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Effect of duration of diabetes mellitus type 1 on properties of Na, K-ATPase in cerebral cortex

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Abstract Time sequence study was performed to characterize the effects of diabetes mellitus type 1 on properties of the Na, K-ATPase in cerebral cortex derived from normal and streptozotocin (STZ)-diabetic rats of both genders. The samples were excised at varying time intervals of diabetes induced by STZ (65 mg kg^{-1}) for 8 days, and 8 and 16 weeks. Expression of α1–3 isoforms of Na, K-ATPase was not altered in statistically significant level during all stages of diabetes neither in female nor in male rats as revealed from Western blot analysis. Studies of kinetic properties of the enzyme resulted in variations in active number of Na, K-ATPase molecules as well as its qualitative properties. Sixteen-week-old control male rats showed better affinity to substrate as indicated by 13 % decrease of K_m value. The effect persisted also in males subjected to 8 days lasting diabetes; however, in males subjected to 8 weeks lasting diabetes, the effect was lost. In 25-week-old rats, the Na, K-ATPase revealed again altered properties in males and females but the mechanism of the variation was different. In females, the number of active molecules of Na, K-ATPase was higher by 32 % in controls and by 17 % in rats with chronic diabetes when comparing to respective male groups as suggested by increased value of V_{max}. So the properties of Na, K-ATPase in cerebral cortex, playing crucial role in maintaining intracellular homeostasis of Na^+ ions, depend on gender, age, and duration of diabetic insult.

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Introduction

Diabetes mellitus type 1 (DM1) is a very common metabolic disorder, which is caused by absolute or relative deficiency of insulin. This is connected with variety of complications in many organs [1]. DM1 is associated with neurophysiological and structural changes in the brain which are considered as "diabetic encephalopathy" [2, 3].

In the brain, one of the components of blood–brain barrier is the Na, K-ATPase, playing a crucial role in maintaining the homeostasis of Na^+ and K^+ ions in the intracellular space and in cerebrospinal fluid [4]. This enzyme located in plasma membrane transports three ions of sodium out of the cell in exchange for two ions of potassium utilizing the energy of one molecule ATP, maintaining thus membrane potential, which is important for excitable tissues, particularly for nerve and muscle tissues [5].

Recent data showed that streptozotocin (STZ)-induced diabetes affects the properties of Na, K-ATPase in many organs, for example, in heart [6–11], kidney [12–14], brain [15], liver [16], skeletal muscle [17], etc. The alterations of Na, K-ATPase may reflect a possible mechanism through which diabetes could affect neuronal excitability, metabolic energy production, and certain systems of neurotransmission [18]. It was documented that in male rats, 8 weeks lasting diabetes induces loss of Na, K-ATPase activity in brain homogenate [18]. In kidney, previously it was shown that diabetes induces various effects on the Na, K-ATPase on gender-specific manner depending on the duration of experimental diabetes [13, 14]. We

hypothesized that in cerebral tissue, the Na, K-ATPase which was shown previously to be sensitive to diabetes might be affected on different way in male and female subjects during various stages of diabetes. However, there is a lack of sufficient information concerning the time course and probable gender differences in properties of cerebral Na, K-ATPase during the development of diabetes. Therefore, the aim of our study was oriented to investigation of functional properties of Na, K-ATPase during development of DM1 during acute, sub-chronic, and chronic states in cerebral cortex derived from normal and STZ-diabetic rats of both genders.

Materials and methods

Animal model

The experimental model of diabetes mellitus in male and female rats was induced by a single intraperitoneal application of STZ in a dose of 65 mg kg^{-1}. STZ was dissolved in 0.1 mol l^{-1} citrate buffer with pH 4.5. The animals were fasted overnight prior to STZ administration.

To follow the development of the disease, three experimental models mimicking the acute, sub-chronic, and chronic states were investigated.

Model for acute DM1

At this model lasting 8 days, we used 15-week-old Wistar rats divided in following groups:

- (1) group—aMC: control males ($n = 9$),
- (2) group—aFC: control females ($n = 9$),
- (3) group—aMDIA: diabetic males ($n = 9$),
- (4) group—aFDIA: diabetic females ($n = 9$).

The animals were sacrificed in the age of 16 weeks.

Model for sub-chronic DM1

At this model lasting 8 weeks, we used 8-week-old Wistar rats divided in following groups:

- (1) group—sMC: control males ($n = 9$),
- (2) group—sFC: control females ($n = 9$),
- (3) group—sMDIA: diabetic males ($n = 9$),
- (4) group—sFDIA: diabetic females ($n = 9$).

The animals were sacrificed in the age of 16 weeks.

Model for chronic DM1

At this model lasting 16 weeks we used 8 weeks old Wistar rats divided in following groups:

- (1) group—chMC: control males ($n = 9$),
- (2) group—chFC: control females ($n = 9$),
- (3) group—chMDIA: diabetic males ($n = 9$),
- (4) group—chFDIA: diabetic females ($n = 9$).

The animals were sacrificed in the age of 24 weeks.

In all three above described models, water and food were available immediately after STZ administration, animals with plasma glucose level higher than 10 mmol l^{-1} were considered diabetic and were included in this study. Control groups received a single dose of 0.1 mol l^{-1} citrate buffer.

During the experiment, the animals were housed in groups of three 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 (ad libitum). The animal room was air-conditioned, and the environment was continually monitored for the temperature of 23 ± 1 °C with relative humidity of 55 ± 10 %. At the end of experiment (after 8 days, 8 weeks, and 16 weeks), rats were killed under thiopental anesthesia; glucose plasma level was measured by commercial glucose GOD 250 kit (PLIVA-Lachema, Brno, Czech Republic). The cerebral cortexes 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, 9th July 2003).

Preparation of plasmalemmal fraction for kinetic measurements

The plasmalemmal membrane fraction from rat cerebral cortex was isolated according to [19] with slight modifications. Briefly, the brain tissue was homogenized in cold isolation medium containing (in mmol l^{-1}) 250 sucrose, 25 imidazole, 1 EDTA (pH 7.4) using a tissue disruptor (3 × 5 s at a setting of 3, Polytron PT-20). The homogenate was centrifuged at 6000 × g for 15 min. The sediment was rehomogenized and centrifuged again at 6000 × g for 15 min. The collected supernatants from both centrifugations were re-centrifuged at 48,000 × g for 30 min, and the final sediment was re-suspended in the isolation medium. An aliquot was removed for determination of proteins by the method of [20] using bovine serum albumin as a standard.

