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Changes in the expression of Na^+/K^+-ATPase isoenzymes in the left ventricle of diabetic rat hearts: effect of insulin treatment

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Summary Na^+/K^+-ATPase related strophanthidin sensitive 3-O-methylfluorescein-phosphatase activity, [^{3}H]ouabain binding and expression of Na^+/K^+-ATPase subunit isoforms were measured in the left ventricle of the heart of normal and streptozotocin-diabetic rats with and without insulin treatment. Compared to control animals, the enzyme activity was 0.75 ± 0.09 and 0.62 ± 0.06 times lower in rats diabetic for 2 and for 4 weeks, respectively. This was associated with a proportional decrease of the [^{3}H]ouabain binding sites. Immunoblots indicated a 0.76 ± 0.08 and 0.61 ± 0.08-fold decrease of $alpha_1$, a 0.68 ± 0.09 and 0.41 ± 0.04-fold decrease of $alpha_2$ subunit in 2- and 4-week diabetic rats, respectively relative to controls. $Beta_1$ subunit decreased proportionally 0.71 ± 0.07 and 0.38 ± 0.06-fold, and $beta_2$ decreased 0.75 ± 0.08 and 0.31 ± 0.06-fold, respectively. Northern blot analysis revealed a significant reduction in mRNA level of Na^+/K^+-ATPase subunit isoforms after 2 and 4 weeks of diabetes (for $alpha_1$ 66.2 ± 8.2 and 55.9 ± 7.8 % of controls for $alpha_2$ 91.7 ± 12.1 and 41.1 ± 7.1 % of controls and for beta subunit 93.4 ± 11.1 and 49.8 ± 6.8 % of controls, respectively). Although, mRNA levels of isoform reverted to even higher levels than the control values after insulin treatment, insulin caused only a partial recovery of enzyme activity, [^{3}H]ouabain binding capacity and protein expression. We have obtained evidence that in cardiac left ventricle there are more than one type of Na^+/K^+-ATPase alpha and beta subunit isoforms which are affected in diabetes and by insulin treatment. The time course of diabetes induced changes and the degree of involvement suggest that the Na^+/K^+-ATPase isoforms are altered individually. [Diabetologia (1997) 40: 1255–1262]

Keywords Rat heart, Na^+/K^+-ATPase, isoenzyme, mRNA, experimental streptozotocin-diabetes, insulin treatment.

Heart failure is the leading cause of death in diabetic patients [1]. Several studies have revealed the presence of cardiac dysfunction, ultrastructural and biochemical abnormalities in human and experimental diabetes [2, 3]. The subcellular defects are suggested to be caused, at least partially, by dysfunction of Na^+/K^+-ATPase [4, 5]. Na^+/K^+-ATPase consists of catalytic alpha and glycosylated beta subunits. Three isoforms of the alpha subunit have been identified, differing in molecular weight, cation-, ATP- and ouabain-binding affinities [6]. The genes of the subunit isoforms are located at different chromosomes and are expressed in a cell- and development-specific manner [7, 8]. The beta subunit contributes to the translocation of the newly synthetized alpha subunit and also protects it from degradation [9]. Three isoforms of the beta subunit have also been described [10]. Although significance of the tissue- and cell-specific distribution of the subunit isoforms is not known,

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Abbreviations: C, Control; D_2, 2 weeks diabetic rats; D_4, 4 weeks diabetic rats; D_2R_2, 2 weeks diabetic + 2 weeks insulin-treated diabetic rats; Na^+/K^+-ATPase, Na^+ and K^+-dependent ATPase; OMFP, 3-O-methylfluorescein-phosphatase; SDS, sodium dodecylsulphate.

evidence is accumulating that alpha subunit isoforms may be differentially regulated by physiological and pathological stimuli [11, 12].

Recently, it has also been demonstrated that in the heart, streptozotocin-induced diabetes caused a conspicuous reduction in enzyme activity with a marked decrease of $alpha_2$ [13]. In an earlier study, Fawzi and McNeill [14] reported a decrease of both low and high affinity ouabain binding sites in left ventricle obtained from chronically diabetic rats. The aims of this study were to examine the Na^+/K^+-ATPase activity and the P_i facilitated [^{3}H]ouabain binding, as well as isoenzyme composition and amount of transcript coding for the Na^+/K^+-ATPase subunits in left ventricles of hearts from normal and diabetic rats with and without insulin treatment.

