogastric gavage [15,16], and the remaining nine diabetic control rats and eight healthy control rats were given distilled water.

All groups were maintained for six weeks with food and water given *ad libitum* through the study period. The study protocol was approved by the local ethics committee for animal care and use.

Blood and urine sampling and laboratory measurements

At the beginning and at the end of the 6-week study period, 24 h urine were collected in individual metabolic cages. At the end of the study period, after overnight fasting, blood samples were collected with cardiac puncture while rats were kept under ether anaesthesia. Fasting blood glucose levels were measured with accutrend test stripes [12]. Intracardiac blood samples were coagulated with Na-EDTA for red blood cell Na^+/K^+ ATPase activity and centrifuged at $1500 \times$ g. After separation of the plasma, erythrocyte pellets were washed three times with 0.9% NaCl [17]. Other blood samples were collected in tubes with heparin for plasma ACE activity and creatinine levels. Blood samples were centrifuged at 3000 rpm for 15 min, plasma separated and stored at $-20°C$ until further use [3].

Membrane preparation

Erythrocytes were hemolyzed with 10 mmol/L Tris, 1 mmol/l EDTA (pH 7.4) at 4°C. Ghosts were sedimented at $27000 \times$ g (20 min, 4°C) and the pellets were washed thrice with 10 mmol/L Tris, pH 7.4. Membranes re-suspended in the buffer were stored at $-70°C$. Membrane proteins were determined according to the biuret method [18].

Assay of ATPase activity

This assay links the hydrolysis of ATP by ATPase with NADH oxidation in the presense of excess pyruvate kinase, lactate dehydrogenase (LDH). The large quantity of NADH produced is oxidized by reducing pyruvate to lactate carried out by LDH and phosphoenolpyruvate (PEP), thus allowing continuous spectrometric recording at 340 nm. The activity of Na^+/K^+ ATPase was measured in the following reaction mixture: 30 mmol/L imidazole (pH 7.3), 100 mmol/L NaCl, 10 mmol/L KCl, 2.5×10^3 mmol/L $MgCl_2$, 0.5×10^3 mmol/L ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 1×10^3 mmol/L Na_2-ATP, 1×10^3 mmol/L PEP, 0.15×10^3 mmol/L NADH, 50 μg/mL LDH with and without 1×10^3 mmol/L ouabain. The enzyme reaction was started by the addition of 25 μL of membrane suspension. NADH oxidation was monitored by measuring the absorbance at 340 nm every 30 s for 10–15 min at 37°C. The results are expressed as unit g (the amount of enzyme which converts 1 μmol substrate to product at 25°C in one minute at optimum conditions) per miligram protein [18].

ACE activity

A commercial kit for spectrophotometric determination of ACE, based on the method Holmquist et al. [19], was used. ACE activity was determined with an ACE kit [Sigma Diagnostics at 340 nm, at using a Shimadzu UV-1238 spectrofotometer, the substrate being 2-furanoacryloyl-L-phenylalanyl-glycyglycine (FAPPG). All analyses at 37°C over an incubation time of 5 min.

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Table I.

	Healthy control	Diabetic control	STZ + losartan	STZ + captopril
Glucose (mg/dL)	120 ± 9.4	$355 \pm 16.6^*$	$347 \pm 32.7^*$	$351 \pm 22.4^*$
Ccr (mL/min)	1.52 ± 0.2	$2.94 \pm 0.4^*$	$2.67 \pm 0.2^*$	$2.70 \pm 0.3^*$
Albumınıria (µg/day)	220 ± 48	$826 \pm 52^*$	$490 \pm 84^{*,**}$	$443 \pm 72^{*,**}$
ACE activity (U/L)	81 ± 10	$125 \pm 15^*$	$130 \pm 10^*$	$94 \pm 13^{*,***}$

Data are means $\pm$ SEM.