Kinetic measurements of Na, K-ATPase

ATP-kinetics of Na, K-ATPase were 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 l^{-1}). The

total volume of medium was 0.5 ml containing (in mmol l^{-1}) 4 $MgCl_2$, 10 KCl, 100 NaCl, and 50 imidazole (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 [21]. 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 l^{-1}) at constant amount of ATP (8 mmol l^{-1}). The kinetic parameters V_{max}, K_m, and K_{Na} were evaluated from obtained data by direct nonlinear regression. 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 ± SEM. The significance of differences between the individual groups was determined with using of two-way ANOVA and Holm–Sidak 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 cerebral cortex were re-suspended in ice-cold buffer containing (in mmol 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. Following this second centrifugation, the supernatants 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 particular membrane fractions. The protein concentrations were estimated by the method of [22].

Electrophoresis and immunochemical Western blot analysis

Samples of particular protein fractions containing equivalent amounts of proteins per lane (30 µg per lane) were separated by sodium dodecyl sulfate–polyacrylamide gel (10 %) electrophoresis. For Western blot assays, separated proteins were transferred from gel to a nitrocellulose membrane. The quality of the transfer was controlled by Ponceau S staining of nitrocellulose membranes after the transfer. Specific antibodies against α1–3 subunits of Na, K-ATPase (all from Millipore) were used for the primary immunodetection using individual membrane for each subunit. Peroxidase-labelled anti-rabbit immunoglobulin (Santa Cruz) was used as the secondary antibody. Bound antibodies were detected by the enhanced chemiluminescence detection method. Densitometrical quantification of protein levels was performed by comparison to loading control beta-actin (from Abcam) using a Carestream program. Data were expressed as mean ± SEM statistical significance of differences between the groups was analyzed by two-way ANOVA using the Holm–Sidak test. Differences were considered as significant at $p < 0.05$.

Results

Single intraperitoneal application of STZ to 15-week-old rats induced a significant increase of the blood glucose after 8 days in both genders. In this acute model characterized by elevation of the glucose in males by 186 % and in females by 298 % (Table 1), a statistically significant loss in body weight gain was observed in diabetic rats of both genders when comparing to corresponding control groups (Table 1).

In vitro activation of the Na, K-ATPase with increasing concentrations of the substrate (ATP) in isolated plasmalemmal membrane fraction from rat cerebral cortex revealed higher activity by 12 % throughout the investigated concentration range in diabetic males when comparing to control males (Fig. 1). Evaluation of kinetic parameters resulted in slight but statistically insignificant increase of V_{max} value together with unchanged value of K_m as a consequence of acute diabetes. In female rats, acute diabetes was followed by stepwise decreasing inhibition, when increasing the concentration of ATP. In the presence of the lowest concentration 0.16 mmol l^{-1} of ATP, the inhibition represented 17 %, and in the presence of the highest 8 mmol l^{-1}, the inhibition amounted 11 % (Fig. 1). Evaluation of kinetic parameters resulted in statistically significant decrease of the V_{max} and statistically insignificant increase of the K_m value in female rats as a consequence of acute diabetes (Fig. 2). Studying the gender specificity of the Na, K-ATPase in cerebral cortex, we observed significant increase of the K_m value in females independently on the physiological state in control as well as in rats subjected to acute diabetes (Fig. 2).

Activation of the Na, K-ATPase with increasing concentration of sodium showed continual increase of its activity in the whole concentration range in diabetic males when comparing to control males. The effect decreased stepwise with increasing concentrations of Na^+ from 15 % observed in the presence of 2 mmol l^{-1} of NaCl to 6 % observed in the presence of 100 mmol l^{-1} (Fig. 3)

Table 1 Body weight of rats and plasma level of glucose in the model of acute diabetes

Experimental groups	BW at the start (g)	BW at the end (g)	BW gain (g)	Glucose (mmol l^{-1})
aMC	234 ± 6	275 ± 7	41 ± 4	7.7 ± 0.5
aFC	173 ± 6	194 ± 6	21 ± 5[c]	4.4 ± 0.5[c]
aMDIA	244 ± 5	221 ± 4	−23 ± 5[a]	22.0 ± 1.3[a]
aFDIA	169 ± 3	162 ± 5	−7 ± 4[b, d]	17.5 ± 0.9[b, d]

At this model lasting 8 days we started with 15 weeks old Wistar rats. The animals were sacrificed in the age of 16 weeks

[a] $p < 0.001$ as compared with the aMC group

[b] $p < 0.001$ as compared with the aFC group

[c] $p < 0.001$ as compared with the aMC group

[d] $p < 0.05$ as compared with the aMDIA group

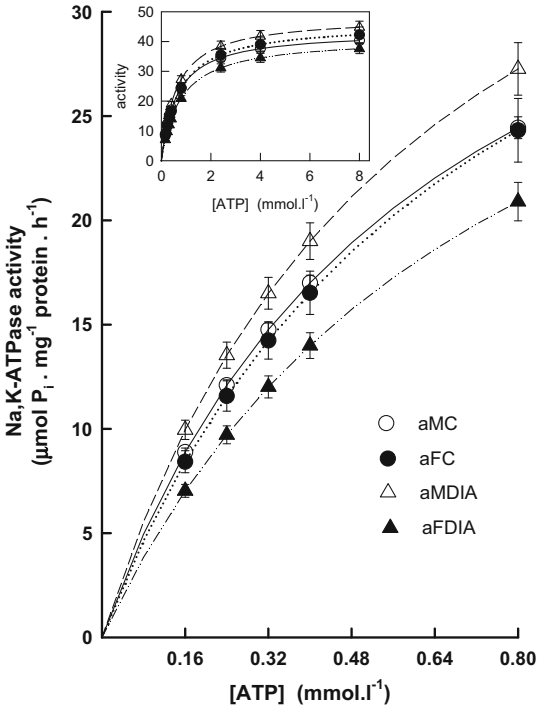

Fig. 1 Activation of Na, K-ATPase from cerebral cortex in acute model of diabetes by low concentrations of substrate ATP in female control rats (aFC), in female rats with STZ-induced diabetes mellitus during (aFDIA), in male control rats (aMC) and in male diabetic rats (aMDIA). *Inset* activation of the enzyme in the whole investigated concentration range of ATP