Materials and methods

Animals. Six-week-old, male Sprague Dawley (Lati, Gödöllõ, Hungary) rats weighing between 190–260 g were randomly assigned to four groups. C, age matched controls; D_2, non-treated diabetes for 2 weeks; D_4, non-treated diabetes for 4 weeks; D_2R_2, diabetic for 2 weeks followed by insulin treatment for another 2 weeks. Rats were made diabetic with a single injection of streptozotocin (Sigma-Aldrich, Budapest, Hungary), buffered with 0.1 mol/l citrate (pH 4.2) at a dosage of 65 mg/kg body weight via the femoral vein as described previously [15]. After hyperglycaemia and glucosuria were detected diabetes was verified 24 h later (Glucose Kit; Boehringer, Mannheim, Germany). All animals were maintained on normal rat chow and water ad libitum. The diabetic and age-matched control animals (citrate buffer injected) were killed 2 and 4 weeks after the injection (groups: D_2, D_4). In insulin replacement studies Ultralente insulin (Novo, Copenhagen, Denmark) was administered subcutaneously, daily, for a 2-week period to the 2-week diabetic rats (groups: D_2R_2). The individual mean daily dosage of Ultralente insulin was 6.4 IU (between 4.1–8.5 IU). All the animals were killed in anaesthesia by decapitation and the trunk blood was collected to determine blood glucose level. The hearts were immediately removed and rapidly frozen in liquid nitrogen for subsequent RNA extraction and preparation of membrane fractions.

Subcellular fractionation. Frozen pieces of heart left ventricle (500 mg) were homogenized in 5 ml of 0.25 mol/l sucrose 10 mmol/l Tris/HCl, pH 7.4 containing 2 mmol/l phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml aprotinin by a homogenizer (Janke and Kungel, Staufen, Germany) for 20 s at half maximal speed [16]. The homogenate was centrifuged at $2500 \times g$ for 10 min and the pellet was discarded. The supernatant was then centrifuged at $100000 \times g$ for 60 min in a Centrikon TFT 70.38 rotor (Kontron, Zurich, Switzerland). The pellet containing plasma membrane and intracellular membranes (microsome) was finally suspended in homogenizing medium and stored at − 80 °C. Protein content was assayed by the method of Bradford [17] using ovalbumin as a standard.

[^{3}H]ouabain binding. [^{3}H]ouabain binding was performed as described earlier with minor modifications [18]. Microsomal fractions (20–100 μg protein) were incubated for 2 h at 37 °C in 1 ml medium containing 3 mmol/l $MgCl_2$, 3 mmol/l imidazole/PO_4, pH 7.25, [^{3}H]ouabain 1.22 Tbq/mmol, (Amersham, Buckinghamshire, UK) and unlabelled ouabain in final concentrations of 2×10^{-8}–5×10^{-4} mol/l (specific activity: 500–15000 cpm/pmol). The dissociation constants (K_d) were calculated according to the methods of Scatchard using a computer program ENZFITTER (version 1.05 EGA single ligand two binding sites model; Elsevier-Biosoft, Cambridge, UK) [19].

Enzyme assay. Na^+/K^+-ATPase activity was assayed in microsomal fractions by measuring the strophanthidin sensitive K^+-dependent 3-O-methylfluorescein-phosphatase activity [20]. Protein was preincubated with 0.1 % Na-deoxycholate pH 7.4 for 30 min at 24 °C. The activity was determined in the presence of 19.5 μmol/l 3-O-methylfluoresceinphosphate, 4 mmol/l $MgCl_2$, 1 mmol/l EDTA, 80 mmol/l Tris/HCl, pH 7.6, 10 mmol/l KCl and 100 μg protein with and without 5 mmol/l strophanthidin. By using a fluorescence spectrofluorimeter (F-4500; Hitachi, Tokyo, Japan) the formation of 3-O-methylfluorescein was monitored by measuring its fluorescence. The experimental data were fitted using ENZFITTER (Biosoft) [21]. 5′-nucleotidase activity was performed as described in detail earlier [22].

Western Blot. SDS-PAGE was performed according to the method of Laemmli [23] with a BioRad Miniprotean II (Hercules, Calif., USA) system. Samples (5–50 μg) were solubilized in a buffer of 0.125 mol/l Tris/HCl, pH 6.7, containing 4.0 % SDS, 1 mmol/l EDTA, 15 % glycerol, 0.1 mol/l dithiotreitol, and 0.01 % bromophenol blue. The samples were loaded on a discontinuous polyacrylamide gel [22]. The proteins were then transferred onto nitrocellulose sheets at 23 °C for 15 min at 250 mA followed by 45 min at 350 mA. The nitrocellulose was fixed for 1 h in a blot solution containing 5 % bovine serum albumin, 0.5 % Tween 20, 150 mmol/l NaCl, 20 mmol/l Tris/HCl, pH 7.5 solution followed by 60 min (23 °C) incubation with polyclonal antibodies of the Na^+/K^+-ATPase subunit isoforms (UBI, Lake Placid, N.Y., USA), diluted to 1 : 2000. Blots were washed and then incubated by peroxidase conjugated goat anti-rabbit secondary antibody (DAKO, Glostrup, Denmark) diluted to 1 : 4000 for 30 min at 23 °C. The blots were washed and developed with the enhanced chemoluminescence Western blotting detection reagent (Amersham). The film negatives were analysed by laser densitometry (Pharmacia, Uppsala, Sweden).