Routine parameters

Urinary albumin excretion was determined by immunoturbidimetric assay with an 911-Hitachi automatic analyzer (Roche diagnostic, Germany). Plasma glucose levels were measured with glucose oxidase by Accutrend Alpha (Roche). Urine and plasma creatinine levels were measured by a colorimetric method with a 911-Hitachi automatic analyzer (Roche diagnostic). Creatinine clearance (mL/min) was calculated using the formulation U.V/P, where U (mg/dL) is the urine creatinine, V (mL) is the urine volume for 24 h and P (mg/dL) is the plasma creatinine.

Statistical analysis

All data are expressed as means $\pm$ SEM. The data were analyzed using analysis of Variance (ANOVA). For the comparision of differences between groups the Tukey HSD test was used. P value < 0.05 was considered significant.

Results

At the end of the six weeks after administration of STZ, the indicator of diabetes mellitus, blood glucose levels were increased significantly in the diabetic control group, STZ + Losartan, STZ + Captopril groups as compared to healthy controls respectively, ($p < 0.001$). The values of creatinine clearance were elevated in all diabetic groups as compared to the healthy control group ($p < 0.001$). Urine albumin excretion rate was significantly elevated in the diabetic control group while it was significantly lower in the STZ + losartan and STZ + captopril groups compared with the diabetic control group. Plasma ACE activity in all diabetic groups was found to be increased in comparison with the healthy control group ($p < 0.001$). It was observed that ACE activity in the STZ + captopril group was decreased significantly as compared to the diabetic control group ($p < 0.001$). (Table I).

Erythrocyte membrane Na⁺/K⁺ ATPase activity in the diabetic control group was lower than in the healthy control group, respectively, ($p < 0.001$). Erythrocyte membrane Na⁺/K⁺ ATPase activity in the STZ + captopril group was decreased in comparision with the STZ + losartan, healthy and diabetic control groups, respectively, ($p < 0.001$, $p < 0.001$, $p < 0,001$), (Table II).

Discussion

The results of the study confirm that serum glucose levels in STZ-diabetic rats are significantly higher than the healthy control [15]. Losartan showed no effect on glucose levels in our study. Our data were similar to those of El Batran et al. [20] who showed that losartan given alone had no obvious effect on serum glucose levels, but that significant decreases in serum glucose level were observed when combined with the oral hypoglycemic drugs, repaglidine or glicliazide. As in the study of Shinoiri et al [21] captopril showed no adverse effect on glucose metabolism in our study.

In our study the albumin excretion rate and creatinine clearance were markedly increased in the diabetic control [22]. We showed here that losartan and captopril reduced the increase in the urinary albumin excretion level, but did not affect creatinine clearance. These results are consistent with previous reports, which investigated the effects of captopril and imidapril, ACE inhibitors, administered for 4 or 8 weeks in STZ-induced diabetic rat models [23] and those of Zandbergen et al [24] who treated the diabetic patients with losartan. However, Gilbert et al. [25] who treated the animals with ramipril, an ACE inhibitor, inhibited glomerular hyperfiltration. Thus, we speculate that the lack of effects of the ACE inhibitors on creatinine clearance may be due to the duration of treatment in our study.

Table II.

	Healthy control	Diabetic control	STZ + losartan	STZ + captopril
Na⁺/K⁺ ATPase (U/ mg protein)	0.147 ± 0.008	$0.072 \pm 0.005^*$	$0.075 \pm 0.008^*$	$0.047 \pm 0.009^{*,**,***}$

Data are means $\pm$ SEM.; $^*p < 0.001$ versus healthy control groups.; $^{**}p < 0.001$ versus diabetic control groups.; $^{***}p < 0.001$ versus STZ + losartan.