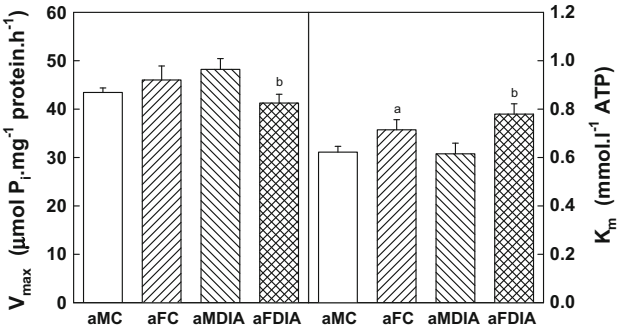

Fig. 2 Kinetic parameters of Na, K-ATPase from cerebral cortex in acute model of diabetes during activation with substrate ATP in female control rats (aFC), in female rats with STZ-induced diabetes mellitus (aFDIA), in male control rats (aMC), and in male diabetic rats (aMDIA). The parameter V_{max} 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 mean ± SEM, $n = 9$ in each group. *a* $p < 0.05$ as compared to the aMC group and *b* $p < 0.05$ as compared to the aMDIA group

resulting in slight but statistically significant decrease of the K_{Na} value as compared to control male group (Fig. 4). Studying the impact of acute diabetes on the Na, K-ATPase in females, we observed a biphasic effect. In the concentration range below 10 mmol l^{-1}, the enzyme activity was higher, and above this concentration, the activity decreased in diabetic rats as compared with control females (Fig. 3). Evaluation of kinetic parameters resulted in slight but statistically significant decrease of the K_{Na} value in females due to acute diabetes (Fig. 4). Concerning the gender specificity of the Na, K-ATPase, the only difference was observed when comparing the V_{max} values in males and females subjected to acute diabetes (Fig. 4).

Quantitative analysis of Na, K-ATPase α-subunits by Western blot (Fig. 5a) showed significant decrease of α1 subunit content as a consequence of acute diabetes in female rats. The expression of α1 subunit in healthy rats was also gender dependent as documented by 27 % decrease in control males as compared to control females (Fig. 5b).

The sub-chronic model of DM1 was characterized by elevation of the glucose by 225 % in males and by 284 % in females (Table 2). The body weight gain was in this model again lower in diabetic animals of both genders when comparing to respective control groups (Table 2).

Activation of the Na, K-ATPase with increasing concentrations of ATP showed increase of the enzyme activity in samples from rats of both genders subjected to sub-chronic model of diabetes. In males, this effect was smaller, representing 13 % increase, while in females, the

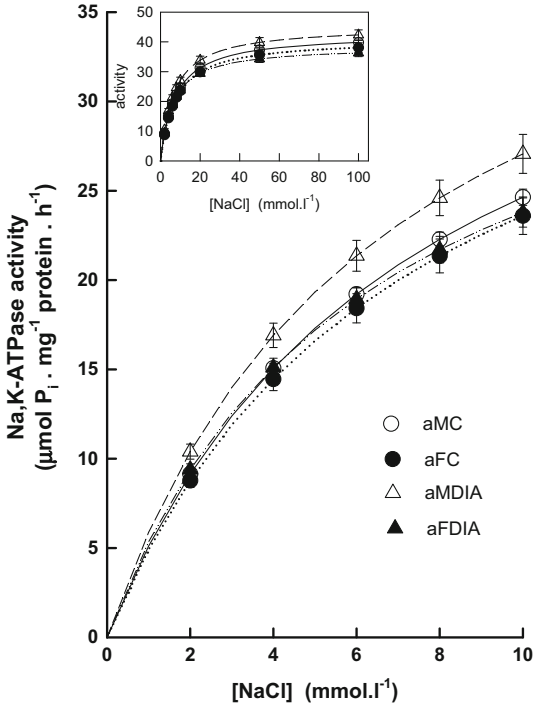

Fig. 3 Activation of Na, K-ATPase from cerebral cortex in acute model of diabetes by low concentrations of cofactor Na^+ in female control rats (aFC), in female rats with STZ-induced diabetes mellitus (aFDIA), in male control rats (aMC), and in male diabetic rats (aMDIA). *Inset* activation of the enzyme in the whole investigated concentration range of ATP

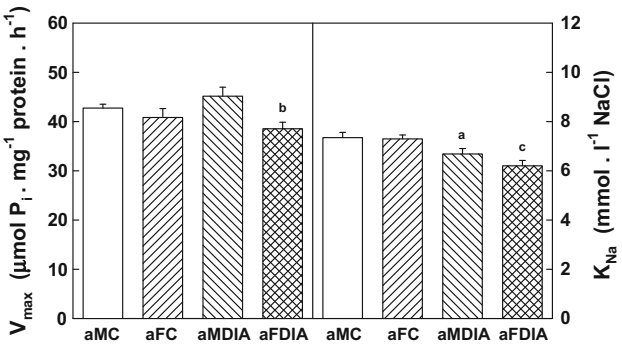

Fig. 4 Kinetic parameters of Na, K-ATPase from cerebral cortex in acute model of diabetes during activation with cofactor Na^+ in female control rats (aFC), in female rats with STZ-induced diabetes mellitus (aFDIA), in male control rats (aMC), and in male diabetic rats (aMDIA). The parameter V_{max} 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 mean ± SEM, $n = 9$ in each group. *a* $p < 0.05$ as compared to the aMC group, *b* $p < 0.01$ as compared to the aMDIA group and *c* $p < 0.001$ as compared to the aFC group

increase represented 34 % in the presence of the lowest ATP concentration (0.16 mmol l^{-1}). In both genders, the effect decreased stepwise with increasing concentration of ATP and in the presence of 8 mmol l^{-1} of substrate activities were similar to those observed in respective controls (Fig. 6). Evaluation of these processes resulted in unaltered V_{max} values, while the K_m value decreased by 30 % explicitly in female rats due to sub-chronic diabetes (Fig. 7).

When activating the enzyme from brain cortex with increasing concentrations of cofactor Na^+, we observed similar activities of the Na, K-ATPase in the whole concentration range in rats of both genders after sub-chronic diabetes as compared to respective controls (Fig. 8). Accordingly the kinetic parameters, V_{max} and K_{Na} were similar in rats subjected to sub-chronic diabetes as compared to corresponding control groups (Fig. 9).

Quantification of Na, K-ATPase α-subunits by Western blot analysis resulted in significant decrease of α3 subunit expression by 35 % in control males as compared to healthy females. By similar gender-specific difference, we observed also in diabetic animals amounting 33 % decrease of α3 subunit expression in males as compared to diabetic females. This model of diabetes was not followed by any significant alterations in expression of α3 subunit in both genders as compared to respective control groups. In expression of α1 and α2 subunits, the sub-chronic model did not induced any statistically significant alterations (Fig. 10).