Northern blot analysis. RNA was prepared from the pooled samples [24]. Northern blot analysis was performed as described [25]. Twenty μg of RNA was analysed by electrophoresis on denaturing 1.2 % agarose-formaldehyde gel and blotted onto Amersham Hybond-N nylon filters by capillary transfer. RNA blots were fixed and cross-linked by ultra-violet irradiation. After prehybridization the samples were hybridized to probes labelled with alpha [^{32}P]dCTP (specific activity: 110 Tbq/mmol) [26]. The Na^+/K^+-ATPase isoform specific probes were a generous gift of Dr. J.B. Lingrel (University of Cincinnati College of Medicine, USA). In hybridization reactions approximately $9–10 \times 10^6$ cpm of labelled probe and 200 μg/ml chicken blood DNA was added to the prehybridization solution. The autoradiographs were obtained by exposing the filters to Medifort RP 90 X-ray films (Forte, Vác, Hungary) using an intensifying screen at − 80 °C for 24–96 h. The films were densitometrically analysed and the area of each hybridization band was related to the area of the corresponding ethidium bromide stained 18S ribosomal band. All the other materials were purchased by Sigma-Aldrich.

Statistical analysis. The results are expressed as a percentage of the controls and are presented as means ± SEM. Unpaired

Table 1. Body, heart weight and blood glucose levels in control (C), streptozotocin-diabetic (D_2, D_4) and insulin-treated streptozotocin-diabetic (D_2R_2) rats

Group	C $n = 9$	D_2 $n = 8$	D_4 $n = 9$	D_2R_2 $n = 9$
Body weight (g) initial	220.5 ± 24.3	229.8 ± 28.5	236.6 ± 20.2	237.9 ± 19.6
2-week	239.9 ± 22.6	181.7 ± 18.7[a]	192.3 ± 13.6[a]	203.2 ± 15.6[a]
4-week	258.8 ± 20.3	–	165.7 ± 19.8[ab]	220.5 ± 20.3[ab]
Heart weight (g)	0.62 ± 0.04	0.51 ± 0.03[ab]	0.46 ± 0.04[ab]	0.61 ± 0.05
Heart/Body weight 10^3	2.63 ± 0.07	2.76 ± 0.10	2.78 ± 0.10	2.77 ± 0.10
Glucose (mmol/l)	7.15 ± 0.52	26.31 ± 3.52[ab]	32.82 ± 3.69[ab]	8.22 ± 0.66[b]

Values are means ± SEM of 8–9 experiments, n = number of experimental animals. [a] $p < 0.05$ for differences between age-matched control and streptozotocin-diabetic or insulin-treated streptozotocin-diabetic rats. [b] $p < 0.05$ for differences between streptozotocin-diabetic (D_2 and D_4) and insulin-treated streptozotocin-diabetic (D_2R_2) rats

Table 2. Strophanthidin sensitive and insensitive 3-O-methylfluorescein-phosphatase (OMFP) and 5′-nucleotidase activities in heart left ventricle microsomes from control (C), streptozotocin-diabetic (D_2, D_4) and insulin-treated streptozotocin-diabetic (D_2R_2) rats

Group	C $n = 9$	D_2 $n = 8$	D_4 $n = 9$	D_2R_2 $n = 9$
OMFP (total) nmol fluorescein · mg protein^{-1} · min^{-1}	89.2 ± 5.9	79.8 ± 6.9[ac]	71.4 ± 8.1[ad]	83.5 ± 8.2[cd]
OMFP + strophanthidin nmol fluorescein · mg protein^{-1} · min^{-1}	50.1 ± 3.9	50.4 ± 3.9	48.9 ± 5.6	50.4 ± 5.9
ΔOMFP nmol fluorescein · mg protein^{-1} · min^{-1}	39.1 ± 2.6[ab]	29.4 ± 2.9[ac]	24.5 ± 3.5[ad]	33.1 ± 3.2[bcd]
5′Nucleotidase nmol phosphate · mg protein^{-1} · min^{-1}	72.1 ± 5.9	68.9 ± 5.9	68.9 ± 5.9	74.5 ± 6.5

