Effect of streptozotocin-induced diabetes on kidney Na^+/K^+-ATPase[1]

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The maximal capacity of low affinity ouabain binding sites in kidney medulla was found to be increased by $20 \pm 3.8\%$ after 2 weeks, and by $35 \pm 4.5\%$ in 4 weeks diabetes. However, in kidney cortex no similar changes could be detected. Western blot analysis of Na^+/K^+-ATPase subunits in kidney medulla indicated a significant enhancement of both the α_1 and β_1 subunit in two and four weeks diabetic rats (α_1: 35 ± 3.1, $51 \pm 5.8\%$ and β_1: 31.3 ± 5.2 and $43.2 \pm 6.8\%$, respectively). However, kidney cortex showed no significant change in any condition tested. In diabetes we could detect a significant change only in the medulla in case of the b subunit mRNA transcript, which showed 1.69 ± 0.59 and 2.89 ± 0.81 times increased in two and four weeks diabetic state, respectively. There was no change in the α_1 subunit mRNA abundance. Insulin treatment of diabetic animals did not result in a complete reversal of diabetes-induced changes in ouabain binding capacity or in the amount of Na^+/K^+-ATPase α_1 and β_1 subunit protein and mRNA levels. Our data indicate a good correlation between changes in low affinity ouabain binding capacity and the level of α_1 isoform in diabetic rats, and suggest an important role of the b subunit in the regulation of enzyme quantity.

Keywords: streptozotocin-induced diabetes, Na^+/K^+-ATPase, α_1 and β_1 subunit, mRNA, kidney

Human and experimental diabetes is accompanied by an early, prominent and persistent renal hypertrophy [6, 22]. However, there are some evidence in experimental animal models of diabetes that early insulin treatment can prevent or reverse renal hypertrophy [15]. Nephromegaly has been associated with a renal hyperfunction (increased glomerular filtration rate of Na^+ and water reabsorption,

increased Na^+/H^+ exchange and Na^+/K^+-ATPase-activity) [6, 14, 31]. The increased activity of Na^+-pump can be considered as a biochemical sign of renal hypertrophy [15]. This phenomenon represents a paradoxical response of kidney to the lack of insulin compared to other tissues [13–15].

Na^+/K^+-ATPase is a transmembrane enzyme primarily responsible for the active transport of sodium and potassium in mammalian cells [25, 28]. The enzyme exists as a heterodimer, there are three isoforms of the catalytic alpha (α) subunit and at least two of the beta (β) chain [29]. The alpha isoforms differ from each other in several biochemical and physiological characteristics and display a cell and tissue specific distribution [19, 29]. The α_1 isoform is widely distributed in all tissues, while α_2 and α_3 expression is mostly limited to excitable cells [12, 21, 23]. The distribution of β subunit isoforms in different cells is less well characterized. β_1 has a broad distribution, while β_2 is localized mostly to glia [24]. The significance of the tissue and cell specific distribution of the subunit isoforms is not known, but evidence is slowly accumulating to suggest that α isoforms may be differentially regulated by physiological and pathological stimuli [2, 10, 19].

Some data show that Na^+/K^+-ATPase isoform ratio changes during development in brain and heart, furthermore certain hormones alter both isoform ratio and isoform abundance [8, 9, 20]. It has been demonstrated that insulin stimulates Na^+/K^+-ATPase activity in a variety of tissues [8, 9, 17, 32].

In rats with experimental and spontaneous diabetes mellitus Na^+/K^+-ATPase activity and maximal ouabain binding capacity have been reported to be decreased in heart, retina muscle and kidney glomeruli but to be increased in kidney tubules and intestines [3, 4, 13, 27, 30]. The aim of this study was to investigate whether the change in P_i facilitated ouabain binding is the consequence of changes in enzyme protein abundance in kidney cortex and medulla derived from normal and streptozotocin-induced diabetic rats with and without insulin treatment. We further examined whether the alteration in protein abundance correlates with the expression of Na^+/K^+-ATPase subunits' mRNAs.