Hyperglycemia causes intraglomerular hypertension and renal hyperperfusion. Increased glomerular pressure results in the deposition of protein in the mesangium, ultimately leading to glomerulosclerosis. These changes may result in proteinuria and renal failure [26]. In our study, high levels of albuminuria determined in the diabetic control group compared to the healthy control group read us to consider that high levels of blood glucose can cause albuminuria. Our data showed that both therapies, losartan and captopril, were equally effective in reducing albuminuria [15,27]. ACE inhibitors slow the progress of diabetic nephropathy by several mechanisms [28]. Edmund et al. [29] support the proposal that captopril slows the progression of diabetic nephropathy by a mechanism that is independent of its antihypertensive properties. Several studies demonstrated that losartan significantly improved albuminuria [30]. The renin angiotensin system, whose important effect on haemodynamic and histopathological changes shown with experimental diabetes studies may cause proteinuria [31]. Glucose and Angiotensin II probably use similar signal tranduction pathways in renal cells in culture. Glucose stimulates de novo synthesis of diacylglycerol (DAG) generated from glycolitic intermediates through the polyol pathway; elevated DAG then leads to activation of protein kinase C (PKC), which then increases transforming growth factor (TGF-β1) matrix protein synthesis in mesangial and tubular cells [32]. This mechanism is the reason for an increase in ACE and the renin angiotensin system (RAS) elements. Erman et al. [33] showed that serum ACE activity is increased in the STZ-diabetic rat and our study showed this in the diabetic control group. This increase in ACE activity was in parallel with the increase in albuminuria. The inhibitory effect of captopril on serum ACE activity which is not shown by losartan leads to the consideration that the renoprotective effect of both agents is related to the inhibition of RAS rather than an ACE mediated effect.

In diabetic humans, serum ACE activity has been reported as being decreased, normal or even elevated. In diabetic rats, renal ACE activity has been reported as being decreased or normal. Thus the results of studies on ACE activity have been conflicting. Recently the importance of tissue ACE activity rather than serum ACE activity on angiotensin II action has been emphasized [34].

The activity of erythrocyte membrane Na^+/K^+ ATPase plays a central role in the regulation of intra- and extra-cellular homeostasis and alteration of this transport system is thought to be linked to several complications of diabetes mellitus [35].

Our study demonstrates that erythrocyte membrane Na^+/K^+ ATPase activity in the diabetic control group was lower than in the healthy control group. A decrease in Na^+/K^+ ATPase activity has been observed in diabetes mellitus that was significantly correlated with glycemia [36]. A diabetes-induced decrease in Na^+/K^+ ATPase activity compromises microvascular blood flow by two mechanisms; by affecting microvascular regulation and by decreasing red blood cell deformability, which leads to an increased blood viscosity. The defect in ATPase is strongly related to diabetic neuropathy; diabetic patients with neuropathy have lower ATPase activity than those without. The diabetes-induced impairment in Na^+/K^+ ATPase activity is identical in red blood cells and neural tissue. The defect in Na^+/K^+ ATPase activity is also probably involved in the development of diabetic nephropathy and cardiomyopathy; physiological C-peptide infusion could be beneficial for the prevention of diabetic complications [5]. Jannot et al. [37] claimed that a decrease in erythrocyte membrane Na^+/K^+ ATPase might be involved in the physiopathology of hypertension.

Our results showed that captopril have an inhibitory effect on Na^+/K^+ ATPase activity. Accetto et al. [38] suggest that captopril can inhibit membrane Na^+/K^+ ATPase activity and vascular smooth muscle cells although the cause of the inhibitory effect of captopril on Na^+/K^+ ATPase activity is uncertain. Captopril has a terminal free-SH group and several sulfhydril agents, most notably *p*-chloromercuribenzenesulfonate and *N*-ethylmaleimide, are known to inhibit Na^+/K^+ ATPase [10]. Losartan has no efffect on Na^+/K^+ ATPase.

In conclusion our study been shown that captopril and losartan have identical potency to prevent albuminuria and this effect may be performed on the renin angiotensin system. It was found that captopril had an inhibitory effect on erythrocyte Na^+/K^+ ATPase activity whereas losartan did not. Our study draws attention to the inhibitory effect of captopril on Na^+/K^+ ATPase activity. Micro and macro vascular complications are preceeding mortality and morbidity causes in diabetes mellitus. There is a strong relationship between the decrease in Na^+/K^+ ATPase activity and hypertension. The non-sulphydryl containing ACEi and ARBs must be the choice of treatment for hypertensive diabetic patients and diabetic nephropathy.

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