Values are means ± SEM of 8–9 experiments, n = number of animals. Activity of each enzyme investigated was not different in the 2- and 4-week control groups, therefore C denotes all the age-matched control groups. The strophanthidin sensitive 3-O-methylfluorescein-phosphatase (ΔOMFP) represents Na^+/K^+-ATPase activity while the strophanthidin insensitive 3-O-methylfluorescein-phosphatase (OMFP + strophanthidine) represents the basal ATPase activity. [a] $p < 0.05$ for differences between control and streptozotocin-diabetic (D_2, D_4). [b] $p < 0.05$ for differences between control and insulin-treated streptozotocin-diabetic (D_2R_2) rats. [c] $p < 0.05$ for differences between 2-week diabetic and insulin-treated diabetic (D_2R_2). [d] $p < 0.05$ for differences between 4-week diabetic and insulin-treated diabetic (D_2R_2)

Student's t-test was used to compare the controls and diabetic groups. A Bonferroni correction for multiple comparisons was used to evaluate the significance of the data. Student's t-test was used throughout this study to compare the differences in various parameters examined. The difference between the means was considered to be significant if p was less than 0.05.

Results

Body and heart weight. The body and the heart weights of streptozotocin-diabetic rats were significantly lower than those of the age-matched non-diabetic controls. Table 1 shows that after 2 and 4 weeks of diabetes the mean final body weight had decreased by an average of 21.1 ± 3.7 % and 30.08 ± 4.1 %, while that of age-matched non-diabetic controls had increased by 8.8 ± 0.93 % and 17.4 ± 2.41 %, respectively ($p < 0.05$). The heart weight of diabetic animals also decreased, but heart weight to body weight ratios did not change. The treatment of diabetic animals with insulin for 2 weeks was found to decrease the blood glucose level with a concomitant enhancement of body and heart weight (Table 1).

Enzyme activities of heart microsomes. Strophanthidin sensitive 3-O-methylfluorescein-phosphatase activity decreased in diabetic heart left ventricle 0.75 ± 0.09-fold within 2 weeks of the onset of diabetes and decreased further 0.62 ± 0.06-fold during 4 weeks of diabetes. Insulin treatment partially restored strophanthidin sensitive 3-O-methylfluorescein-phosphatase activity (Table 2). The strophanthidin concentration producing half maximal inhibition was not significantly different among the groups tested: it was 0.81 ± 0.06 μmol/l for controls, 0.78 ± 0.032 μmol/l for 4-week diabetic rats and 0.83 ± 0.032 μmol/l for diabetic insulin-treated animals (D_2R_2). The strophanthidin independent 3-O-methylfluorescein-phosphatase and 5′-nucleotidase activity was found to be equal in all groups examined. The activities of all the examined enzymes were the same in the 2- and 4-week control groups, therefore control (C) represents all the age-matched controls in the presentation of the results.

[^{3}H]ouabain binding capacity. The Scatchard type plot gives two populations of binding sites with

Table 3. Maximal ouabain binding capacity of the left heart ventricle microsomes from control (C), streptozotocin-diabetic (D_2, D_4) and insulin-treated streptozotocin-diabetic (D_2R_2) rats

Group	C $n = 6$	D_2 $n = 6$	D_4 $n = 7$	D_2R_2 $n = 5$
$B_{max\ total}$ (pmol/mg protein)	69.24 ± 7.02^{ab}	53.18 ± 5.26^{ac}	45.92 ± 5.18^{ad}	59.99 ± 7.2^{bcd}
$B_{max\ low\ affinity}$ (pmol/mg protein)	56.85 ± 4.31^{ab}	43.69 ± 4.42^{ac}	39.51 ± 3.73^{ad}	48.58 ± 2.4^{bcd}
Contribution (%)	82.1 ± 8.1	82.15 ± 8.4	86.05 ± 8.7	81 ± 8.5
$B_{max\ high\ affinity}$ (pmol/mg protein)	12.39 ± 1.32^{a}	9.49 ± 0.87^{ac}	6.41 ± 0.82^{ad}	11.41 ± 1.21^{cd}
Contribution (%)	17.9 ± 1.9	17.7 ± 1.6	13.9 ± 1.7^{ad}	19 ± 1.9^{d}
Turnover (min^{-1})	564.7	552.8	533.54	551.8

Maximal ouabain binding capacity designated as ($B_{max\ total}$). $B_{max\ low\ affinity}$ refers to the low affinity binding sites, $B_{max\ high\ affinity}$ to the high affinity binding sites. Values are means ± SD of 5–9 experiments, n is equal with the number of animals. The B_{max} values were not different in the 2 and 4-week control groups, therefore C denotes all the age-matched control groups. Turnover rate was calculated as the ratio of strophanthidin sensitive 3-O-methylfluorescein-phosphatase (ΔOMFP) and $B_{max\ total}$. [a] $p < 0.05$ for differences between control (C) and streptozotocin-diabetic (D_2, D_4) rats. [b] $p < 0.05$ for differences between control (C) and insulin-treated streptozotocin-diabetic (D_2R_2) rats. [c] $p < 0.05$ differences between 2-week diabetic (D_2) and insulin-treated diabetic (D_2R_2) rats. [d] $p < 0.05$ differences between 4-week diabetic (D_4) and insulin-treated diabetic (D_2R_2)