In the chronic model of experimental diabetes lasting 16 weeks, the 24-week-old rats were characterized by 120 % elevation of the blood glucose in males and by 203 % in females. The significant decrease of body weight gain was observed again in both genders in consequence of this model of diabetes (Table 3).

When activating in vitro Na, K-ATPase from brain cortex with substrate in the range 0.16–8 mmol l^{-1} ATP, we did not observe significant changes in the enzyme activity in response to chronic diabetes either in male or in female rats (Fig. 11). Adequately the kinetic parameters V_{max} and K_m remained unaltered after chronic model of diabetes (Fig. 12). However, when comparing the enzyme properties from the point of view of gender specificity, we observed significant increase of enzyme activity throughout the investigated concentration range of ATP (Fig. 11) resulting in higher V_{max} (Fig. 12) in both female groups independently on their physiological state when comparing to respective male groups.

Similar trend was observed also when activating the enzyme with increasing concentrations of sodium. Chronic diabetes did not alter significantly the enzyme activities (Fig. 13) and also the kinetic parameters neither in males nor in females (Fig. 14). On the other hand, the gender specificity was manifested again independently on the physiological state. Control as well as diabetic females showed higher activities throughout the investigated concentration range of sodium (Fig. 13). Consequently, the

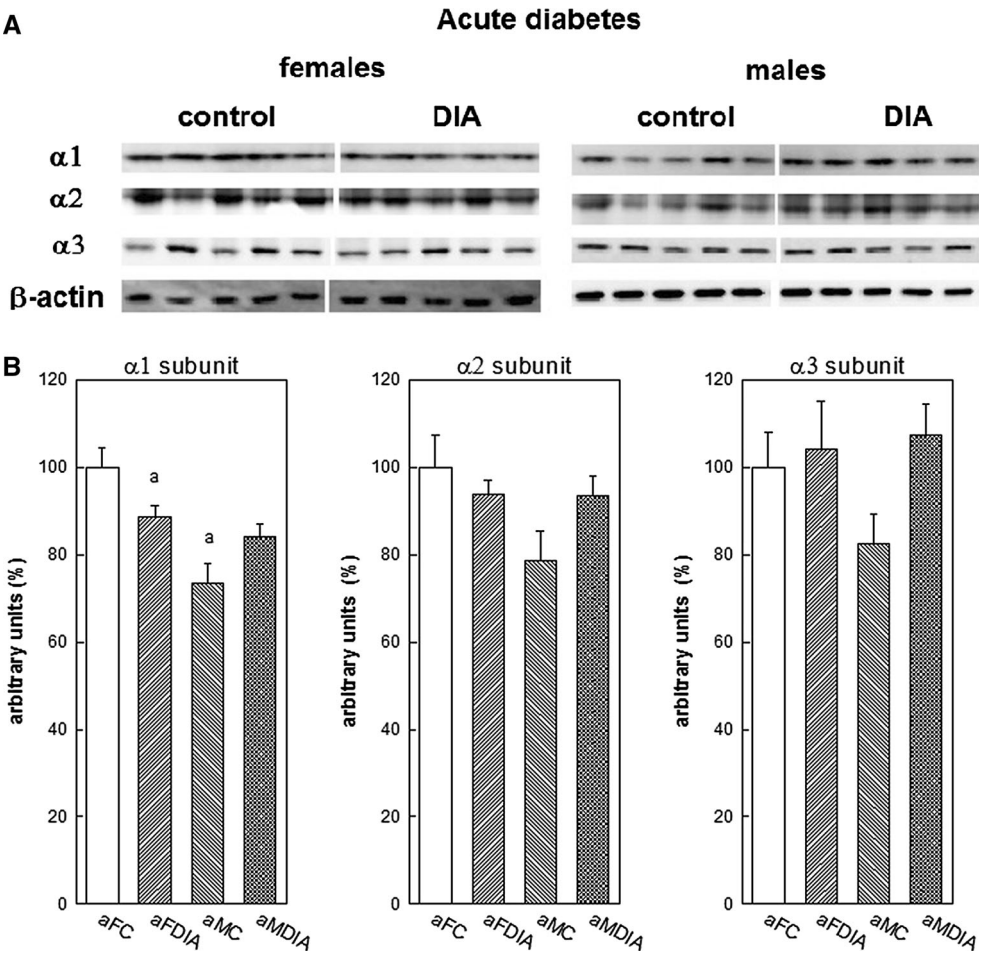

Fig. 5 **a** Immunoblot analysis of *α1–3* subunits of Na, K-ATPase from cerebral cortex in acute model of diabetes in female control rats (aFC), in female rats with STZ-induced diabetes mellitus (aFDIA), in male control rats (aMC), and in male diabetic rats (aMDIA). **b** Relative abundance of *α1–3* subunits of Na, K-ATPase. Relative densities of bands. Data represent mean ± SEM, $n = 5$ in each group. *a* $p < 0.05$ as compared to the aFC group

Table 2 Body weight of rats and plasma level of glucose in the model of sub-chronic diabetes

Experimental groups	BW at the start (g)	BW at the end (g)	BW gain (g)	Glucose (mmol l^{-1})
sMC	149 ± 5	387 ± 16	238 ± 17	7.7 ± 0.5
sFC	106 ± 2	212 ± 7	106 ± 9	4.4 ± 0.5[c]
sMDIA	154 ± 4	263 ± 10	109 ± 11[a]	25.0 ± 1.6[a]
sFDIA	108 ± 3	171 ± 7	63 ± 5[b, d]	16.9 ± 1.8[b, d]

At this model lasting 8 weeks we started with 8 weeks old Wistar rats. The animals were sacrificed in the age of 16 weeks

[a] $p < 0.001$ as compared with the sMC group

[b] $p < 0.001$ as compared with the sFC group

[c] $p < 0.001$ as compared with the sMC group

[d] $p < 0.01$ as compared with the sMDIA group

V_{max} values were significantly higher when comparing to respective male groups (Fig. 14).

Quantifying the expression of Na, K-ATPase by Western blot analysis showed that no statistically changes for α1 and α2 subunits were observed in rats of both genders subjected to chronic diabetes. The level of α3 subunits was altered only in healthy animals in gender specific manner revealing 39 % decrease in control males as compared to control females (Fig. 15).