Methods*

One group of non-diabetic male rats was used as control (C). 60 mg/kg i.v. streptozotocin (STZ) was given to an other group and diabetes was verified 24 h later by the appearance of hyperglycaemia and glucosuria. A group of the diabetic animals was sacrificed after two weeks (D_2), another group after 4 weeks (D_4) of STZ administration. In insulin replacement studies insulin was administered to diabetic rats in an individual dose to normalize blood glucose level 2 and 4 weeks after STZ injection for 2 weeks before the time of the assay (D_2R, D_4R) [30]. Kidneys were removed and the cortex and medulla layers were separated and stored at $-80\ °C$ until use for the assays. Crude microsomal fractions were prepared by

* **Abbreviations:** C, control rats; D_2, 2 weeks diabetic rats; D_4, 4 weeks diabetic rats; D_2R, 2 weeks diabetic + 2 weeks insulin reverted rats; D_4R, 4 weeks diabetic + 2 weeks insulin reverted rats; STZ, streptozotocin; Na^+/K^+-ATPase, Na^+ and K^+-dependent ATPase (EC3:6.1.37)

differential centrifugation [30]. Protein content was assayed by the method of Bradford using ovalbumin as standard [5]. (^{3}H)ouabain binding was measured as previously described [3, 18]. (^{3}H)ouabain (1.22 TBq/mmol) and unlabelled ouabain were applied in final concentrations of 2×10^{-8}–10^{-5} M (spec. act. 500–15 000 cpm/pmol). Nonspecific (^{3}H)ouabain binding was measured in the presence of 1 mM unlabelled ouabain. The samples were filtered through Whatman GFC filters. Filters were washed and radioactivity counted by liquid scintillation method. Maximal binding (B_{max}) expressed in picomol per milligram of protein, and the dissociation constant (K_d) expressed in nanomol or micromol per liter, were calculated according to the methods of Scatchard using a computer program ENZFITTER (version 1.05 EGA single ligand one binding site model, Elsevier-Biosoft, Cambridge, UK) [11]. Solubilized microsomal proteins were analyzed on 10% SDS polyacrylamide gels [16] and transferred to nitrocellulose sheets for Western blot analysis [23]. These membranes were incubated with isoform specific Na$^+$/K$^+$-ATPase antibodies (UBI, USA), and a peroxidase conjugated second antibody. The reaction was detected using an ECL Western blotting detection kit (Amersham), and the film negatives were analyzed by laser densitometry. Total cellular RNA was purified by the guanidium isothiocyanate method [7]. RNA electrophoresis, blotting and hybridization were performed according to general protocols [1]. Autoradiograms were analyzed by laser densitometry, and each peak of Na$^+$/K$^+$-ATPase gene transcript was related to the area of the ethidiumbromide stained 18S rRNA band. cDNA probes specific for α_1 and β isoforms were a generous gift of Dr. J. B. Lingrel (Univ. Cincinnati). For the statistical analysis a 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 $p < 0.05$. Data were analyzed by non-linear least squares procedure.

Results

All animals treated with STZ, developed hyperglycaemia on the following day (the blood glucose level of the animals was minimum 25 mM). Body weight of STZ-diabetic rats was significantly lower after 2 and 4 weeks diabetic state than that of the age-matched non-diabetic controls (Table I). The kidney shows a significant hyperplasia both in 2 and 4 weeks diabetes. The kidney/body weight ratio was also enhanced during diabetes and was not reversed on insulin treatment. The blood glucose levels of the STZ-diabetic groups were 3.7- and 4.2-fold higher than those of age-matched non-diabetic controls. Blood glucose was decreased close to the control level by insulin administration and a relative body weight gain was also observed during two weeks of insulin administration.

The basic characteristics of [^{3}H]ouabain binding to kidney cortex and medulla microsomes from control, diabetic and diabetic insulin-treated groups were assessed in a series of experiments. We defined relative [^{3}H]ouabain affinities over the concentration of 2×10^{-8} to 10^{-5} M to evaluate the binding sites and to determine the K_d values. In this concentration range a Scatchard type plot gives one population of binding sites with apparent dissociation constant in the control group ($K_d = 52.4 \pm 5.9$, 59.6 ± 4.8 μmol in the case of kidney cortex and medulla, respectively). Maximal [^{3}H]ouabain binding capacity of the kidney medulla layer was increased in STZ-induced diabetes compared to the control and to the insulin-treated diabetic animals. Changes in ouabain binding capacity proved to be significant in kidney medulla of both two- and four-week diabetic animals. The enhancement in

ouabain binding was partly reversed by insulin treatment. The K_d values did not change significantly (Table II). No significant changes were detected in kidney cortex between the control, two- and four-week diabetic as well as insulin-treated diabetic groups.