apparent dissociation constants K_{d1}: 3.12 ± 0.39 μmol/l in the case of low affinity binding sites and K_{d2}: 56.15 ± 4.8 nmol/l in the case of high affinity binding sites in the control group. The K_d values did not change significantly during diabetes and insulin treatment ($p > 0.1$). The maximal [^{3}H]ouabain binding capacities of heart left ventricle microsomes was 69.24 ± 7.02 pmol/mg protein (100 %) in the control and 53.18 ± 5.26 pmol/mg protein (76.85 ± 7.92 %) and 45.92 ± 5.18 pmol/mg protein (66.32 ± 7.37 %) in 2- and 4-week diabetic animals, respectively. Table 3 also shows the contribution of the isoenzymes in terms of low ($alpha_1$) and high affinity ouabain binding sites ($alpha_2$). In 2-week diabetic animals the reduction in ouabain binding was about 20 % in the case of both types of ouabain binding sites. However, in 4-week diabetic animals the reduction was more pronounced in the number of high affinity ouabain binding sites (48.26 ± 6.4 %) than in the low affinity sites (30.5 ± 4.4 %). The maximal [^{3}H]ouabain binding capacity was partially reversed by insulin treatment (D_2R_2) compared to the controls (C), yet it was higher than the 4-week diabetic values. The turnover rate of the enzyme (strophanthidin sensitive 3-O-methylfluorescein-phosphatase activity per maximal [^{3}H]ouabain binding) did not significantly differ in the groups examined (Table 3).

Western blot analysis of heart microsomes. In heart left ventricle two alpha and beta isoforms could be detected in all the groups examined. The $alpha_1$ and $alpha_2$ protein levels indicated that $alpha_1$ represented the majority (> 70 %) of Na^+/K^+-ATPase in the control left ventricles. No $alpha_3$ specific band was detected. The $beta_1$ and $beta_2$ protein levels suggested that $beta_1$ is the predominant isoform of beta (> 60 %). A representative immunoblot of control and diabetic heart left ventricles is shown in Figure 1. The control level of each species is designated as 100 % in Figure 2. The quantity of the $alpha_1$ isoform decreased significantly after 2 weeks of diabetes to 76.2 ± 8.2 % of the controls, $alpha_2$ isoform decreased to 78.1 ± 8.7 % of the controls. Also the level of $beta_1$ was 71.18 ± 6.91 % and $beta_2$ 75.1 ± 6.9 % of the controls in 2-week diabetic heart left ventricle. Moreover in 4-week diabetes $alpha_1$ isoform decreased to 61.3 ± 7.8 % and $alpha_2$ isoform decreased to 41.1 ± 4.1 % of the controls. The level of $beta_1$ subunit decreased to 38.12 ± 5.75 % and $beta_2$ subunit decreased to 31.42 ± 5.75 % of the controls during 4 weeks of diabetes. Two weeks' insulin treatment of the 2-week diabetic animals (D_2R_2) did not completely restore the original isoform pattern. The relative amount of $alpha_1$ isoform in diabetic insulin-treated animals was lower compared to the controls (86.8 ± 7.9 %), but it was 1.14 ± 0.2 times higher than the 2-week diabetic and it was 1.42 ± 0.15 times higher than the 4-week diabetic values. The amount of $alpha_2$ was 1.12 ± 0.1 times higher than the 2-week diabetic and 2.19 ± 0.3 times higher than 4-week diabetic values. Insulin treatment increased the $beta_1$ level to 105.1 ± 12.7 % and the $beta_2$ level to 127.3 ± 11.7 % of control values, respectively. This was a 1.47 ± 0.2-fold and 1.7 ± 0.2-fold increase compared to the 2-week diabetic values and 2.7 ± 0.32 and 4.1 ± 0.5 times higher than 4-week diabetic values, respectively.