Discussion

Our present study oriented to the time course of development of complications induced by DM1 brought interesting results concerning the gender-specific differences in physiological status of rats. Difference relating to variability of blood glucose in control rats observed in our experiment showing lower glucose content in control female rats as compared to control males is in disagreement

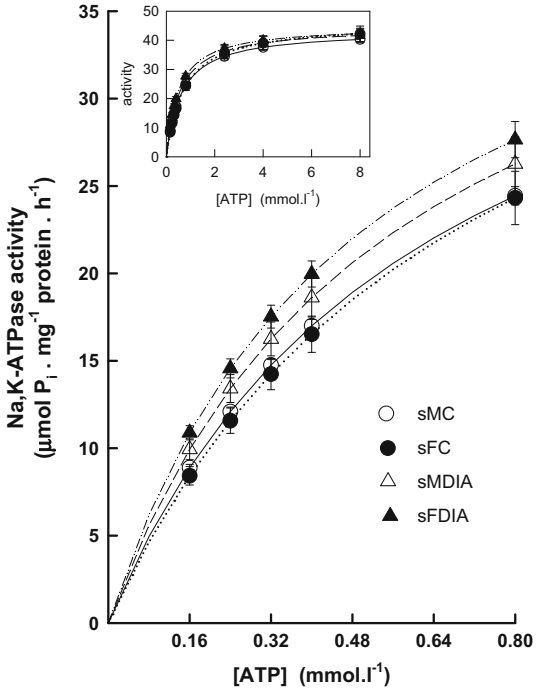

Fig. 6 Activation of Na, K-ATPase from cerebral cortex in sub-chronic model of diabetes by low concentrations of substrate ATP in female control rats (sFC), in female rats with STZ-induced diabetes mellitus (sFDIA), in male control rats (sMC), and in male diabetic rats (sMDIA). *Inset* activation of the enzyme in the whole investigated concentration range of ATP

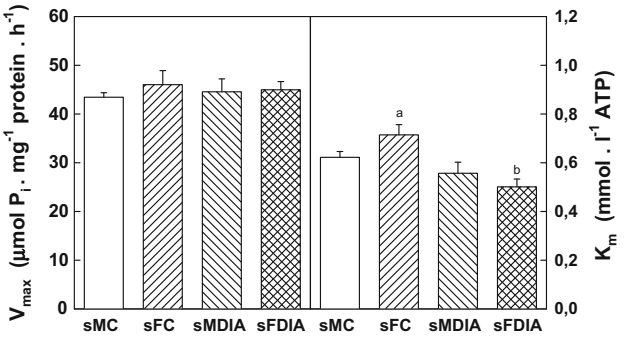

Fig. 7 Kinetic parameters of Na, K-ATPase from cerebral cortex in sub-chronic model of diabetes during activation with substrate ATP in female control rats (sFC), in female rats with STZ-induced diabetes mellitus (sFDIA), in male control rats (sMC), and in male diabetic rats (sMDIA). The parameter V_{max} 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 mean ± SEM, $n = 9$ in each group. *a* $p < 0.05$ as compared to the sMC group and *b* $p < 0.001$ as compared to the sFC group

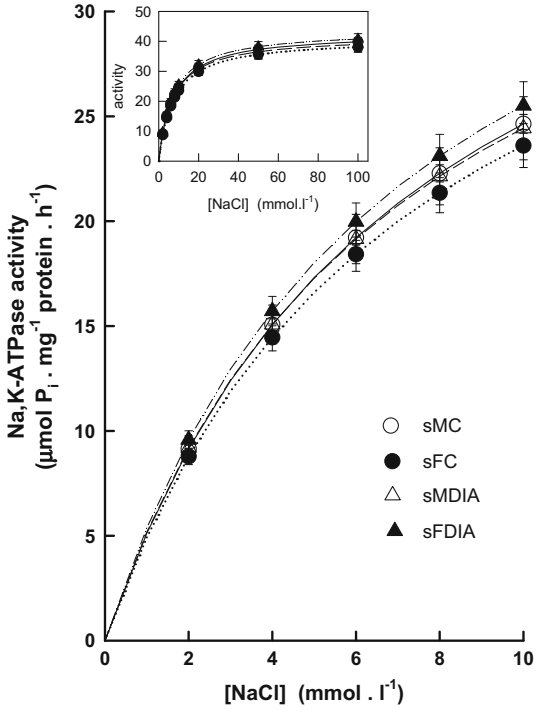

Fig. 8 Activation of Na, K-ATPase from cerebral cortex in sub-chronic model of diabetes by low concentrations of cofactor Na^+ in female control rats (sFC), in female rats with STZ-induced diabetes mellitus (sFDIA), in male control rats (sMC), and in male diabetic rats (sMDIA). *Inset* activation of the enzyme in the whole investigated concentration range of ATP

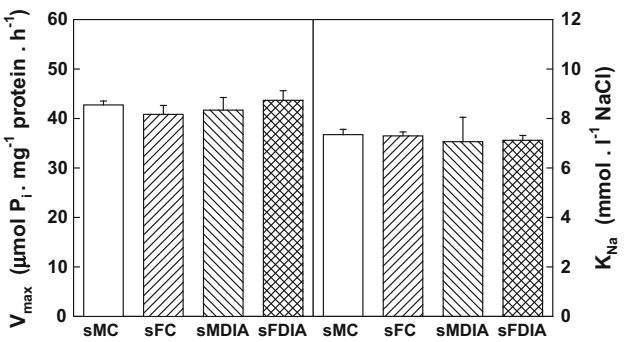

Fig. 9 Kinetic parameters of Na, K-ATPase from cerebral cortex in sub-chronic model of diabetes during activation with cofactor Na^+ in female control rats (sFC), in female rats with STZ-induced diabetes mellitus (sFDIA), in male control rats (sMC), and in male diabetic rats (sMDIA). The parameter V_{max} 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 mean ± SEM, $n = 9$ in each group

with the data of Choi et al. [23] who did not observe gender-specific differences in controls. This difference may be ascribed to strain-specific variations, as in our study, Wistar rats were used and in the study of Choi et al. [23] Sprague–Dawley rats were investigated. However, the higher content of glucose in control male Wistar rats, as a model, seems to be from this point of view nearer to human population as shown by slightly higher glucose level in men as compared to women without any history of diabetes [24]. Within this context, it is interesting that men develop

