Acute diabetes mellitus and its influence on renal Na,K-ATPase in both genders

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Abstract. Due to the importance of renal Na,K-ATPase in maintaining the sodium homeostasis in the organism, its activity and abundance is intensively studied in condition of diabetes mellitus. The main subject of this study was the investigation of properties of renal Na,K-ATPase and abundance of its α1 subunit in view of possible gender-dependent differences in male and female diabetic rats. Diabetes was induced by a single intraperitoneal dose of streptozotocin in a dose of 65 $mg \cdot kg^{-1}$. The acute diabetes lasting 8 days induced a significant increase in Na,K-ATPase activity accompanied by significant gender specific increase in K_m value indicating a worsened affinity of ATP-binding site in female rats. In addition, our present experiments, revealed a significantly higher abundance of renal Na,K-ATPase α1 subunit in diabetic rats of both genders amounting 94% increase in males and 107% in females. But, not all of the newly synthesized enzyme molecules are fully active, as the increase in the number of active molecules is smaller (representing 23% in males and 20% in females) as indicated by lower increase in V_{max} values.

Key words: Sodium pump — Hyperglycemia — Streptozotocin — Kidney

Introduction

In accordance to the world health organization (WHO), 180 million people had diabetes mellitus in 2007, and one of them died approximately every six seconds. Diabetes is accompanied by many complications such as hyperglycemia, ketoacidosis and non-ketotic hyperosmolar coma. Serious long term complications include cardiovascular disease, retinal damage, nerve damage, microvascular damage and chronic renal failure. Diabetic nephropathy is the leading cause of death representing more than 40% of mortality in diabetic patients. Diabetes induces progressive trends of electrolyte abnormalities as a consequence of failure of chief transport mechanisms in kidney leading to the end-stage renal disease in patients (Shahid and Mahboob 2008). The disease is also often accompanied by hypertension (Landsberg 1994) and altered sodium homeostasis is also a consistent finding in diabetes, as both insulin-dependent and non-insulin-dependent diabetic patients have a significant increase in total exchangeable sodium (O'Hare et al. 1985; Weidmann and Ferrari 1991). The kidney plays an important role in the regulation of blood pressure through modulation of sodium transport across the proximal tubules by the aid of Na,K-ATPase called also as sodium pump. This enzyme is an oligomeric transmembrane protein that establishes and maintains the high internal K^+ and low internal Na^+ concentrations typical of most animal cells. By using the energy from the hydrolysis of one molecule of ATP, it transports three Na^+ ions out in exchange for two K^+ ions that are taken in. Na,K-ATPase consists of two main subunits, α and β, and in some tissues including kidney is associated with the third subunit, γ. The α subunit is a membrane multispanning protein that is responsible for the catalytic and transport properties of the enzyme. This subunit contains binding sites for the cations, ATP, and the inhibitor, ouabain (Pedemonte and Kaplan 1990; Mercer 1993; Lingrel and Kuntzweiler 1994; Pressley 1996; Blanco and Mercer 1998). The β subunit is needed for stability, functional maturation, and/or exit of α subunit from the endoplasmatic reticulum (McDonough et al. 1990; Pedemonte and Kaplan 1990; Mercer 1993). Four α isoforms (α1, α2, α3, and α4) and two β isoforms (β1, β2) have been identified in mammals, α1 and β1 are the main isoforms of rat kidney (Shull et al. 1986; Sverdlov et al. 1987;

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Martin-Vasallo et al. 1989; Gloor et al. 1990; Shamraj and Lingrel 1994; Appel et al. 1996).

Studies in streptozotocin (STZ)-induced diabetic rats have shown, that changes in the function of renal Na,K-ATPase depend on the development and the seriousness of the disease in time-dependent manner. In the acute phase of disease after 10 days, the activity and expression of the Na,K-ATPase was significantly higher in diabetic male rats as compared to the control group (Lal et al. 2000). Previous studies have documented that Na,K-ATPase is involved in the gender specific protection from the detrimental effects of various diseases and injuries in kidney (Fekete et al. 2004) as well as in the cardiovascular system (Dzurba et al. 1997; Vlkovicova et al. 2005; Sudar et al. 2008). Due to the absence of data concerning the possibility of Na,K-ATPase involvement in possible gender specific protection during diabetes, this present study was designed to investigate the influence of acute diabetes on properties of Na,K-ATPase. Characterization of kinetic properties of the enzyme and estimation of α1 subunit abundance in male and female rats were used as a tool.