Northern blot analysis. RNA samples were subjected to Northern blot analysis to examine the integrity of the RNA preparation and the amount of the mRNA

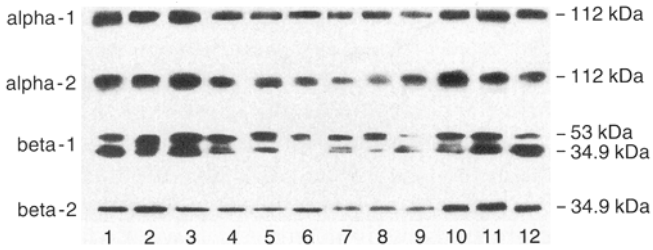

Fig. 1. Immunoblot analysis of Na^+/K^+-ATPase alpha and beta subunit isoforms of heart left ventricle. A representative photo of an enhanced chemoluminescence developed Western blot of control (C, lines 1–3), 2-week diabetic (D_2, lines 4–6), 4-week diabetic (D_4, lines 7–9) and 2-week diabetic and 2-week insulin-treated rats (D_2R_2, lines 10–12). Molecular weight markers run on the same gel, were phosphorylase B (112), ovalbumin (53), and carbonic anhydrase (34.9 kDa)

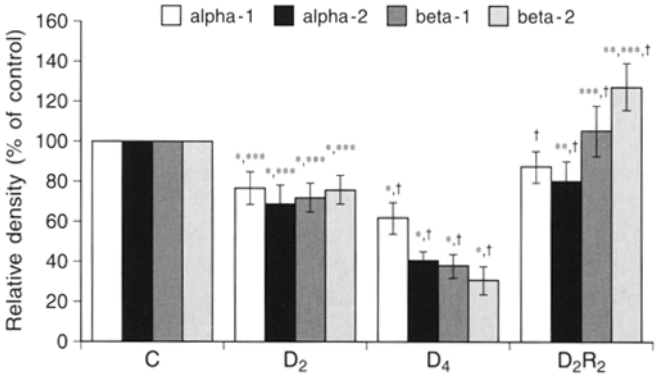

Fig. 2. Densitometric analysis of Western blots of Na^+/K^+-ATPase subunit isoforms in rat heart left ventricle. The control level of each species is designated as 100 %. Data are mean ± SEM of samples from 7 animals. * $p < 0.05$ for differences between controls and streptozotocin-diabetic (D_2, D_4). ** $p < 0.05$ differences between control and insulin-treated streptozotocin-diabetic (D_2R_2) rats. *** $p < 0.05$ differences between 2-week diabetic (D_2) and insulin-treated diabetic (D_2R_2) rats. + $p < 0.05$ differences between 4-week diabetic (D_4) and insulin-treated diabetic (D_2R_2) rats

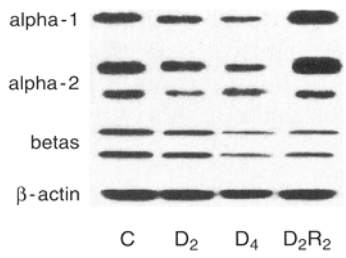

Fig. 3. A representative photo of Northern blot analysis of Na^+/K^+-ATPase subunit isoforms and β actin mRNAs from left heart ventricles of control (C), 2-week diabetic (D_2), 4-week diabetic (D_4) and 2-week diabetic and insulin-treated (D_2R_2) rats

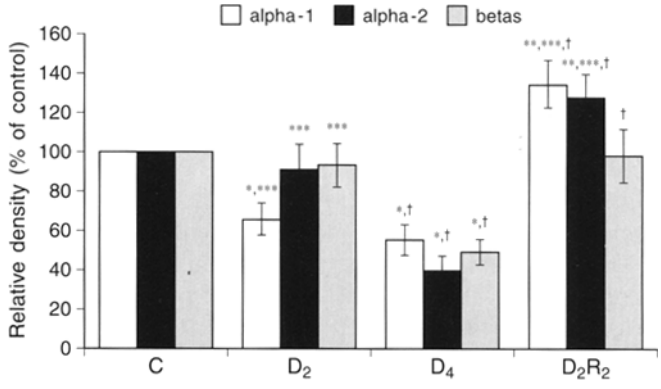

Fig. 4. Densitometric analysis of Northern blot of Na^+/K^+-ATPase $alpha_1$, and $alpha_2$, beta subunits mRNAs prepared from heart left ventricle of control (C), 2-week diabetic (D_2), 4-week diabetic (D_4) and 2-week diabetic and 2-week insulin-treated (D_2R_2) rats. Data are means ± SEM of 5 experiments. The control level of each species is designated as 100 %. * $p < 0.05$ for differences between controls (C) and streptozotocin-diabetic (D_2, D_4). ** $p < 0.05$ for differences between control and insulin-treated streptozotocin-diabetic (D_2R_2) rats. *** $p < 0.05$ differences between 2-week diabetic and insulin-treated diabetic (D_2R_2) rats. + $p < 0.05$ differences between 4-week diabetic and insulin-treated diabetic (D_2R_2) rats