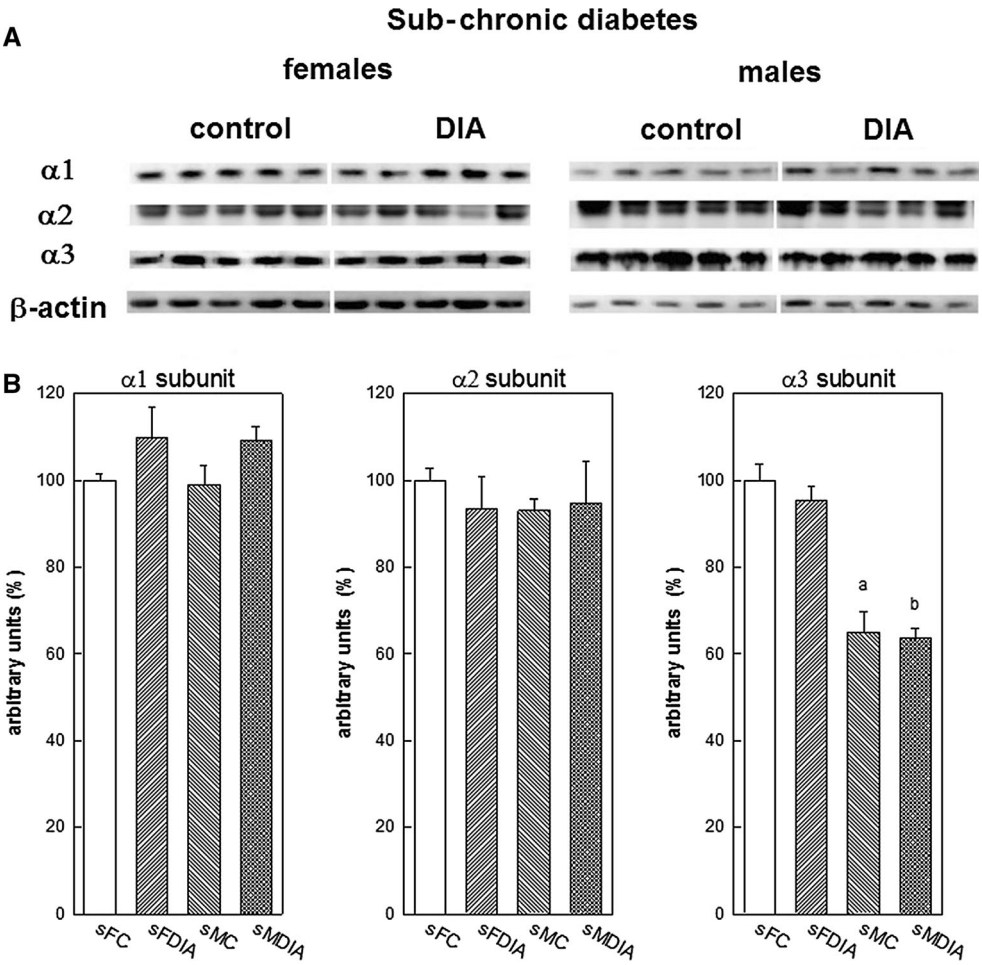

Fig. 10 **a** Immunoblot analysis of *α1–3* subunits of Na, K-ATPase from cerebral cortex in sub-chronic model of diabetes in female control rats (sFC), in female rats with STZ-induced diabetes mellitus (sFDIA), in male control rats (sMC), and in male diabetic rats (sMDIA). **b** Relative abundance of *α1–3* subunits of Na, K-ATPase. Relative densities of bands. Data represent mean ± SEM, $n = 5$ in each group. *a* $p < 0.001$ as compared to the sFC group and *b* $p < 0.001$ as compared to the sFDIA group

Table 3 Body weight of rats and plasma level of glucose in the model of chronic diabetes

Experimental groups	BW at the start (g)	BW at the end (g)	BW gain (g)	Glucose (mmol l^{-1})
chMC	129 ± 4	358 ± 9	229 ± 9	9.9 ± 0.5
chFC	125 ± 5	245 ± 5	120 ± 6	7.3 ± 0.2[c]
chMDIA	133 ± 4	245 ± 25	112 ± 26[a]	21.8 ± 2.5[a]
chFDIA	110 ± 5	152 ± 9	42 ± 7[b, d]	22.1 ± 2.0[b]

At this model lasting 16 weeks we started with 8 weeks old Wistar rats. The animals were sacrificed in the age of 24 weeks

[a] $p < 0.001$ as compared with the chMC group

[b] $p < 0.001$ as compared with the chFC group

[c] $p < 0.005$ as compared with the chMC group

[d] $p < 0.001$ as compared with the chMDIA group

diabetic neuropathy earlier than women [25] and suffer from it more often [26].

Despite the above gender-specific difference of glucose content in control Wistar and Sprague–Dawley rats, they reacted similarly after 1 week to comparable dose of STZ. Prolongation of the period of diabetes to 8 or 16 weeks in our experiments showed relatively stable overload of glucose in both genders. Even though the glucose level was more or less constant during the development of diabetes, the Na, K-ATPase in brain cortex was affected on different way at various stages of disease development.

In acute phase of diabetes, the number of active Na, K-ATPase molecules was not changed as indicated by unaltered V_{max} values for both types of enzyme activations with substrate ATP and cofactor Na^+. However, in this stage of the disease, the enzyme binds better Na^+ as

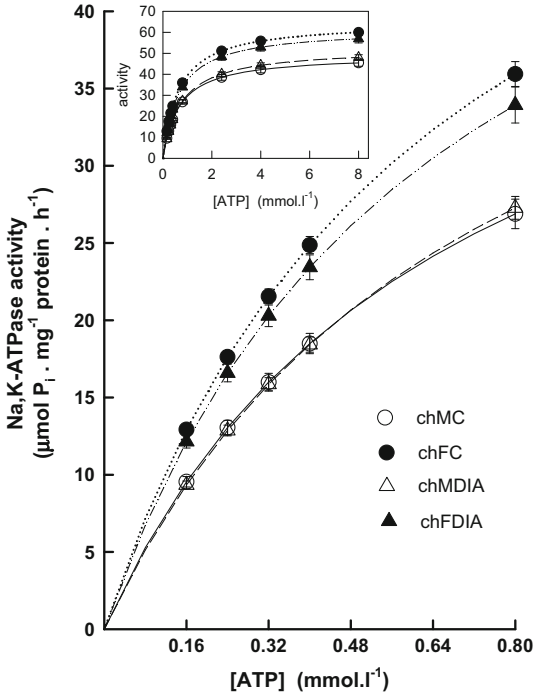

Fig. 11 Activation of Na, K-ATPase from cerebral cortex in chronic model of diabetes by low concentrations of substrate ATP in female control rats (chFC), in female rats with STZ-induced diabetes mellitus (chFDIA), in male control rats (chMC), and in male diabetic rats (chMDIA). *Inset* activation of the enzyme in the whole investigated concentration range of ATP