Na^+/K^+-ATPase isoforms. Immunoblotting with anti-α_1, α_2 and α_3 as well as β_1 and β_2 monospecific antibodies were performed on protein from kidney cortex and medulla microsomal membrane prepared from control and diabetic rats. We have found α_1 and β_1 subunits but not α_2, α_3 and β_2 isoforms in the cortex and medulla layers. The sensitivity of the antibody probes for α_2 and α_3 as well as β_2 was checked on immunoblots containing different amounts of partially purified brain (α_1, α_2, α_3, β_1 and β_2), heart (α_1 and α_2 β_1 and β_2), liver and kidney (α_1 and β_1) Na^+/K^+-ATPase preparations. Anti-α_2 and anti-α_3 antibodies did not cross react with the α_1 subunit in liver and kidney preparations (not shown). Isoenzyme specificity of the kidney did not change after streptozotocin and/or insulin treatment. Figure 1A shows a representative Western blot of α_1 and β_1 subunits of kidney medulla, while panel B contains the results of the densitometric analysis of all the data in kidney medulla and cortex.

Table I

Body and kidney weight and blood glucose levels in control, STZ-diabetic and insulin-treated STZ-diabetic rats

Groups (n)	C (17)	D₂ (13)	D₄ (16)	D₂R (14)	D₄R (14)
Body weight (g)					
Initial	190 ± 24	196 ± 18	218 ± 20	187 ± 19	198 ± 20
2 weeks	211 ± 24	176 ± 18*	196 ± 19*	167 ± 15*	178 ± 15*•
4 weeks	227 ± 24	----	183 ± 19.8*•	181 ± 20*•	166 ± 18*•
6 weeks	236 ± 24	----	----	----	188 ± 21*•
Kidney weight (g)	1.25	1.52*	1.49*	1.42*	1.45*
Kidney/body	0.0053	0.0086*	0.0081*	0.0079*	0.0077*
Glucose (mM)	7.8 ± 1.5	28.7 ± 3.5*•	32.8 ± 3.6*•	8.8 ± 2.6•	9.8 ± 2.5•

Experimental diabetes was induced by streptozotocin injection (60 mg/kg body weight i.v.). Diabetic rats were killed either after 2 weeks (D₂ group) or after 4 weeks (D₄ group). Insulin treatment was started after 2 or 4 weeks of STZ administration (groups D₂R, D₄R, respectively) and continued for 2 weeks as described in Materials and Methods. Data are means ± SE, n = number of experimental animals. Statistically significant ($p < 0.05$) differences between the age-matched controls and either STZ-diabetic or insulin-treated STZ-diabetic rats are denoted by *. Statistically significant ($p < 0.05$) differences between STZ-diabetic (D₂ and D₄) and insulin-treated STZ diabetic (D₂R, D₄R) rats are denoted by •

Table II

*Maximal ouabain binding capacity and K_d values of kidney cortex and medulla microsomes from control,
STZ-diabetic and insulin-treated STZ-diabetic rats*

| Groups | Kidney cortex | | Kidney medulla | |
	K_d (μmol)	B_{max} (pmol/mg protein)	K_d (μmol)	B_{max} (pmol/mg protein)
C (n = 16)	52.4 $\pm$ 5.9	43.1 $\pm$ 4.3	59.3 $\pm$ 8.8	35.9 $\pm$ 4.3
D$_2$ (n = 6)	54.6 $\pm$ 5.9	39.1 $\pm$ 4.9	61.3 $\pm$ 5.3	43.3 $\pm$ 3.9*•
D$_4$ (n = 7)	50.6 $\pm$ 9.7	41.2 $\pm$ 3.9	57.7 $\pm$ 7.2	48.6 $\pm$ 3.6*••
D$_2$R (n = 5)	49.9 $\pm$ 8.3	43.8 $\pm$ 4.9	64.6 $\pm$ 7.8	37.7 $\pm$ 7.2•
D$_4$R (n = 5)	47.2 $\pm$ 8.3	44.8 $\pm$ 4.2	62.5 $\pm$ 8.3	41.9 $\pm$ 4.6*•