level of different Na^+/K^+-ATPase isoforms. The $alpha_1$ probe hybridized to a single 3.7 kb band. Two bands of mRNA were detected by hybridization with $alpha_2$ cDNA clone at 3.4 and 5.3 kb, respectively. No significant hybridization was found in the case of $alpha_3$ cDNA clone either in the control or in the diabetic heart samples. The beta subunit probe which contained both $beta_1$ and $beta_2$ isoforms cDNA hybridized to two major species (Fig. 3). The mRNA of beta actin is also shown as control. The $alpha_1$ and $alpha_2$ mRNA levels indicated that $alpha_1$ represented the majority (> 75 %) of Na^+/K^+-ATPase transcript in the left ventricle of the control rat hearts. The relative levels of alpha and beta subunit mRNAs are shown in Figure 4. The results are expressed as the percentage relative to the mRNA level measured in the age-matched, control animals. Northern blot analysis of the total RNA samples revealed that mRNA levels of $alpha_1$ subunit significantly decreased to 66.2 ± 8.2 % of the controls during the first 2 weeks of diabetes and was 55.9 ± 6.9 % of the controls in 4 weeks of diabetes. The $alpha_2$ mRNA did not significantly change in the first 2 weeks of the diabetic state (91.7 ± 12.1 % of the controls, $p > 0.5$), but decreased to 41.1 ± 7.1 % of the controls in the 4-week diabetic animals. The mRNA level of the beta subunit was 93.4 ± 11.1 % in 2-week diabetic and decreased to 49.8 ± 6.32 % of the controls in the 4-week diabetic animals. The mRNA level of beta actin did not change significantly in diabetes. The insulin treatment increased the amount of $alpha_1$ over the control (134.7 ± 13.6 %) and it was 2.0 ± 0.3 and 2.4 ± 0.25 times higher than the 2- and 4-week diabetic left ventricle. $Alpha_2$ mRNA level was 128.7 ± 14.1 % of the controls. It was 1.4 ± 0.2 and 3.1 ± 0.4 times higher than the 2- and 4-week diabetic

left ventricle. The level of the beta subunit mRNA was not significantly changed compared to the 2 weeks' diabetic heart; however, it exceeded the level of the 4-week diabetic rats by 1.9 ± 0.25-fold.

Discussion

In this study we examined diabetes-induced alterations in Na^+/K^+-ATPase activity, in ouabain binding capacity, isoenzyme composition and mRNA expression in the early phase of diabetes. We also assessed the reversibility of the diabetes-induced abnormalities by insulin administration. It was reported that insulin stimulated the sarcolemmal Na^+-pump activity or the sarcolemmal ATPase activity in cardiac muscle [27]. Therefore, the chronic defect of circulating insulin must depress the enzyme activity of cardiac sarcolemma. Indeed, an increased intracellular Na^+ concentration was also observed in diabetic heart muscle [28]. As shown earlier, the Na^+/K^+-ATPase activity was reduced in the diabetic heart [29]. Similar results were obtained in the present study. Moreover, we showed that the enzyme activity decreased progressively during diabetes. The decrease involved both the low affinity and the high affinity components of ouabain binding sites similar to that published earlier [14]. Nevertheless the time course of the alteration of the individual components of ouabain binding sites differ. The low affinity binding sites (referring to $alpha_1$ isoform of Na^+/K^+-ATPase) decreased significantly during the first 2 weeks of diabetes and remained near to this level. But the high affinity sites (referring to $alpha_2$ isoform of Na^+/K^+-ATPase) decreased continuously. The fact that both isoenzymes are affected in diabetic heart alterations is contrary to earlier dogma that it is usually the $alpha_2$ isoenzyme which is responsive to diabetes [30, 31]. While this manuscript was in preparation in agreement with our data, Gerbi et al. [32] published that the activity of the low affinity isoenzyme is decreased in diabetes [32]. Since we found that the turnover rate of Na^+/K^+-ATPase (ATPase activity/[^{3}H]ouabain binding) does not change significantly during diabetes and insulin treatment, there is a possibility that the decrease in ATPase activity arises from the decrease in the amount of ATPase molecule [33], rather than inactivation or modification of the enzyme as suggested by others [32]. The results of our Western blot analysis of the samples supported the above assumption. Both isoforms of catalytic subunit ($alpha_1$ and $alpha_2$) decrease in 2 and 4 weeks of diabetes in the left ventricle. The $alpha_1$ decreases mainly in the first 2 weeks of diabetes. On the other hand $alpha_2$ decreases continuously, similarly to the high affinity ouabain binding sites. However, contrary to our results, Gerbi et al. [32] found that $alpha_1$ isoform protein enhanced in 8 weeks of diabetes [32]. These discrepancies may be the result of the difference of the heart weight/body weight ratio in 4- and 8-week diabetic animals [34]. Similarly to the earlier report, our results show that the heart weight/body weight ratio does not change compared to controls in the first 4 weeks of experimental diabetes [34]. Yet, there is a significant enhancement in heart weight/body weight ratio in 8-week diabetes [32, 34]. In the light of the above data we can only assume that as opposed to 8 weeks' duration of diabetes, the 4-week duration of untreated experimental diabetes is not associated with cardiac hypertrophy [34]. Interestingly, it is the right ventricle which is dominant in diabetic hypertrophy [35]. Furthermore, we have to take into account that the right ventricle of the rat heart has 2.6 times more [^{3}H]ouabain binding site density than the left ventricle [36]. The above-mentioned differences might explain the contradictions.