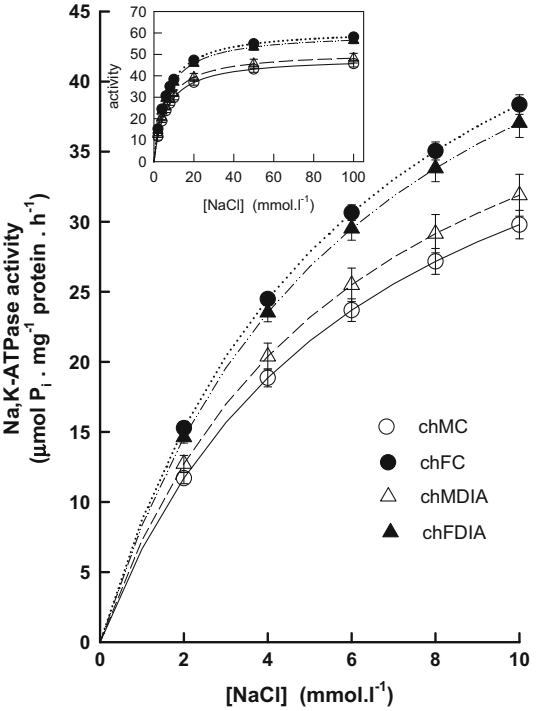

Fig. 13 Activation of Na, K-ATPase from cerebral cortex in chronic model of diabetes by low concentrations of cofactor Na^+ in female control rats (chFC), in female rats with STZ-induced diabetes mellitus (chFDIA), in male control rats (chMC), and in male diabetic rats (chMDIA). *Inset* activation of the enzyme in the whole investigated concentration range of ATP

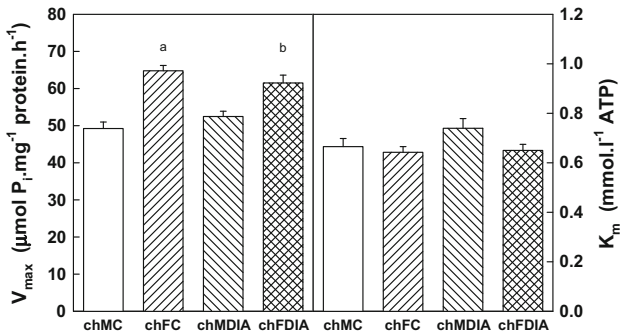

Fig. 12 Kinetic parameters of Na, K-ATPase from cerebral cortex in chronic model of diabetes during activation with substrate ATP in female control rats (chFC), in female rats with STZ-induced diabetes mellitus (chFDIA), in male control rats (chMC), and in male diabetic rats (chMDIA). The parameter V_{max} 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 mean ± SEM, $n = 9$ in each group. *a* $p < 0.001$ as compared to the chMC group and *b* $p < 0.01$ as compared to the chMDIA group

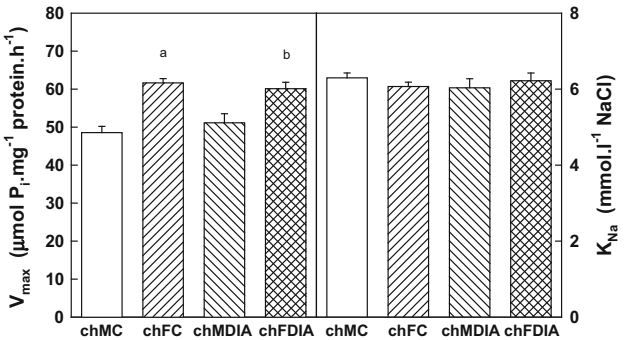

Fig. 14 Kinetic parameters of Na, K-ATPase from cerebral cortex in chronic model of diabetes during activation with cofactor Na^+ in female control rats (chFC), in female rats with STZ-induced diabetes mellitus (chFDIA), in male control rats (chMC), and in male diabetic rats (chMDIA). The parameter V_{max} 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 mean ± SEM, $n = 9$ in each group. *a* $p < 0.001$ as compared to the chMC group and *b* $p < 0.01$ as compared to the chMDIA group

indicated by lowered K_{Na} values in both genders. So the Na, K-ATPase is able to extrude the excessive Na^+ more efficiently in the early phase of DM1 as compared to controls. The way of enzyme adaptation to acute diabetes seems to be organ specific as, in the same model of acute DM1, the renal Na, K-ATPase was adapted on different way. In the kidney, the enzyme improved its activity by increasing the number of active Na, K-ATPase molecules without any alterations in sodium-binding properties [13]. Prolongation of the duration of diabetes to 8 weeks was