Materials and Methods

Animal model

Diabetes mellitus in male and female rats was induced by a single intraperitoneal dose of STZ of 65 $mg \cdot kg^{-1}$. STZ was dissolved in 0.1 $mol \cdot l^{-1}$ citrate buffer, pH 4.5. The animals were fasted overnight prior to STZ administration. Water and food were available immediately after dosing. Eight days after STZ administration, animals with plasma glucose level higher than 10 $mmol \cdot l^{-1}$ were considered diabetic and were included in this study. Control groups received a single dose of 0.1 $mol \cdot l^{-1}$citrate buffer. We had four groups. Group FD was acute diabetic group of female rats. Group FC served as a female control. Group MD was acute diabetic of male rats and MC was control group to diabetic male rats.

During the experiment, the animals were housed in groups of 3 in cages of the type T4 Velaz (Prague, Czech Republic) with bedding composed of wood shaving (exchanged daily). All rats were allowed free access to food and drinking water. The animal room was air-conditioned and the enviroment was continiously monitored for the temperature of 23 ± 1°C with relative humidity of 55 ± 10%. All the experiments were terminated in the age of animals 16 weeks.

At the end of experiment, glucose plasma level was measured by commercial glucose GOD 250 kit (PLIVA-Lachema, Brno, Czech Republic). The kidneys were immediately frozen in liquid nitrogen and stored for further investigations of Na,K-ATPase properties. All experiments were approved by the Veterinary Council of the Slovak Republic (Decree No. 289, part 139, July 9th 2003) and they conform with Principles of Laboratory Animals Care (NIH publication 83-25, revised 1985).

Preparation of tissue fractions for kinetic measurements

The plasmalemmal membrane fraction from kidney was isolated according to Jorgensen (1974). Amount of proteins was determined by the procedure of Lowry et al. (1951) using bovine serum albumin as a standard.

Kinetic measurements of Na,K-ATPase

ATP-kinetics of Na,K-ATPase was estimated at a temperature of 37°C measuring the hydrolysis of ATP by 10 μg plasmalemmal proteins in the presence of increasing concentrations of substrate ATP (0.16–8.0 $mmol \cdot l^{-1}$). The total volume of medium was 0.5 ml containing (in $mmol \cdot l^{-1}$): $MgCl_2$ 4, KCl 10, NaCl 100 and imidazole 50 (pH 7.4). After 20 min of pre-incubation in substrate-free medium, the reaction was started by addition of ATP and after 20 min the reaction was stopped by addition of 0.3 ml 12% ice-cold solution of trichloroacetic acid. The liberated inorganic phosphorus was determined according to Taussky and Shorr (1953). In order to establish the Na,K-ATPase activity, the ATP hydrolysis that occurred in the presence of Mg^{2+} only was subtracted.

The Na,K-ATPase kinetics for cofactor Na^+ was determined by the same method, in the presence of increasing concentration of NaCl (2.0–100.0 $mmol \cdot l^{-1}$). The amount of ATP was constant (8 $mmol \cdot l^{-1}$).

From obtained data by direct nonlinear regression, the following kinetic parameters were evaluated: V_{max}, K_m, K_{Na}. The parameter V_{max} represents the maximal velocity, K_m and K_{Na} values represent the concentrations of ATP or Na^+ necessary for half maximal activation of the enzyme. All results were expressed as mean ± S.E.M. The significance of differences between the individual groups was determined with using of ANOVA and Bonferroni test. A value of $p < 0.05$ was regarded as significant.