Another major goal of this study was to investigate the possible involvement of beta subunits in diabetic alterations. We found that the left heart ventricle contains two types of beta subunits similarly to brain and skeletal muscle [10]. The presence of mRNA of $beta_2$ subunit in rat heart has already been reported [37]. It is also suggested that under some conditions its expression is regulated together with $alpha_2$ [37]. In contrast to others, we found by using rat specific polyclonal antibodies that the left ventricle contained both $beta_1$ and $beta_2$ subunits [32]. In addition, both types of beta subunit proteins decreased in diabetes. This was especially true in 4-week diabetes, where the protein level of beta subunits decreased more than that of the alpha subunits. Insulin administration reverted the beta subunit protein levels over the controls. The role of the beta subunit is still highly questionable [9] and it is also debated whether the alpha/beta ratio might modify the ATPase activity [32, 38].

Streptozotocin-induced diabetes in rats is characterized by hyperglycaemia, a decrease in serum insulin and an increase in serum immunoreactive glucagon concentration [39]. It is also associated with the increase in serum concentrations of mineralo- and glucocorticoids [40], with a decrease in serum concentrations of thyroid hormones [40], an increase in serum K^+ and decrease in total body K^+ [41]. It is well documented that aldosterone alone is sufficient to induce $alpha_1$ and $beta_1$ gene expression in cardiomyocytes [42]. In hypokalaemia, $alpha_2$ isoform expression is depressed in the heart [11]. In the heart, it is mainly the $alpha_2$ and $beta_1$ mRNA level that is influenced by hypothyroid state [43, 44]. According to our data, in 2-week diabetes only the $alpha_1$ mRNA level decreased, as opposed to the hypothyroid state and to aldosterone effect. However, 4 weeks of diabetes altered both $alpha_1$, $alpha_2$ catalytic isoforms and the beta subunit at the transcription level. We cannot

exclude the possibility that these changes in hormonal and ionic status may also play a role in altered expression of Na^+/K^+-ATPase isoforms in this model of diabetes [45, 46]. Furthermore, the diabetes induced metabolic abnormalities might also alter the ATPase functions [47].

In agreement with our previous reports insulin enhances Na^+/K^+-ATPase activity [48]. The insulin-induced enhancement of Na^+/K^+-ATPase activity is a consequence of the increase of protein and mRNA level, but the insulin effect is not isoform specific [22, 26]. It seems that in the chronic insulin deficient state the regulation of $alpha_1$ catalytic isoform is primarily arranged at the transcription level both in 2- and 4-week diabetes. This is suggested by the correlation among the changes of low affinity ouabain binding sites, the changes of $alpha_1$ protein and the $alpha_1$ mRNA level. The replacement of insulin results in a marked increase of mRNA and of protein level. However, the amount of $alpha_2$ mRNA is not significantly influenced in the first 2 weeks of the insulin-deficient state, but it is decreased by a great amount after 4 weeks of insulin deficiency. In contrast to mRNA, the protein level of $alpha_2$ isoform and consequently the enzyme function (high affinity binding sites) also decreased significantly in 2-week diabetes. This suggests that the regulation of $alpha_2$ isoform might occur at a post-transcriptional level in the early state of diabetes. However, in 4 weeks of experimental diabetes the decrease in the high affinity binding sites ($alpha_2$ isoform) is the result of the decrease of Na^+/K^+-ATP-ase isoform which itself is the consequence of the lower level of its mRNA. Insulin administration enhanced both mRNA and protein levels of the subunits. The mRNA level of alpha subunits increased at a higher rate than the beta subunits; however, in protein level the enhancement of beta subunits is more pronounced than alphas.

In conclusion, we have obtained evidence that in cardiac left ventricle there are more than one type of Na^+/K^+-ATPase alpha and beta subunit isoforms which are affected in diabetes and by insulin treatment. The time course of diabetes induced changes and the degree of involvement suggest that the Na^+/K^+-ATPase isoforms are altered individually. Furthermore, our results also suggest that beside the changes in transcription of Na^+/K^+-ATPase the alterations in translation, and/or degradation of the enzyme might also influence the level and function of the enzyme. We are at present further researching the above-mentioned aspects of diabetes induced changes in Na^+/K^+-ATPase.

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