Maximal ouabain binding capacity (B_{max}) of kidney cortex and medulla microsomes was determined as
described in Section 2. Values are means $\pm$ SE, n = number of experiment. The B_{max} and K_d values were
the same in the two, four and six weeks control groups, therefore C denotes all the age-matched control
groups. Statistically significant ($p < 0.05$) differences between control and STZ-diabetic (D$_2$, D$_4$) are
denoted by *. Statistically significant ($p < 0.05$) differences between control and insulin-treated STZ-
diabetic (D$_2$R, D$_4$R) rats are denoted by •. Statistically significant ($p < 0.05$) differences between STZ-
diabetic (D) and insulin-treated STZ-diabetic (DR) rats are denoted by •

After two weeks of streptozotocin treatment there was a significant (35 $\pm$ 3.1%,
$p < 0.05$) increase in the amount of the a1 subunit and a 31 $\pm$ 5.2% ($p < 0.05$)
increase in the case of β_1 subunit in kidney medulla. The enhancement of the amount
of α_1 and β_1 subunit proteins was more predominant in the four-week diabetic state
(51 $\pm$ 5.8% and 3.2 $\pm$ 6.8%, respectively $p < 0.01$). Insulin treatment decreased the
amount of both subunits in two-week diabetic animals and restored the relative
abundance of both α_1 and β_1 subunits to the control level in case of four-week
diabetes. The relative amount of α_1 and β_1 isoform did not change significantly in
cortex in any conditions tested.

Northern blots. To determine whether the increase in ouabain binding capacity
and α_1 and β_1 subunit abundances seen in diabetes are associated with increases in
mRNA transcripts encoding the subunits, total cellular RNA was probed with ^{32}P
labelled isoform specific cDNAs. As shown in Fig. 2 we found differences in the
isoform specific mRNA transcription between cortex and medulla. In diabetes the only
significant change could be detected in the medulla in case of the β isoform transcript,
which showed a 169 $\pm$ 35.8% and 289 $\pm$ 43.1% ($p < 0.05$) increase in the two- and
four-week diabetic state compared to the control, respectively. Insulin administration
could totally (D$_2$) or partially (D$_4$) reverse the alterations observed. There was no
difference in α_1 subunit mRNA abundance in any state of diabetes. The α_1 mRNA
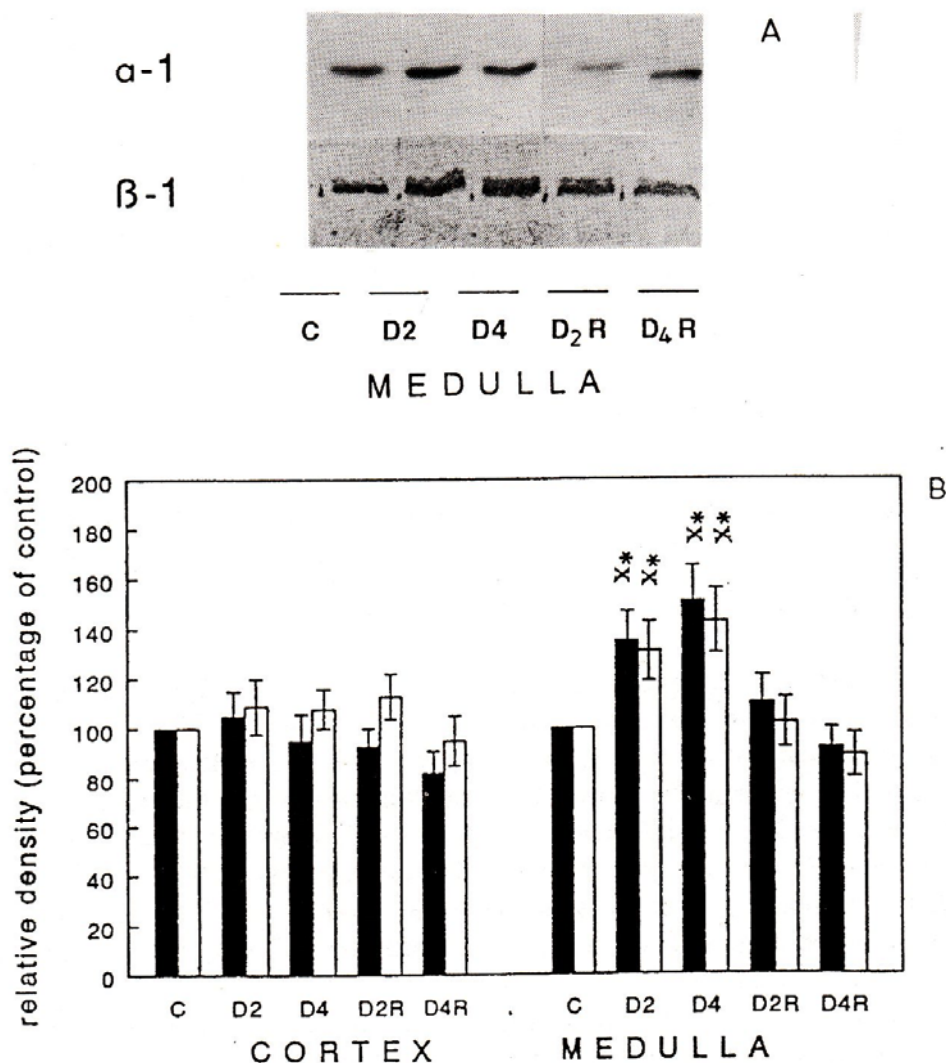transcription was not significantly affected.