Preparation of tissue fractions for electrophoresis and immunochemical Western blot analysis

The tissue samples from kidneys were crushed in liquid nitrogen and consequently re-suspended in ice-cold buffer containing (in $mmol \cdot l^{-1}$): 50 Tris-HCl, 250 sucrose, 1.0 dithiothreitol, 1.0 phenylmethylsulfonylfluoride (pH 7.4) and homogenized with a glass-teflon homogenizer. The homogenates were centrifuged at 800 × *g* for 5 min at 4°C, pellets after this centrifugation were discarded and the supernatants were centrifuged again at 9300 × *g* for 30 min. The supernatants after this second centrifugation were discarded again and the pellets were re-suspended in homogenizing buffer supplemented with 0.2% Triton X-100 and centrifuged

at 9300 × *g* for 1 min. The Triton X-100 soluble supernatants represented the particulate fractions. The protein concentrations were estimated by the method of Lowry (1951).

Electrophoresis and immunochemical Western blot analysis

Samples of particular protein fractions containing equivalent amounts of proteins (90 μg) per lane were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (1970). Total contents of α1 subunits of Na,K-ATPase were estimated by Western blot analysis. For primary immunodetection of α1 subunit of Na,K-ATPase, specific anti-α1 antibody (from Sigma) was used. After electrophoretic separation, proteins were transferred to nitrocellulose membrane (Western blot assays). As the secondary antibodies peroxidase-labelled anti-mouse imunoglobulins (Amersham Biosciences) were used. Bound antibodies were detected by the enhanced chemiluminescent method.

Quantification of protein levels was performed using ImageJ program. Data were expressed as means ± S.E.M. Statistical significance of differences between the groups was analyzed by the unpaired Student's *t*-test. Differences were considered as significant at $p < 0.05$.

Results

Body weight, kidney weight

The gain in body weight was lower in both diabetic groups. In MD group this reduction represented 22% and in FD group 14% as compared to the respective controls. The kidney weight was unchanged in both diabetic groups investigated. On the other hand, the ratio of kidney weight versus body weight was significantly higher in both diabetic rats as comparing with adequate control groups. In FD group, the ratio was higher by 31% and in MD group it was higher by 29% (Table 1).

Table 1. Influence of the STZ-induced diabetes mellitus on weight parameters of males and females measured at the end of acute experiments (8 days)

Groups of rats	Bw (g)	Kw (L+R) (mg)	Kw (L+R)/Bw ($mg \cdot g^{-1}$)
FC	190 ± 6	1457 ± 85	7.7 ± 0.3
FD	163 ± 7[a]	1638 ± 114	10.0 ± 0.4[a]
MC	278 ± 6	1913 ± 91	7.3 ± 0.3
MD	216 ± 4[b]	2176 ± 80	9.4 ± 0.7[b]

Data represent means ± SEM at the end of experiment, $n = 7$ in all groups. Bw, body weight; Kw, kidney weight; L+R, left+right; FC, female control rats; FD, female rats with STZ-induced diabetes mellitus; MC, male control rats; MD, male diabetic rats; [a] $p < 0.005$ as compared to FC group, [b] $p < 0.005$ as compared to MC group.

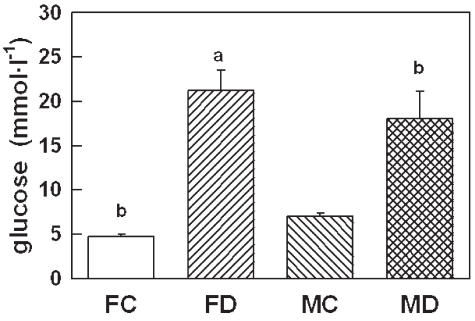

Figure 1. Plasma level of glucose in female control rats (FC), in female rats with STZ-induced diabetes mellitus (FD), in male control rats (MC) and in male diabetic rats (MD). [a] $p < 0.005$ as compared to FC group; [b] $p < 0.005$ as compared to MC group; $n = 7$ in all groups.

Level of plasma glucose

In FC group, the glucose level was lower by 33–45% as compared to MC group. Administration of STZ resulted in significantly higher level of plasma glucose in both genders. In FD group, the glucose concentration was increased by 351%, and in MD group the increase represented 159% (Fig. 1).

Kinetic measurements

Comparison of FC with MC group did not result in variations of kinetic properties of Na,K-ATPase molecule. When activating the enzyme with increasing concentration of ATP or Na^+ we did not observe any significant alterations of the enzyme activity between the FC and MC groups (Figs. 2 and 4). Consequently, the kinetic parameters for activation of the enzyme with ATP or Na^+ did not reveal gender-related changes in control animals (Figs. 3 and 5).