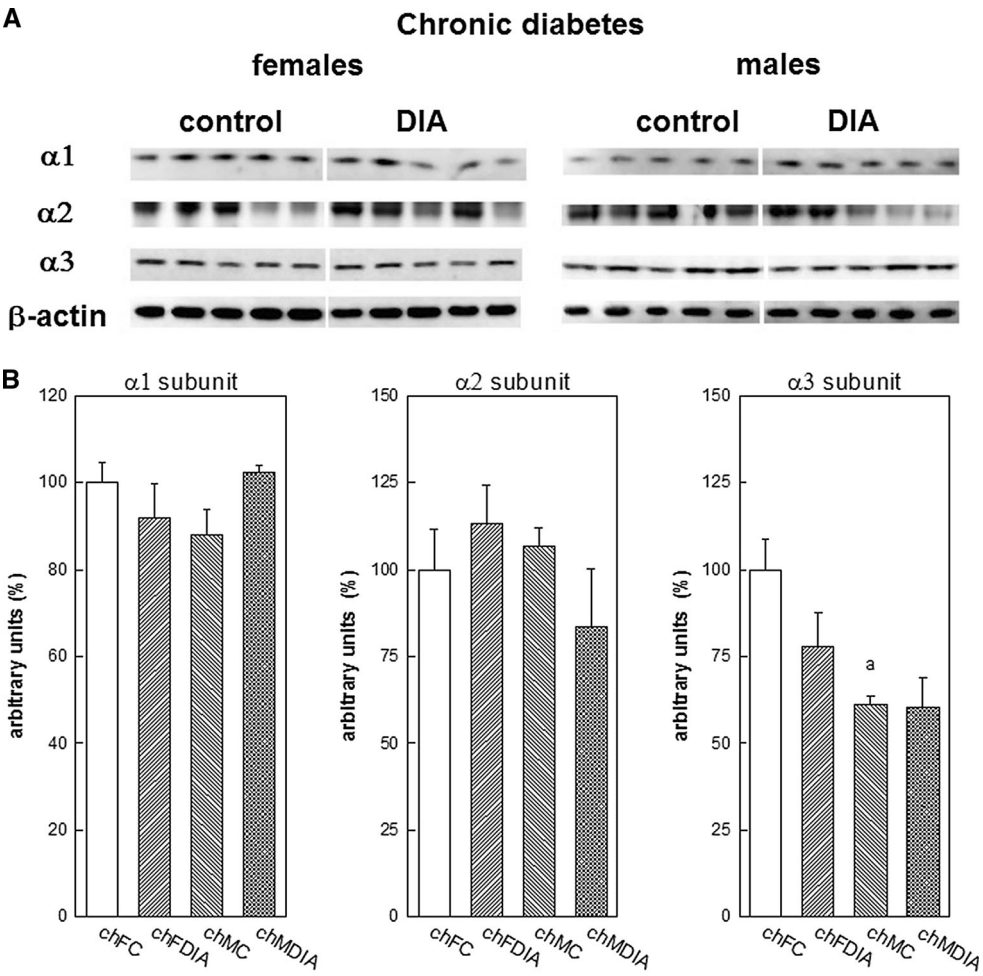

Fig. 15 **a** Immunoblot analysis of *α1–3* subunits of Na, K-ATPase from cerebral cortex in chronic model of diabetes in female control rats (chFC), in female rats with STZ-induced diabetes mellitus (chFDIA), in male control rats (chMC), and in male diabetic rats (chMDIA). **b** Relative abundance of *α1–3* subunits of Na, K-ATPase. Relative densities of bands. Data represent mean ± SEM, $n = 5$ in each group. *a* $p < 0.01$ as compared to the chFC group

followed by loss of enzyme adaptation in brain cortex of males as documented by similarities of V_{max}, K_m and K_{Na} values in control, as well as STZ-treated rats. On the other hand, in female rats, we observed still an adaptation of the enzyme to sub-chronic diabetes especially in the vicinity of ATP-binding site resulting in better affinity to substrate as indicated by lowered K_m value. This finding is contradictory to the observation in kidney where the Na, K-ATPase showed decrease of affinity to ATP after 8 weeks lasting diabetes [14]. Thus, the Na, K-ATPase from brain cortex might be able to utilize better the substrate ATP and consequently extrude better excessive intracellular sodium ions out of the cells especially in the lower physiologically relevant concentration range of ATP.

In the chronic stage of the disease represented in our study by 16 weeks lasting diabetes, the properties of Na, K-ATPase in diabetic rats of both genders returned to the level comparable in respective control groups as shown by similarities in all investigated kinetic parameters of the enzyme, i.e., V_{max}, K_m, and K_{Na}. So the Na, K-ATPase which is one of the components of the blood–brain barrier [4] seems to be protected against the complications induced by glucose overload in the organism during diabetes securing, thus, the more or less normal intracellular homeostasis of sodium ions in the tissue of brain cortex. The protection of the Na, K-ATPase in brain cortex against complications induced by chronic diabetes seems to be bound especially to the brain, while in cardiac and also in renal tissue, deterioration of the enzyme from the point of quantitative and also qualitative properties was observed [9, 27, 28]. This specificity of Na, K-ATPase in brain as compared to heart and kidney may be ascribed to the role of insulin. It is known that insulin stimulates the Na, K-ATPase via binding to specific insulin receptors [29] or by direct binding to Na, K-ATPase molecule [30].

Our finding concerning the resistance of Na, K-ATPase in brain cortex to diabetes is in disagreement with previous observations showing decreased activity as well as expression of the enzyme during diabetes [15, 31, 32]. In our experiments, relatively young rats were subjected to diabetes showing no significant effect on the enzyme in the brain cortex. In other experimental model of diabetes induced by alloxan, when even younger rats were investigated in acute state of diabetic injury, increased activity of

Na, K-ATPase in brain cortex was observed [33]. On the other hand, in experiments showing depression of Na, K-ATPase activity, older rats were subjected to similar models of STZ-induced diabetes. So, the above findings suggest that Na, K-ATPase in brain responds to diabetic insult differently depending on the age of animals. In young animals, the enzyme seems to be resistant to diabetic insult; however, in old animals, the diabetes induces significant loss of enzyme activity. This fact may be ascribed to age-dependent alterations of respiratory chain in cerebral mitochondria as it was shown that in young animals, the energy production was increased after 4 weeks lasting diabetes [34]; however, in adult animals, the activity of respiratory chain in mitochondria was significantly depressed [35]. It might be hypothesized that mitochondria produce enough energy for Na, K-ATPase expression and function in young animals, while in old animals subjected to diabetes, the energy production in mitochondria is insufficient for proper function of the enzyme in cerebral tissue.

Other interesting findings of the present study pointed out to gender specificity of Na, K-ATPase properties in brain cortex and its variations during the aging of rats. In 16 weeks old control male rats, the Na, K-ATPase extrudes the sodium ions better as compared to females especially in physiologically relevant lower concentrations of ATP due to better affinity to substrate as indicated by lower value of K_m. This effect persisted also in males subjected to 8 days lasting diabetes; however, in males subjected to 8 weeks lasting diabetes, this effect was lost as suggested by similarities of K_m values in diabetic rats of both genders in the sub-chronic model of diabetes. In older rats aged 24 weeks, the Na, K-ATPase in brain cortex revealed again different properties in males and females, but the mechanism of the variation was connected to quantitative changes of the enzyme. In females, the number of active molecules of Na, K-ATPase was significantly higher independently on the physiological state as suggested by increased value of V_{max} in control as well as diabetic group when comparing to respective male groups. So the enzyme in 24 weeks old female rats probably extrudes better the excessive sodium ions as compared to respective male groups. This effect may be ascribed to estradiol because several previous studies have shown that this hormone is one of the primary Na, K-ATPase regulators increasing the activity/expression of the enzyme in the cardiovascular system [36–40]. The above hypothesis is supported also by our Western blot data which indicate higher expression of α3 subunit in healthy females and also in female rats subjected to sub-chronic as well as to chronic diabetes. This finding may be of physiological relevance due to the fact that the presence of this subunit varies between different neuronal cells [41], revealing a unique and essential role following suprathreshold synaptic activity [42].

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