Fig. 1. Abundance of Na^+/K^+-ATPase $\alpha_1 \beta_1$ subunit isoforms in kidney of control, STZ-diabetic insulin-treated rats. Microsomal membranes (50 μg) of kidney cortex and medulla from control (C), 2 weeks STZ-diabetic rats without (D_2) and with insulin treatment (D_2R), 4 weeks STZ-diabetic rats without (D_4) and with insulin treatment (D_4R) were subjected to SDS-PAGE. Relative abundance of α_1 and β_1 were quantitated by immunoblotting using isoform specific antisera and peroxidase conjugated antirabbit IgG as primary and secondary antibodies, respectively. A: a representative photo of an ECL developed Western blot of α_1 and β_1 subunit isoform detected in kidney medulla. B: relative densities of Na^+/K^+-ATPase α_1 and β_1 subunit isoform in kidney medulla and cortex. Closed bars: α_1, open bars: β_1. Data are means ± SE of 5 experiments. Statistically significant (p < 0.05) differences between controls and either STZ-diabetic or insulin-treated STZ-diabetic rats are denoted by *. Statistically significant (p < 0.05) differences between STZ-diabetic and insulin-treated STZ-diabetic rats are denoted by x
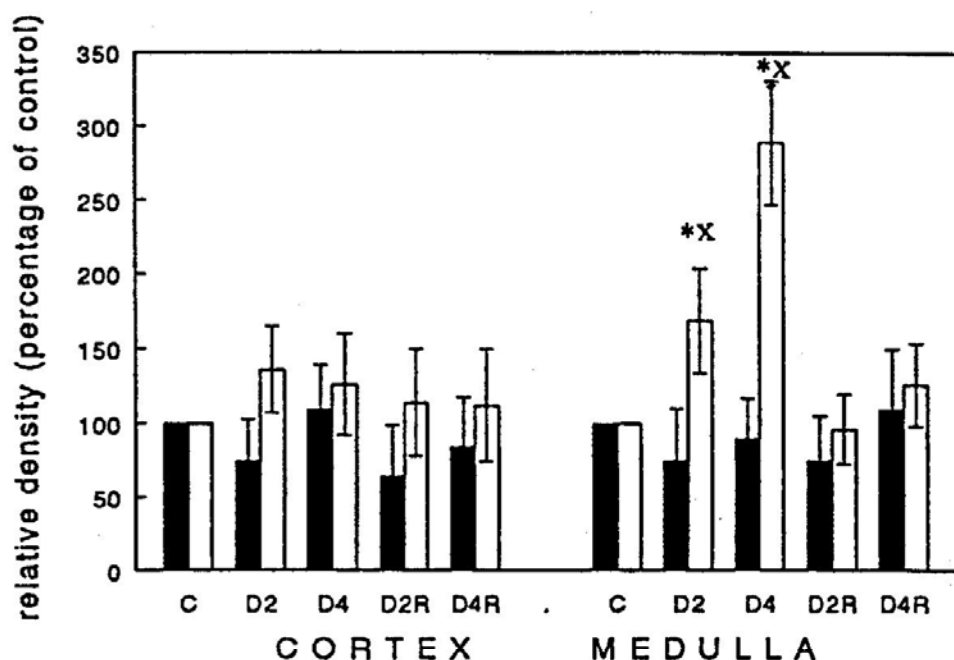
Fig. 2. Densitometric analysis of Northern blot of Na$^+$/K$^+$-ATPase α_1 and β subunit mRNAs prepared from kidney cortex and medulla of control, STZ-diabetic and STZ-diabetic insulin-treated rats. Relative densities of Na$^+$/K$^+$-ATPase α_1 and β subunit isoform. Closed bars: α_1, open bars: β_1. Data are means $\pm$ SE of 5 experiments. Statistically significant (p < 0.05) differences between controls and either STZ-diabetic or insulin-treated STZ-diabetic rats are denoted by *. Statistically significant (p < 0.05) differences between STZ-diabetic and insulin-treted STZ-diabetic rats are denoted by x