Acute diabetes induced significant increase in Na,K-ATPase activities in both genders. When activating the enzyme with increasing concentration of ATP we observed a slight increase in the enzyme activity in FD as well as in the MC group, as compared to respective controls. However, the above stimulation of Na,K-ATPase revealed gender specific variations. In female rats, the effect rose up gradually with increasing concentration of ATP, reaching the maximum (17%) at 8 $mmol \cdot l^{-1}$of ATP. On the other hand, in diabetic males the stimulatory effect decreased with increasing concentration of substrate from 18% at 0.16 $mmol \cdot l^{-1}$ to 12% at 8.0 $mmol \cdot l^{-1}$of ATP (Fig. 2). Evaluation of the above data by the method of nonlinear regression resulted in statistically significant increase in V_{max} values by 20% in FD and by 23% in MD group, as compared to respective controls. The K_m value

was significantly increased by 24% in FD group but in MD group it remained unchanged (Fig. 3).

Direct comparison of FD and MD groups showed higher stimulation of Na,K-ATPase activity in males. The effect decreased from 38 to 12% with increasing concentration of substrate in the range 0.16–8.0 mmol·l^{-1} of ATP (Fig. 2). The V_{max} value was increased by 10% and the K_m value was lower by 24% in the MD, as compared to FD group (Fig. 3).

When activating the enzyme with increasing concentrations of NaCl we observed an increase in the enzyme activity in both diabetic groups. In the FD group, the stimulation was constantly 12% throughout the investigated concentration of NaCl. In the MD group, the increase represented 20% at the lowest concentration of NaCl (2 mmol·l^{-1}). With growing concentrations of the cofactor, the effect slightly decreased to 12% in the presence of the highest concentration of NaCl (100 mmol·l^{-1}) (Fig. 4). Evaluation of the above data by the method of nonlinear regression showed that acute diabetes in rats resulted in increased V_{max} by 21% in FD and by 24% in MD group. The value of K_{Na} remained unchanged by diabetes in both genders (Fig. 5). Direct comparison of FD and MD groups showed slightly higher stimulation (5–9%) of Na,K-ATPase activity in males resulting in increased V_{max} value by 10% in the MD group (Fig. 4). The K_{Na} value did not show gender-dependent changes in FD and MD groups (Fig. 5).

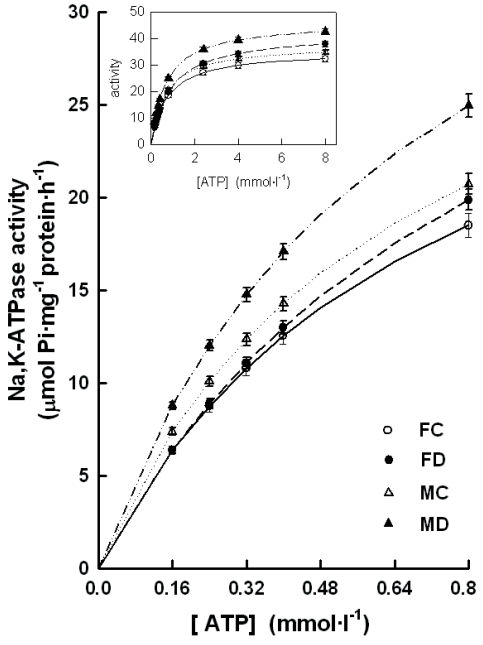

Figure 2. Activation of renal Na,K-ATPase by low concentrations of substrate ATP in female control rats (FC), in female rats with STZ-induced diabetes mellitus (FD), in male control rats (MC) and in male diabetic rats (MD). Insert: activation of the enzyme in the whole investigated concentration range of ATP.

Quantification of Na,K-ATPase α1 subunit

Direct comparison of abundance of Na,K-ATPase in control groups of both genders resulted in significantly lower presence of α1 subunit by about 16–21% in FC group. Similarly, comparison of diabetic groups FD vs. MD resulted in significant decrease by about 20% in the female group as compared to male group. Comparison of particular diabetic groups with their respective controls yielded the following data. Diabetes induced an increase in relative amount of Na,K-ATPase α1 subunit in both genders. In males (MD vs. MC), the increase represented 94% and in females 107% (FD vs. FC) (Fig. 6).

Discussion

Recent studies have shown that diabetes induces many different complications throughout the organism, e.g. neuropathy accompanied with decrease in nerve conduction velocity (Skalska et al. 2008), alterations in endothelium and ultrastructure in femoral and mesenteric arteries (Sotnikova et al. 2006), disturbances of plasma lipid metabolism (Vojtasakova et al. 2007; Soulimane-Mokhtari et al. 2008), increase in aldose reductase activity in lens (Djoubisie et al. 2006), decrease in oxidative energy production in the cardiac tissue (Ferko et al. 2006), impaired function of ion transporting Na,K-ATPase in heart (Vlkovicova et al. 2006) and in kidney (Vrbjar et al. 2007).

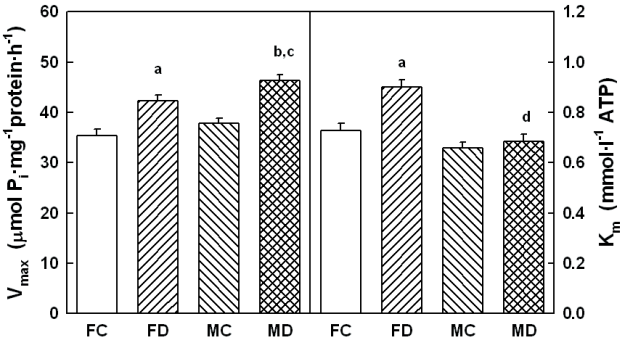

Figure 3. Kinetic parameters of renal Na,K-ATPase during activation with ATP in female control rats (FC), in female rats with STZ-induced diabetes mellitus (FD), in male control rats (MC) and in male diabetic rats (MD). The parameter V_{max} represents the maximal velocity of enzyme reaction, K_m value refers to the concentration of ATP necessary for half maximal activation of the enzyme. Data represent means ± S.E.M, $n = 9$ in each group. [a] $p < 0.001$ as compared to the FC group; [b] $p < 0.001$ as compared to the MC group; [c] $p < 0.01$ as compared to the FD group; [d] $p < 0.001$ as compared to the FD group.

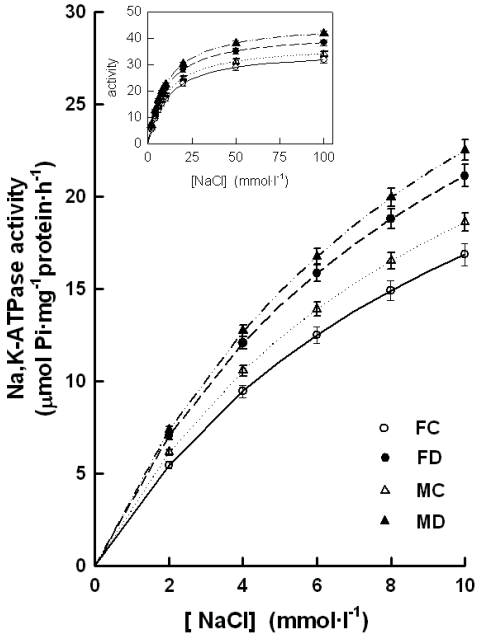

Figure 4. Activation of renal Na,K-ATPase by low concentrations of cofactor Na^+ in female control rats (FC), in female rats with STZ-induced diabetes mellitus (FD), in male control rats (MC) and in male diabetic rats (MD). Insert: Activation of the enzyme in the whole investigated concentration range of NaCl.