Discussion

Streptozotocin-induced diabetes in rats results in the increase in kidney mass. Kidney weight gain developes within the first two weeks of STZ-caused experimental diabetes. The main histological characteristic of this state is medullar hyperplasia [6]. The Na$^+$/K$^+$-ATPase activity is concomitantly increased [14] and as our experiments proved so is the maximal ouabain binding capacity. The α subunit of Na$^+$/K$^+$-ATPase is responsible for ouabain binding, and the different isoforms of α subunit show different ouabain binding affinities [29]. There are only one low affinity ouabain binding isoform (α_1) and two high affinity ouabain binding isoform (α_2 and α_3) [12, 29]. According to our results in agreement with the earlier data in kidney cortex and medulla only the low affinity binding sites (α_1 isoform) are present. In kidney cortex the number of ouabain binding sites did not change significantly in diabetes, however, there is a significant enhancement in kidney medulla.

The question arises whether the changes in ouabain binding capacity in diabetes is due to alterations of biochemical properties of the enzyme or derive from the quantitative changes of the transport molecule. To evaluate the expression of Na$^+$/K$^+$-ATPase isoforms, antisera specific for α_1, α_2, α_3, β_1, and β_2 subunits were used to probe Western blots of kidney cortex and medulla microsomes prepared from control,

STZ-treated diabetic and STZ-treated insulin injected rats. The major changes were observed in kidney medulla, while the cortex in accordance with the histological findings turned to be less affected. We could detect a concomitant increase in both α_1 and β_1 protein level in diabetic states while was could only prove a parallel change in β mRNA level. Ouabain binding data confirm our results concerning the α_1 protein level. There is other report showing that the synthesis and degradation rate of α_1 isoform are enhanced in the nervous system of spontaneously diabetic rats [27]. In contrast to other tissues, where $\alpha_2\beta_1$ isoform composition was regulated by insulin [17], our results show that $\alpha_1\beta_1$ isoform level changes in diabetic kidney medulla and is affected by insulin similarly as we reported on brain cortex [26, 30].

The question is whether the observed alterations in experimental diabetes are derived from the absence of insulin, from the relative overflow of glucagon and other hormones or from other metabolic disorders is not resolved in this study. From our data we concluded that the activity changes of Na^+/K^+-ATPase as well as the alteration of ouabain binding capacity in experimental diabetes and in case of insulin administration is a result of the change of protein amount. The mRNA transcription showed a good correlation with the β but not with the α_1 protein subunit. There are also data showing a prolonged latency of changes in the synthesis of Na^+/K^+-ATPase catalytic subunit after insulin administration [27], and these results suggest that the regulation of Na^+/K^+-ATPase activity in diabetes does not directly take place through insulin response elements in case of α subunit. However, these are the first data concerning the changes in expression of β subunit, which is supposed to have a regulatory function in the enzyme activity.

Acknowledgements

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