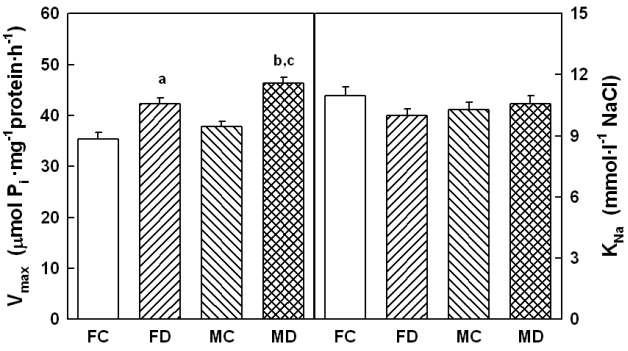

Figure 5. Kinetic parameters of renal Na,K-ATPase during activation with Na^+ in female control rats (FC), in female rats with STZ-induced diabetes mellitus (FD), in male control rats (MC) and in male diabetic rats (MD). The parameter V_{max} represents the maximal velocity of enzyme reaction, K_{Na} value refers to the concentration of Na^+ necessary for half maximal activation of the enzyme. Data represent means ± S.E.M, $n = 9$ in each group. [a] $p < 0.001$ as compared to the FC group; [b] $p < 0.001$ as compared to the MC group; [c] $p < 0.01$ as compared to the FD group.

In our experiments, in rats suffering STZ-induced diabetes for 8 days, significant changes in body weight were observed. Previously published studies documented that in acute 7 days lasting STZ-induced diabetes the reduction represented 10% in male rats when STZ was used in a dose 50 mg·kg^{-1} (Davel et al. 2000). The 22% decrease in body weight observed in our acute form of experiment for male diabetic rats may be explained by the higher dose of 65 mg·kg^{-1} of STZ. The present study brings an indication about a probable gender specific effect in female rats as the loss in weight gain was significantly lower in female diabetic rats amounting 14% only. The explanation of the mechanism of the above effect remains unclear and needs further investigation. Even if the relative kidney weight (kidney weight / body weight ratio) was significantly increased by acute form of diabetes, the unchanged kidney weight in both genders excluded the suspicion of renal hypertrophy. The relative kidney weight seems to be changed only due to lower body weight in diabetic groups of both genders.

The main aim of the present study was to establish whether acute STZ-induced diabetes in rats is followed by

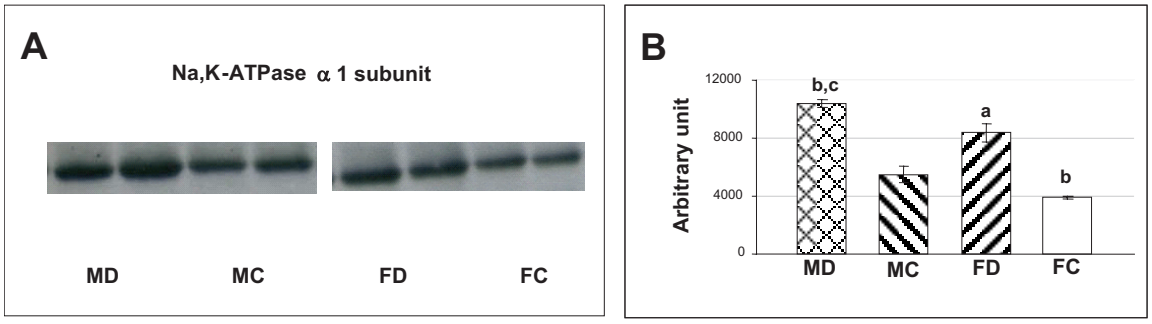

Figure 6. A. Immunoblot analysis. **B.** Relative densities of bands. Relative abundance of Na,K-ATPase α1 subunit in kidney after acute diabetes in female control rats (FC), in female rats with STZ-induced diabetes mellitus (FD), in male control rats (MC) and in male diabetic rats (MD). [a] as compared to the FC group; [b] as compared to the MC group; [c] $p < 0.01$ as compared to the FD group; $n = 4$ in all groups.

any functional changes of renal Na,K-ATPase. Renal epithelial cells contain high densities of mitochondria necessary to produce sufficient ATP for the active transport of Na^+ ions. Approximately 90% of oxygen extracted by mitochondria in the kidney is used for the work required for Na^+ reabsorption in the nephron (Welch 2006). Due to the importance of renal Na,K-ATPase in maintaining the sodium homeostasis in the organism, its activity and abundance is intensively studied in condition of diabetes.

Our studies of the kinetics of Na,K-ATPase revealed gender specific changes in qualitative properties of the enzyme during the acute diabetes. In female rats, hyperglycemia induced deterioration of the affinity of the ATP binding site in the enzyme molecule as indicated by increased K_m value. In male rats, the ATP binding site of the enzyme was resistant to diabetes-induced complications as suggested by unchanged value of K_m. In this context may be interesting the fact that in female rats the same dose of STZ was followed by two-fold increase in blood glucose level as compared to male rats (351% in FD group vs. 159% in MD group). This gender specific difference in hyperglycemia may be responsible for the worsening of ATP binding properties of Na,K-ATPase in female rats. The diabetes-induced conformational changes of the enzyme molecule in female rats are probably restricted to the cytoplasmic loop of the enzyme containing the ATP binding site. The sodium binding site localized nearby the intracellular surface of the cell membrane seems to be unaffected as suggested by unchanged value of K_{Na}.

Concerning the quantity of enzyme our study offers two series of information about Na,K-ATPase during acute diabetes in the renal tissue. First information is given by Western blot analysis and the second information is given by analysis of V_{max} values obtained from kinetic studies. Since V_{max} is independent of substrate concentration, it provides information about the changes in number of active enzyme molecules in the renal tissue. Acute diabetes induced higher enzyme activity accompanied with significant increase in the V_{max} value suggesting enhanced number of active Na,K-ATPase molecules in both genders. This hypothesis is supported by increased abundance of Na,K-ATPase α1 subunit in diabetic male as well as female rats as documented by presented Western blots. Up to our knowledge there is a lack of information about diabetes-induced alterations of the Na,K-ATPase in females. Our data of significant increase in the level of α1 subunit induced by acute form of diabetes in male rats are in agreement with previous observations about a significant increase in the activity and expression of the enzyme as a consequence of acute diabetes (Lal et al. 2000; Scherzer and Popovtzer 2002). The above changes of the enzyme in kidney seem to be a tissue specific process, as the enzyme in cardiac tissue as well as in the vascular smooth muscle revealed significant decrease in activity and the amount of α1 subunit (Ziegelhoffer et al. 1996; Ver et al. 1997; Davel et al. 2000). The observed accumulation of Na,K-ATPase α1 subunit in kidney supports its importance in adaptation to complications induced by acute diabetes, as it was hypothesized by Ku et al. (1986). Since Na,K-ATPase-mediated ion transport is the major consumer of metabolic energy in the kidney, utilizing about 20–30% of ATP production (Jorgensen and Pedersen 2001), the enzyme is critically important to renal function. It may be suggested that the increase in activity and abundance of renal Na,K-ATPase observed in acute form of diabetes is an essential component of the renal hyperfunction seen in this disease and may represent an important adaptive change in response to hyperglycemia.

An other important subject of this study was the investigation of amounts of Na,K-ATPase α1 subunit in view of possible gender dependent differences in male and in female rats. Our data showed that the abundance of α1 subunit is significantly higher in control male rats as compared to females. This may be explained by probable contribution of 17-β estradiol in regulation of Na,K-ATPase synthesis in the renal tissue. Previously it was shown that administration of 17-β estradiol to non-diabetic ovariectomized rats decreased the abundance of Na,K-ATPase α1 subunit in the outer medulla (Riazi et al. 2006). In view of higher expression of Na,K-ATPase α1 subunit in males, it could be expected that the number of active enzyme molecules would be also increased. However, the present data demonstrated, that the number of active enzyme molecules seems to be comparable in both genders as suggested by similarities of V_{max} values in control male and female rats. So, the excessively expressed molecules of α1 subunit in male rats are probably not fully active.

The diabetes-induced increased expression of α1 subunit in both genders (amounting 94% in males and 107% in females) is followed by increased activity of the enzyme. But in this elevation of the activity not all of the newly synthesized enzyme molecules are involved as the increase in the number of active molecules is smaller (representing 23% in males and 20% in females) as indicated by increased V_{max} values.

In summary, our present data provide an evidence for higher abundance of Na,K-ATPase α1 subunit in renal tissue of control male rats as compared to respective female group. Acute diabetes induced higher expression of Na,K-ATPase α1 subunit in both genders, but not all of the newly synthesized enzyme molecules were fully active, as suggested by our kinetic studies.

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